尿病患者から分離したコクサッキーウイルスが実験動物の糖尿病を発症したとする報告は稀である．ケトアシドーシスで死亡した小児から分離されたコクサッキーB4ウイルスバリアントが，SJL/Jマウスで糖尿病を誘発したとされている．

最近，劇症のコクサッキーウイルス感染で死亡した新生児の膵島細胞にエンテロウイルス抗原が出現していること，外分泌細胞にはウイルス抗原は認められないこと，さらに，ヒト培養膵島細胞にエンテロウイルスの受容体として機能するポリオウイルスレセプターや，インテグリン$\alpha V\beta_3$が発現していることが示されている．このことからも，コクサッキーウイルスがヒト膵島細胞に比較的選択的に感染しうると考えられる．ただしヒトコクサッキーウイルスアデノウイルスレセプター(CAR)と1型糖尿病感受性との関連の報告はなく，ウイルスレセプターがヒト1型糖尿病の感受性，発症制御に関与しているかどうかは不明である．このように多くの臨床的な知見の蓄積にもかかわらず，エンテロウイルス感染による1型糖尿病発症メカニズムの全貌は解明されていない．

Ⅲ．実験的ウイルス糖尿病の発症機構

1．ウイルスおよび宿主の糖尿病誘発性の遺伝的決定因子

コクサッキーB4ウイルスは感受性の実験動物に糖尿病を誘発するわけではないが，感受性マウス(SJL/J)のマウス膵島細胞で継代を続けることで糖尿病誘発性を獲得することが知られている．一方，糖尿病誘発性マウス脳心筋炎(EMC)ウイルスのM variantをさらにクローニングし，特定のマウス系統に糖尿病誘発性の高いEMC-Dウイルスと，誘発しないEMC-Bが分離された[9]．マウスの系統としては，SJL/J，DBA/2が感受性で，Balb/C，NODが中等度，C57BL/6は抵抗性である．このマウスの感受性には性差もあり，糖尿病発症の感受性は，オスのほうが高く，膵島細胞の障害が著しい．マウスの系統依存性の感受性の差については，単一の遺伝子であると報告されているが，感受性を決定する遺伝子は同定されていない．

なお，ウイルス糖尿病高誘発性のEMC-Dウイルスと，誘発しないEMC-Bウイルスの遺伝子配列研究により，それぞれの株間は14核酸の違いしかないことが示された[10]．さらに，遺伝子改変ウイルスを用いた検討により，誘発性を決定しているのは，ウイルスカプシド(VP1)の776番目のアミノ酸がアラニンであること(Thr776Ala)が重要であることが明らかとなった[11]．この部位はウイルスの膵島細胞への接着に重要であることから，ウイルスとウイルスレセプターの関係が，少なくとも一部は，ウイルス糖尿病の誘発を制御していると考えられる．

一方，エンテロウイルス感染症の病態について，ポリオウイルス研究が進展し，ポリオウイルスの組織特異的神経細胞障害メカニズムが解明された．すなわち，ウイルスレセプターの発現，ウイルス蛋白質の翻訳開始機構(Internal Ribosome Entry Site；IRES)による組織選択的翻訳活性，神経細胞におけるインターフェロン誘導能の低さによるウイルス感受性亢進の機序で，その発症機構の大要が説明できる[12]．

しかしながら，エンテロウイルスは，神経皮膚向性とされるコクサッキーA群ウイルス，臓器障害をきたすコクサッキーB群ウイルス，また，エンテロウイルス70は出血性結膜炎をきたすなど，その臨床像は多彩であり，それぞれの発症メカニズムはさらに多様であることが推測される．

2．実験的ウイルス糖尿病発症の制御機構

ウイルス糖尿病については，既述のようにEMCウイルスを用いた研究が最も広く行われている．感受性のSJL/Jマウスでは，オスでは急激に糖尿病を発症し，死に至るが，メスではいったん糖尿病を発症するもののその後は回復する．また，比較的大量(10^5PFU/mouse)のウイルス量の投与では，3日以内の早期に発症するのに対し，少量(<10^2PFU/mouse)のウイルス投与では，マクロファージの活性化が膵島細胞障害に働くことが知られている．一方，われわれは，マクロファ

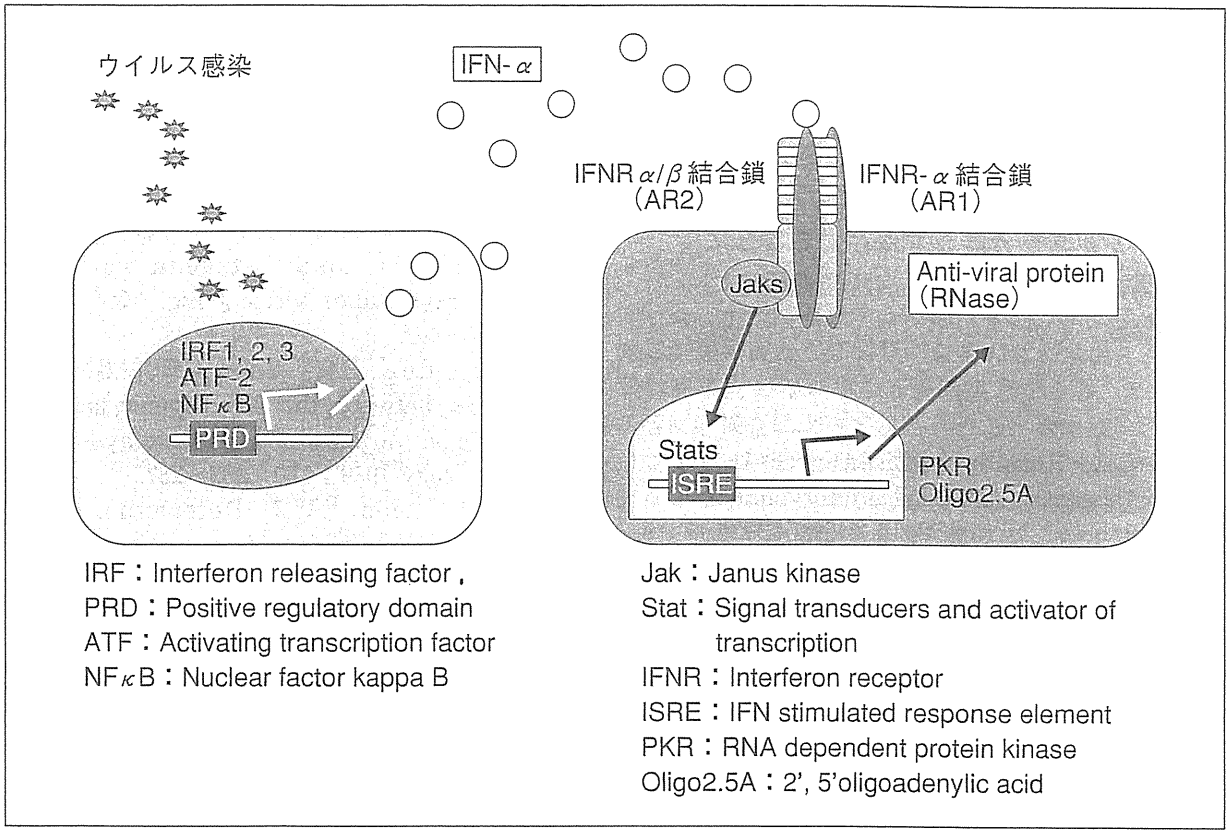

図2 ウイルス感染によるインターフェロン(IFN)の産生誘導とインターフェロンによる抗ウイルス作用の発揮

ージ賦活剤である*Corynebacterium parvum*を前投与することにより，ウイルス糖尿病発症を完全に阻止できることを明らかにした．T細胞欠損マウス，B細胞欠損マウス，T細胞もB細胞も欠損するRag-1ノックアウトマウスいずれも糖尿病発症に影響なく，また，中和抗体の投与は感染後36時間以内の早期移入した場合のみで有効であるので，インターフェロン(図2)やマクロファージの活性化など，自然免疫の働きがその防御に重要であると考えている[13]．

興味深いことに，トランスジェニックマウスを用いた研究で，膵島細胞特異的にサイトカイン抑制分子を発現したマウスではウイルス増殖が促進され，NK細胞による膵島細胞障害が進行し，糖尿病に対する感受性が亢進することが示された[14]．すなわち，膵島細胞におけるサイトカイン防御反応が重要であることが示唆されたが，果たして自然感染でもこのメカニズムが働いているのか，さらに検討が必要であろう．

おわりに

さまざまな臨床的知見，実験研究の成果から，エンテロウイルス感染がヒトにおける糖尿病の発症に関わっていることが強く示唆されている．しかしながら，ウイルス側の要因，宿主側の感受性因子についての決定的な証拠は乏しい．ウイルス感染による糖尿病として発症するかどうかの要因として，ウイルスの臓器親和性，感染のレベル(重症度)，宿主の自然免疫，獲得免疫の質と速度，応答のレベル，さらには膵島細胞の障害感受性など，多くの要素が関与すると考えられ，高度に複雑な発症機構であることが推測される．

現時点では，ウイルスが糖尿病発症の原因であることを証明するために必要なウイルスの糖尿病誘発性を検定できるシステムがないことが問題であると考える．一方，ウイルスの病原性検定システムの開発には，宿主要因の解明が進まなければならない．ウイルス感染症による膵島障害と糖尿

病発症メカニズムの全貌を明らかにするためには，ウイルスと宿主要因いずれをも明らかにするための総合的なアプローチが必要である．感染症専門家におかれては，ウイルス感染症と糖尿病発症が関連していることが疑われる場合には，病因の候補となるウイルスを分離し，保存することにより，将来の当該ウイルスの糖尿病誘発性検定に備える努力をお願いしたい．

参考文献

1) Bluestone JA, Herold K, Eisenbarth G：Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010；464：1293～1300.
2) 永淵正法，大澤　進，和泉賢一，小川秀一郎，栗崎宏憲，高上悦志：1型糖尿病の成因③環境因子．Diabetes Frontier 2008；19(2)：180～184.
3) Jenson AB, Rosenberg HS, Notkins AL：Pancreatic islet-cell damage in children with fatal viral infections. Lancet 1980；2：354～358.
4) 永淵正法，近藤しおり：自己免疫性1型糖尿病とウイルス感染．Diabetes Frontier 印刷中.
5) 楠原浩一：先天性風疹症候群と糖尿病．月刊糖尿病　2009；1(7)：116～124.
6) Filippi CM, Estes EA, Oldham JE, von Herrath MG：Immunoregulatory mechanisms triggered by viral infections protect from type 1 diabetes in mice. J Clin Invest 2009；119：1515～1523.
7) Nagafuchi S, Katsuta H, Anzai K：Rituximab, B-lymphocyte depletion, and Beta-cell function. N Engl J Med 2010；362：761.
8) 永淵正法，小川秀一郎，栗崎宏憲：エンテロウイルス感染症と臓器障害のメカニズム―膵島障害と糖尿病誘発―．臨床とウイルス　2007；35：146～151.
9) Yoon JW, McClintock PR, Onodera T, Notkins AL：Virus-induced diabetes mellitus. XVIII. Inhibition by a nondiabetogenic variant of encephalomyocarditis virus. J Exp Med 1980；152：878～892.
10) Bae YS, Eun HM, Yoon JW：Genomic differences between the diabetogenic and non-diabetogenic variants of encephalomyocarditis virus. Virology 1989；170：282～287.
11) Bae YS, Yoon JW：Determination of diabetogenicity attributable to a single amino acid, Ala-776, on the polyprotein of encephalomyocarditis virus. Diabetes 1993；42：435～443.
12) Ida-Hosonuma M, Iwasaki T, Yoshikawa T, et al.：The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus. J Virol 2005；79：4460～4469.
13) Kounoue E, Izumi K, Ogawa S, et al.：The significance of T cells, B cells, antibodies and macrophages against encephalomyocarditis (EMC)-D virus-induced diabetes in mice. Arch Virol 2008；153：1223～1231.
14) Flodström M, Maday A, Balakrishna D, et al.：Target cell defense prevents the development of diabetes after viral infection. Nat Immunol 2002；3：373～382.

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Tyrosine kinase 2 interacts with the proapoptotic protein Siva-1 and augments its apoptotic functions

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ABSTRACT

Siva-1 is a molecule that has the potential to induce both extrinsic (receptor-mediated) and intrinsic (non-receptor-mediated) apoptosis. Siva-1 binds to CD27, a member of the tumor necrosis factor receptor (TNFR) family, Abl-related gene (ARG), and BCL-X_L, and these partner molecules reportedly enhance the apoptotic properties of Siva-1. In this study, we show that Siva-1 also interacts with a member of the Jak family protein kinases, tyrosine kinase 2 (Tyk2). Siva-1 bound to Tyk2 via its N-terminal region, and Tyk2 phosphorylated Siva-1 at tyrosines 53 and 162. In murine pro-B cells, Ba/F3 cells, expression of Tyk2 augmented Siva-1-induced apoptosis. This augmentation of Siva-1-induced apoptosis was retained regardless of the phosphorylation of Siva-1, but was almost completely prevented by the abrogation of the Tyk2-Siva-1 association. These findings indicate that the interaction between Siva-1 and Tyk2 directly augments the apoptotic activity of Siva-1. Our novel observations suggest that Siva-1 forms a functional complex with Tyk2 and participates in the transduction of signals that inhibit B lymphocyte growth.

1. Introduction

Apoptosis is a cell suicide mechanism that plays a central role both in development and in homeostasis of almost all multicellular organisms [1–3]. In the adult animal, cells die by apoptosis during tissue turnover or at the end of an immune response. Because the physiological role of apoptosis is crucial, aberration of this process can cause such as cancers, viral infections or autoimmune disorders [4,5].

Siva-1 is a molecule that has been reported to have a potential ability both in extrinsic (receptor-mediated) and intrinsic (non-receptor-mediated) apoptosis [6–14]. Siva-1 was originally cloned and identified as an intracellular ligand of CD27, which is a member of the TNFR superfamily that is expressed on both T and B lymphocytes [6]. Unlike Fas or TNFR1, CD27 lacks a death domain (DD) in its cytoplasmic tail but can induce apoptosis suggesting that Siva-1, which contains a death domain homology region (DDHR), has an important role in CD27-mediated apoptotic signaling [6,7,15]. Exogenous overexpression of Siva-1 alone can induce apoptosis in various cells, whereas an alternate splicing variant of the *SIVA* gene, Siva-2, lacks DDHR and seems much less apoptotic [6,7]. This observation supports the idea that DDHR is the effector region of Siva-1 as a proapoptotic protein. It is also shown that Siva-1 is overexpressed in various pathological circumstances, such as acute ischemic injury and Coxsackie virus infection and that Siva-1 is possibly involved in these apoptotic processes [16–19].

Several groups have so far identified Siva-1 as a partner of distinct signaling molecules other than CD27, such as the glucocorticoid-induced TNFR family-related gene (GITR/TNFR18), which is also a member of the TNFR family [8], the Abl-related gene (ARG), which is a non-receptor tyrosine kinase [9], and BCL-X_L, which belongs to the BCL family and promotes cell survival [10,11]. Siva-1 is also reported to inhibit NF-κB activity in T lymphocytes, suggesting that Siva-1 is involved in T cell receptor-mediated activation-induced cell death (AICD) [12]. Recently, Fei Chu et al. examined the role of Siva-1 from a clinical perspective. They showed that Siva-1 protein expression enhanced cisplatin-induced apoptosis in breast cancer cells (MCF7 cells) and proposed that Siva-1 could be used as a potentiator of chemotherapy [13].

We and others previously reported that Tyk2 is essential for the transduction of the IFN-α-induced inhibition of B lymphocyte growth [20–23]. This inhibitory signaling pathway downstream of Tyk2 still remains unknown. In order to identify the novel molecules that interact with Tyk2 and inhibit B lymphocyte growth, we performed a yeast two-hybrid screen using Tyk2 as bait. Then, we identified Siva-1, and according to the reported apoptotic nature of Siva-1, we further investigated the relationship between Siva-1 and Tyk2 both biophysically and functionally.

In this report, we show that Tyk2 interacts with and phosphorylates Siva-1. Furthermore, we identify not only the region within Siva-1 that binds to Tyk2 but also the specific tyrosines in Siva-1 that are phosphorylated by Tyk2. Tyk2 augments the apoptotic effects of Siva-1 in murine pro-B cells (Ba/F3 cells) through a process

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that requires its association with Siva-1 but not tyrosine phosphorylation of Siva-1.

2. Materials and methods

2.1. Yeast two-hybrid screen

A yeast two-hybrid screen was performed as previously described [24]. Briefly, cDNA encoding the kinase domain (aa 833–1187) of human Tyk2 was subcloned into the Gal DNA-binding domain plasmid pGBKT7 (BD Clontech). This construct was used as the bait in a yeast two-hybrid screen of a human B lymphocyte cDNA library that was constructed in pACT (BD Clontech). Approximately 1.6×10^6 colonies were screened for activation of the ADE2, HIS3, and *lacZ* reporter genes using the host strain AH109. The inserts from positive library plasmids were PCR amplified and mapped by *AluI* digestion. The plasmids were transformed into bacteria, isolated, and then sequenced.

2.2. Plasmid constructions

Human Tyk2 cDNA was kindly provided by Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN), and subcloned into the pCMV-Tag2 vector (N-terminal Flag tag) (Stratagene, Heidelberg, Germany). The full-length human Siva-1 cDNA was generated by RT-PCR from human bone marrow mononuclear cells using the oligonucleotide primers 5′-cgg ccc cgc ggc cat gcc caa gcg gag ctg ccc ctt-3′ and 5′-ctt gag cca gcc tca ggt ctc gaa cat ggc aca g-3′, and subcloned into pCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA). Siva-1 in the pCR2.1-TOPO construct was digested with *EcoRI* and ligated into the pCMV-Tag2 vector or the pEGFP-C1 vector (BD Clontech) to obtain the Flag-Siva-1 or GFP-Siva-1 constructs, respectively.

The GFP-Siva-1 deletion mutants, A, C and E–G, indicated in Fig. 2, were generated by PCR with specific primers, and the amplified fragments were inserted into the *EcoRI* and *BamHI* sites of the pEGFP-C1 vector. The deletion mutants B and D were obtained by self-ligation after digesting full-length GFP-Siva-1 with *ApaI* or *PstI*, respectively.

To substitute tyrosine with phenylalanine at residues 34, 53, and 162 of wild-type Siva-1, oligonucleotide-directed mutagenesis was performed. The transformer site-directed mutagenesis kit (BD Clontech) was used according to the manufacturer's protocol with the following oligonucleotides: Y34F oligo, gcc gag cgc ttc tcg cag gag; Y53F oligo, gcc cag gcc ttc ctg gac cac; and Y162F oligo, gca gtg aca tgt tcg aga aag tgc.

All plasmid constructs were confirmed by sequencing the coding region.

2.3. Cell culture and transfections

HEK293T cells were maintained in DMEM (Sigma–Aldrich) supplemented with 10% FCS (ICN, Osaka, Japan) at 37 °C in 5% CO_2. HEK293T cells were transiently transfected with plasmid DNA using the calcium phosphate precipitation protocol.

An interleukin (IL)-3-dependent murine pro-B cell line, Ba/F3, was cultured in RPMI 1640 medium (Sigma–Aldrich) containing 10% FCS and 5% conditioned medium from WEHI 3B cells as a source of IL-3. The Ba/F3 cell lines stably expressing either wild-type Tyk2 (Ba/F3/Tyk2 WT) or constitutively activated Tyk2 with valine at position 678 replaced by phenylalanine (Ba/F3/Tyk2 V678F) were established as previously described [25]. Plasmid DNA was transiently transfected into Ba/F3 cells using the Nucleofector system (Amaxa Biosystems GmbH, Cologne, Germany). Nucleofections were performed using the Cell Line Nucleofector Solution V and the X-001 program according to the manufacturer's instruction (Amaxa Biosystems GmbH).

2.4. Immunoprecipitations and Western blotting

Immunoprecipitations and Western blot analysis were performed as previously described [24] except that RIPA buffer (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% DOC and 0.1% SDS) was used to lyse cells and to wash the immunoprecipitates that examined the interaction between Tyk2 and Siva-1. The following antibodies were used to detect Tyk2, phospho-tyrosine, Flag and GFP, respectively: anti-Tyk2 Ab (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine Ab (4G10) (Upstate, Lake Placid, NY), anti-Flag M2 mAb (Sigma–Aldrich, St. Louis, MO), and the BD Living Colors A.v. Peptide Ab (BD Clontech).

2.5. Flow cytometry assays

Flow cytometry analyses were performed on GFP-positive cells with a FACSCalibur cytofluorimeter (BD Biosciences, San Jose, CA). Apoptotic cells were detected by staining the cell surface with APC-conjugated Annexin V (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

3. Results

3.1. Tyk2 interacts with Siva-1

To identify novel Tyk2-interacting proteins, a yeast two-hybrid screen was performed using the kinase domain of Tyk2 fused to the Gal DNA-binding domain as the bait. A human B lymphocyte cDNA library was screened. One of a number of positive clones was found to be the previously reported Siva-1, which was originally cloned as a CD27 binding protein.

To further investigate this potential interaction with Siva-1, we first examined the association between Tyk2 and Siva-1 in mammalian cells by performing coimmunoprecipitation experiments. Full-length Flag-tagged Siva-1 was transiently transfected into 293T cells with or without the full-length Tyk2 expression vector. Twenty-four hours post transfection, cell lysates were immunoprecipitated with an anti-Tyk2 Ab and subsequently analyzed by immunoblotting with an anti-Flag Ab or immunoprecipitated with an anti-Flag Ab and subsequently immunoblotted with an anti-Tyk2 Ab. As shown in Fig. 1, Siva-1 coimmunoprecipitated with Tyk2 and vice versa when both Tyk2 and Siva-1 were expressed in 293T cells, indicating that Tyk2-Siva-1 complexes exist within these cells.

3.2. The N-terminus region of Siva-1 is required to bind to Tyk2

We next determined which region of Siva-1 was responsible for binding to Tyk2. Siva-1 consists of the three distinct regions: an N-terminus region, a death domain homology region (DDHR, aa 48–114) in the central part of the protein, and a Cys-rich region (Fig. 2A). The unique putative amphipathic helical structure of the Siva-1 protein (SAH) is formed from amino acid residues 36–55 in the N-terminus (Fig. 2A). Based on these structural features of Siva-1, we made seven Siva-1 deletion mutants that were fused to GFP (GFP-Siva-1) (Fig. 2A). Wild-type GFP-Siva-1 or each GFP-Siva-1 deletion mutant was transiently transfected into 293T cells along with Flag-tagged Tyk2. Twenty-four hours post transfection, cell lysates were immunoprecipitated with an anti-Flag Ab and analyzed by immunoblotting with an anti-GFP Ab. As shown in Fig. 2B, wild-type Siva-1 and the Siva-1 deletion mutants A, B, C,

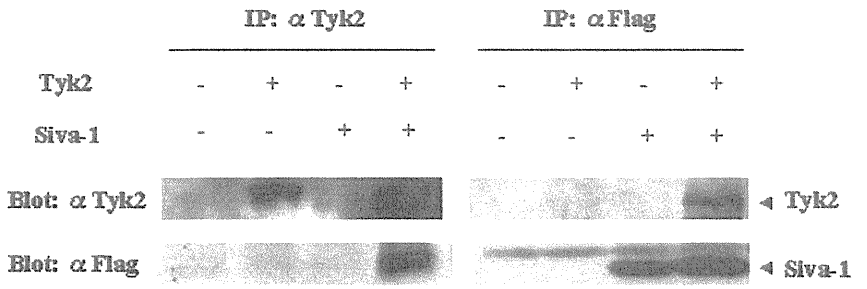

Fig. 1. Tyk2 interacts with Siva-1. 293T cells were transfected with Tyk2 and/or Flag-Siva-1. *Left*, Cell lysates were immunoprecipitated with an anti-Tyk2 Ab and then immunoblotted with an anti-Tyk2 (*upper panel*) or anti-Flag Ab (*lower panel*). *Right*, Cell lysates were immunoprecipitated with an anti-Flag and then immunoblotted with an anti-Tyk2 Ab (*upper panel*) or anti-Flag Ab (*lower panel*).

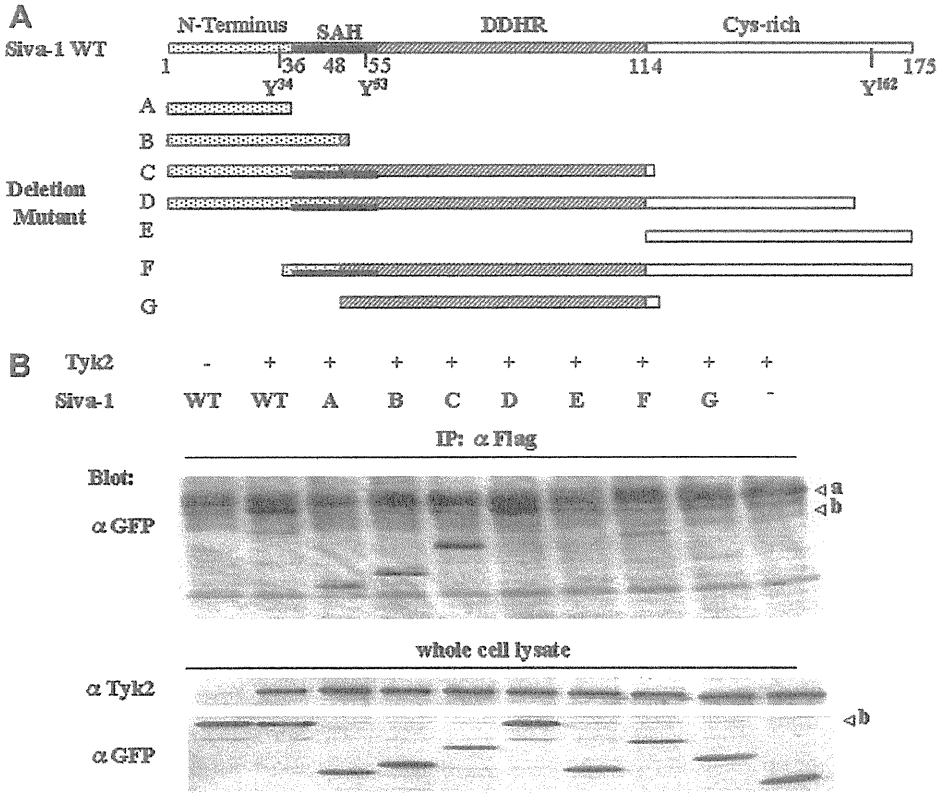

Fig. 2. The N-terminus region of Siva-1 is required for Tyk2 binding. (A) A schematic of the domain structure of Siva-1 and the Siva-1 deletion mutants. (B) GFP-Siva-1 deletion mutants were coexpressed with Flag-tagged Tyk2 in 293T cells. After 24 h, the cell lysates were immunoprecipitated with an anti-Flag Ab and then immunoblotted with an anti-GFP Ab (*upper panel*). As a control, whole cell lysates were blotted with an anti-Tyk2 Ab or anti-GFP Ab (*lower panel*). a: immunoglobulin heavy chain (55 kDa), b: GFP-Siva-1 (Wt, D) (46 kDa).

and D, which all contain the N-terminus region of Siva-1, bound to Tyk2, whereas the Siva-1-E and -G mutants, which both lack the N-terminus region of Siva-1, did not bind to Tyk2. These findings demonstrate that Siva-1 associates with Tyk2 through its N-terminus region. Furthermore, the fact that even the Siva-1-A mutant, which only contains amino acid residues 1–36, bound to Tyk2 indicates that the N-terminus region of Siva-1 is both necessary and sufficient to bind to Tyk2. The Siva-1-F mutant weakly associated with Tyk2, suggesting that the partial N-terminus region corresponding to residues 34–48 that is present in Siva-1-F contributed to weak binding to Tyk2.

3.3. Tyk2 phosphorylates Siva-1 on Tyr^{53} and Tyr^{162}

Because Tyk2 is a tyrosine kinase, we next assessed whether Tyk2 phosphorylates Siva-1. Wild-type Flag-Siva-1 was expressed in 293T cells with or without Tyk2, and the cell lysates were immunoprecipitated with an anti-Flag Ab and subsequently immunoblotted with an anti-phospho-tyrosine Ab. The results shown in Fig. 3A demonstrate that Tyk2 phosphorylates Siva-1 in cells.

Next, we determined the specific tyrosine residue(s) in Siva-1 that is phosphorylated by Tyk2. There are three tyrosine residues in Siva-1: Tyr^{34}, Tyr^{53} and Tyr^{162}. We substituted these individual tyrosines or a combination of these tyrosines with phenylalanine to obtain seven Siva-1 mutants (Fig. 3B). Then, wild-type Flag-Siva-1 or each Flag-Siva-1 mutant was expressed in 293T cells with or without Tyk2, and the cell lysates were immunoprecipitated with an anti-Flag Ab and subsequently immunoblotted with an anti-phospho-tyrosine Ab. As shown in Fig. 3C, the Siva-1 mutants containing phenylalanine at both positions 53 and 162 as well as the triple tyrosine Siva-1 mutant (indicated with * in Fig. 3C) were not phosphorylated by Tyk2. Therefore, we identified

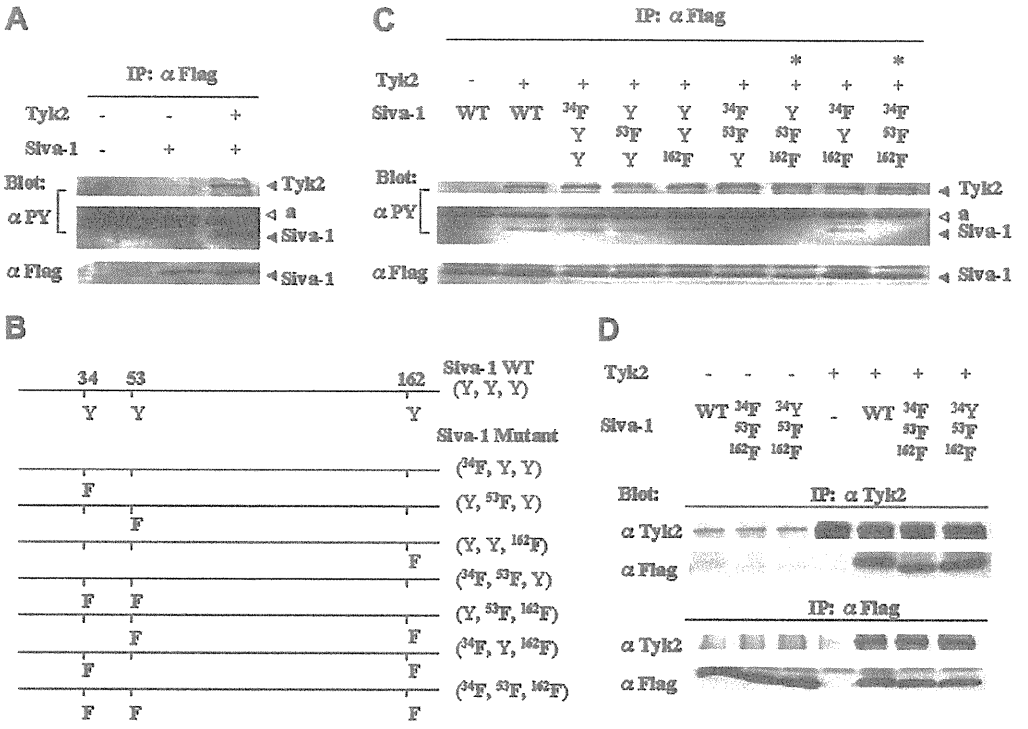

Fig. 3. Tyk2 phosphorylates Siva-1 at residues Tyr^{53} and Tyr^{162} independently of the Tyk2-Siva-1 interaction. (A) Tyk2 and/or Flag-Siva-1 were transfected into 293T cells. Twenty-four hours post transfection, the cell lysates were immunoprecipitated with an anti-Flag Ab and then immunoblotted with an anti-phospho-tyrosine Ab (*upper and middle panels*) or anti-Flag Ab (*lower panel*). a: immunoglobulin light chain (23 kDa). (B) Site-directed mutagenesis of Siva-1 was performed to substitute individual or multiple tyrosine residues with phenylalanine. (C) A series of wild-type or Siva-1 mutants were coexpressed with Tyk2 in 293T cells. Twenty-four hours post transfection, the cell lysates were immunoprecipitated with an anti-Flag Ab and then immunoblotted with an anti-phospho-tyrosine Ab (*upper and middle panels*) or anti-Flag Ab (*lower panel*). a: immunoglobulin light chain (23 kDa). (D) Two Flag-Siva-1 mutants, Siva-1 (^{34}F, ^{53}F, ^{162}F) and Siva-1 (^{34}Y, ^{53}F, ^{162}F), which cannot be phosphorylated by Tyk2, were transfected with or without Tyk2. Wild-type Siva-1 was used as a positive control. After 24 h, the cell lysates were immunoprecipitated with an anti-Tyk2 Ab (*upper panels*) or anti-Flag Ab (*lower panels*) and subsequently immunoblotted with an anti-Tyk2 or anti-Flag, respectively.

two tyrosines, Tyr^{53} and Tyr^{162} in Siva-1 that are phosphorylated by Tyk2.

3.4. Tyrosine phosphorylation of Siva-1 is not involved in the Tyk2-Siva-1 interaction

Based on our findings that Tyk2 associates with Siva-1 and that Tyk2 phosphorylates two specific tyrosines in Siva-1, Tyr^{53} and Tyr^{162}, we next examined whether this tyrosine phosphorylation of Siva-1 by Tyk2 is involved in the interaction between Tyk2 and Siva-1. We investigated the association between Tyk2 and the Siva-1 mutants that were not tyrosine phosphorylated by Tyk2 because Tyr^{53} and Tyr^{162} had been substituted with phenylalanines. As shown in Fig. 3D, both of these Siva-1 mutants coimmunoprecipitated with Tyk2 and vice versa. In addition, both the wild-type and the tyrosine mutants were immunoprecipitated to equivalent levels. These data indicate that Siva-1 can bind to Tyk2 independently of being phosphorylated by Tyk2 and demonstrate that phosphorylation of tyrosines 53 and 162 in Siva-1 is not required for the Tyk2-Siva-1 interaction.

3.5. Tyk2 augments Siva-1-induced apoptosis through the Tyk2-Siva-1 interaction

Data to date indicate that Siva-1 is a proapoptotic protein [6–8,14]. Therefore, we next examined whether Tyk2 affected Siva-1-mediated apoptosis. We established Ba/F3 cell (IL-3 dependent murine pro-B cell) lines stably expressing either wild-type Tyk2 (Ba/F3/Tyk2 WT) or constitutively activated Tyk2 with valine at position 678 replaced by phenylalanine (Ba/F3/Tyk2 V678F) [25]. In Ba/F3/Tyk2 V678F cells, Tyk2 was constitutively phosphorylated without cytokine stimulation (Fig. 4A).

To assess whether Tyk2 has any influence on the apoptotic function of Siva-1, we transiently transfected either GFP-Siva-1 or control GFP vector into Ba/F3, Ba/F3/Tyk2 WT and Ba/F3/Tyk2 V678F cells (Fig. 4B). Forty-eight hours post transfection, we examined the prevalence of Annexin V-positive cells by flow cytometry after gating on GFP-positive cells. Siva-1 expression induced apoptosis approximately fourfold in Ba/F3 cells, and the presence of Tyk2 drastically augmented the apoptotic function of Siva-1. The degree of apoptosis induced by Siva-1 in Ba/F3/Tyk2 V678F cells was highly similar to that in Ba/F3/Tyk2 WT cells (Fig. 4B).

We next transfected the Siva-1 mutant (^{34}Y, ^{53}F, ^{162}F), which contains phenylalanine at both positions 53 and 162 and cannot be phosphorylated by Tyk2, into Ba/F3 and Ba/F3/Tyk2 V678F cells (Fig. 4C). Similarly to wild-type Siva-1, the Siva-1 mutant (^{34}Y, ^{53}F, ^{162}F) showed enhanced apoptotic activity when expressed in Ba/F3/Tyk2 V678F cells, indicating that the Tyk2 augmented the apoptotic function of Siva-1 independently of the phosphoylation of Siva-1. These results suggested that the association of Siva-1 with Tyk2, not the phosphorylation of Siva-1 by Tyk2, augmented Siva-1-induced apoptosis in Ba/F3 cells.

To confirm this, we next transfected either Ba/F3 or Ba/F3/Tyk2 WT cells with the Siva-1-G mutant, which contains only the DDHR and cannot bind to Tyk2 (Fig. 4D). Expression of the Siva-1-G mutant in Ba/F3 cells induced apoptosis comparable to wild-type Siva-1 whereas the presence of Tyk2 hardly augmented the apoptosis induced by the Siva-1-G mutant in Ba/F3/Tyk2 WT cells. These findings demonstrate that the apoptotic function of Siva-1 is signif-

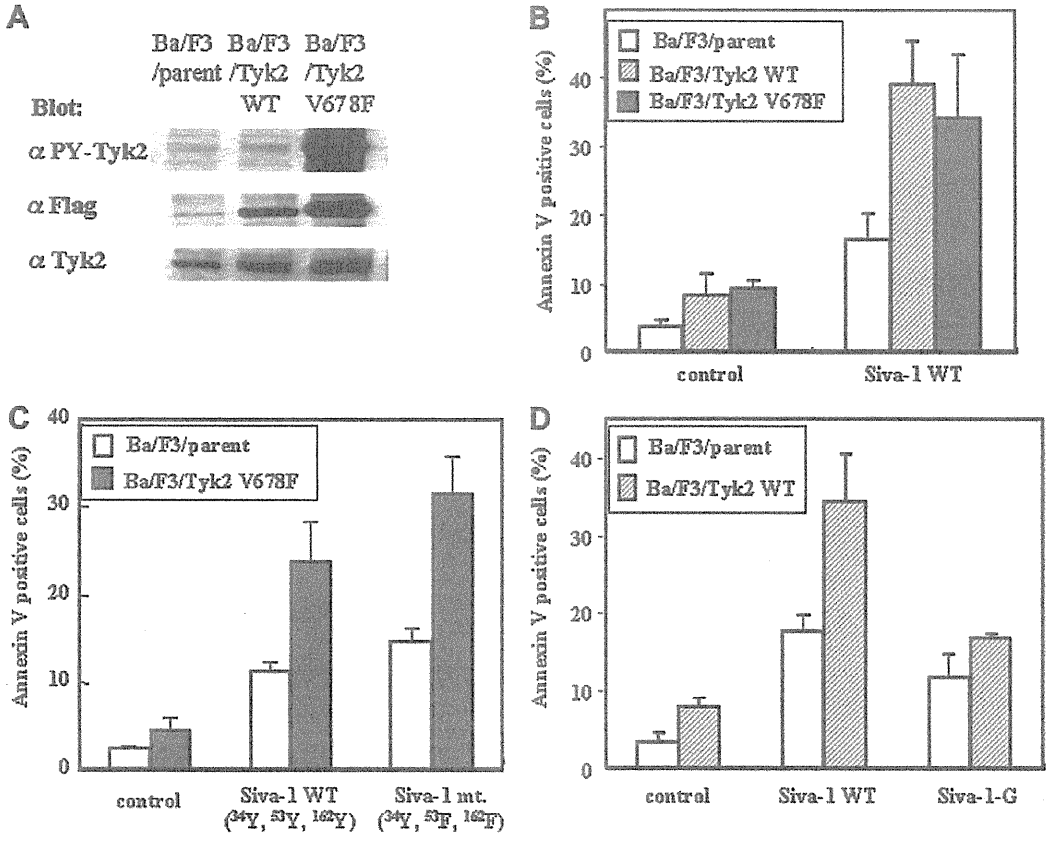

Fig. 4. Tyk2 augments Siva-1-induced apoptosis through the Tyk2-Siva-1 interaction. (A) The Ba/F3 cell lines stably expressing either wild-type Tyk2 (Ba/F3/Tyk2 WT) or constitutively activated Tyk2 (Ba/F3/Tyk2 V678F) were prepared. Cells were lysed and the phosphorylation status of Tyk2 was assessed by Western blot. (B) Either control GFP vector or GFP-Siva-1 was transiently transfected into Ba/F3, Ba/F3/Tyk2 WT and Ba/F3/Tyk2 V678F cells. (C) Either a control GFP vector, wild-type Siva-1 (^{34}Y, ^{53}Y, ^{162}Y) or the Siva-1 mutant (^{34}Y, ^{53}F, ^{162}F), which could not be phosphorylated by Tyk2, was transiently transfected into Ba/F3 and Ba/F3/Tyk2 V678F cells. (D) Either a control GFP vector, wild-type Siva-1, or Siva-1-G mutant was transiently transfected into Ba/F3 and Ba/F3/Tyk2 WT cells. (B–D) Forty-eight hours post transfection, Annexin V-positive cells were measured by FACS after gating on the GFP-positive cells. Values represent the mean of four independent experiments. Error bars represent 1 SD from the mean.

icantly augmented through the Tyk2-Siva-1 association, not by the phosphorylation of Siva-1.

4. Discussion

Siva-1 was originally identified as a ligand for CD27, a member of the TNFR family [6]. Functionally, Siva-1 was shown to exhibit an apoptotic property and to play an important role in the CD27-mediated apoptosis [6]. To date, Siva-1 is considered to be involved both in extrinsic and intrinsic apoptotic pathway [6–14], and several signaling molecules other than TNFR, such as ARG and BCL-X_L have been reported to interact with Siva-1 [9–11].

Tyk2 was the first member of the Jak family kinases to be cloned as an essential molecule in IFN-α signaling [26]. Tyk2 is essential for B cell growth inhibition by IFN-α in addition to its dispensable role in helper T cell differentiation by IL-12 [20–23]. We sought to identify the molecule that is downstream of Tyk2 by performing a yeast two-hybrid screen. Through these analyses we identified Siva-1 as a novel Tyk2-interacting protein (Fig. 1).

Siva-1 bound to Tyk2 through its N-terminus region (aa 1–36) (Fig. 2). Although another Siva-1 binding partner, BCL-X_L, requires the SAH domain of Siva-1, which forms a helix that spans from the N-terminus tail to the DDHR head (aa 36–55), to associate with Siva-1 [10,11], Tyk2 did not require this helical structure. Tyk2 is a tyrosine kinase that belongs to Jak family, we then examined whether Tyk2 phosphorylates Siva-1 or not. When Tyk2 and Siva-1 were cotransfected into 293T cells, Siva-1 was phosphorylated by Tyk2 (Fig. 3A). Among the three tyrosine residues in Siva-1, Tyk2 specifically phosphorylated Tyr^{53} and Tyr^{162} (Fig. 3B and C) unlike ARG, which was associated with Siva-1 and phosphorylated the third tyrosine of Siva-1, Tyr^{34} [9]. These findings suggest that Siva-1 has many binding partners, but that the interacting site and the affected amino acid residues in Siva-1 depend upon the specific partner. The phosphorylation of Siva-1 by Tyk2 was not required for the interaction between Tyk2 and Siva-1 (Fig. 3D). The two tyrosines in Siva-1, Tyr^{53} and Tyr^{162}, that are phosphorylated by Tyk2 are not located in the N-terminus region (Fig. 3B and C), which is the domain that is necessary and sufficient to bind to Tyk2 (Fig. 2A and B). Therefore, it is reasonable to assume that the Tyk2-Siva-1 interaction is independent of Tyk2-mediated tyrosine phosphorylation of Siva-1.

Siva-1 itself induced apoptosis in Ba/F3 cells, and Tyk2 augmented the proapoptotic effects of Siva-1 in Ba/F3 cells (Fig. 4B–D). As a mutant Siva-1 that cannot bind to Tyk2 did not enhance apoptosis, even in the presence of Tyk2, the direct interaction between Siva-1 and Tyk2 plays a pivotal role in augmenting the apoptotic activity of Siva-1 (Fig. 4D). Contrary to our expectation, the phosphorylation of Siva-1 by Tyk2 has little effect on the augmentation of Siva-1-induced apoptosis (Fig. 4B and C). The association of Siva-1 with Tyk2, not the phosphorylation of Siva-1 by Tyk2, sufficiently promoted Siva-1-induced apoptosis. This differs from the ARG-Siva-1 interaction. The association between ARG and Siva-1 was dependent on ARG-mediated Siva-1 phosphorylation, and Siva-1-induced apoptosis was also dependent on ARG kinase activity [9]. Although both Tyk2 and ARG bound to Siva-1 and

promoted the Siva-1-induced apoptotic response, the molecular mechanisms involved in these two interactions appear to be different.

Several evidences have been accumulated that Siva-1 plays some role in apoptosis. ARG enhanced Siva-1-induced apoptosis [9]. The function of BCL-X_L, which is a well-known antiapoptotic protein, was inhibited in the presence of Siva-1 [10,11]. Recently, Gudi et al. showed that Siva-1 inhibits NF-κB activity, which is an essential pro-survival transcription factor that is activated upon TCR ligation [12]. Considering these reports as well as our results that Tyk2 augmented Siva-1-induced apoptosis, Siva-1 seems to have constitutive apoptotic activity that helps cells maintain the equilibrium between cell survival and programmed cell death (apoptosis). Furthermore, this apoptotic function of Siva-1 may be strengthen by its upstream partner molecule(s).

In conclusion, we have shown that Tyk2 specifically interacts with the proapoptotic protein Siva-1 via its N-terminus region and that Tyk2 augments the apoptotic effects induced by Siva-1 through the Tyk2-Siva-1 interaction independently of the phosphorylation of Siva-1 by Tyk2.

Acknowledgments

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References

[1] H. Steller, Mechanisms and genes of cellular suicide, Science 267 (1995) 1445–1449.
[2] M.D. Jacobson, M. Weil, M.C. Raff, Programmed cell death in animal development, Cell 88 (1997) 347–354.
[3] D.L. Vaux, S.J. Korsmeyer, Cell death in development, Cell 96 (1999) 245–254.
[4] A. Roulston, R.C. Marcellus, P.E. Branton, Viruses and apoptosis, Annu. Rev. Microbiol. 53 (1999) 577–628.
[5] R.W. Johnstone, A.A. Ruefli, S.W. Lowe, Apoptosis: a link between cancer genetics and chemotherapy, Cell 108 (2002) 153–164.
[6] K.V. Prasad, Z. Ao, Y. Yoon, M.X. Wu, M. Rizk, S. Jacquot, S.F. Schlossman, CD27, A member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein, Proc. Natl. Acad. Sci. USA 94 (1997) 6346–6351.
[7] Y. Yoon, Z. Ao, Y. Cheng, S.F. Schlossman, K.V. Prasad, Murine Siva-1 and Siva-2, alternate splice forms of the mouse Siva gene, both bind to CD27 but differentially transduce apoptosis, Oncogene 18 (1999) 7174–7179.
[8] S. Spinicelli, G. Nocentini, S. Ronchetti, L.T. Krausz, R. Bianchini, C. Riccardi, GITR interacts with the pro-apoptotic protein Siva and induces apoptosis, Cell Death Differ. 9 (2002) 1382–1384.
[9] C. Cao, X. Ren, S. Kharbanda, A. Koleske, K.V. Prasad, D. Kufe, The ARG tyrosine kinase interacts with Siva-1 in the apoptotic response to oxidative stress, J. Biol. Chem. 276 (2001) 11465–11468.
[10] L. Xue, F. Chu, Y. Cheng, X. Sun, A. Borthakur, M. Ramarao, P. Pandey, M. Wu, S.F. Schlossman, K.V. Prasad, Siva-1 binds to and inhibits BCL-X(L)-mediated protection against UV radiation-induced apoptosis, Proc. Natl. Acad. Sci. USA 99 (2002) 6925–6930.
[11] F. Chu, A. Borthakur, X. Sun, J. Barkinge, R. Gudi, S. Hawkins, K.V. Prasad, The Siva-1 putative amphipathic helical region (SAH) is sufficient to bind to BCL-XL and sensitize cells to UV radiation induced apoptosis, Apoptosis 9 (2004) 83–95.
[12] R. Gudi, J. Barkinge, S. Hawkins, F. Chu, S. Manicassamy, Z. Sun, J.S. Duke-Cohan, K.V. Prasad, Siva-1 negatively regulates NF-kappaB activity: effect on T-cell receptor-mediated activation-induced cell death (AICD), Oncogene 25 (2006) 3458–3462.
[13] F. Chu, J. Barkinge, S. Hawkins, R. Gudi, R. Salgia, P.V. Kanteti, Expression of Siva-1 protein or its putative amphipathic helical region enhances cisplatin-induced apoptosis in breast cancer cells: effect of elevated levels of BCL-2, Cancer Res. 65 (2005) 5301–5309.
[14] B. Py, C. Slomianny, P. Auberger, P.X. Petit, S. Benichou, Siva-1 and an alternative splice form lacking the death domain, Siva-2, Similarly induce apoptosis in T lymphocytes via a caspase-dependent mitochondrial pathway, J. Immunol. 172 (2004) 4008–4017.
[15] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (1998) 1305–1308.
[16] B.J. Padanilam, A.J. Lewington, M.R. Hammerman, Expression of CD27 and ischemia/reperfusion-induced expression of its ligand Siva in rat kidneys, Kidney Int. 54 (1998) 1967–1975.
[17] A. Henke, H. Launhardt, K. Klement, A. Stelzner, R. Zell, T. Munder, Apoptosis in coxsackie virus B3-caused diseases: interaction between the capsid protein VP2 and the proapoptotic protein Siva, J. Virol. 74 (2000) 4284–4290.
[18] A. Henke, M. Nestler, S. Strunze, H.P. Saluz, P. Hortschansky, B. Menzel, U. Martin, R. Zell, A. Stelzner, T. Munder, The apoptotic capability of coxsackie virus B3 is influenced by the efficient interaction between the capsid protein VP2 and the proapoptotic host protein Siva, Virology 289 (2001) 15–22.
[19] K. Mihatsch, M. Nestler, H.P. Saluz, A. Henke, T. Munder, Proapoptotic protein Siva binds to the muscle protein telethonin in cardiomyocytes during coxsackie viral infection, Cardiovasc. Res. 81 (2009) 108–115.
[20] K. Shimoda, K. Kato, K. Aoki, T. Matsuda, A. Miyamoto, M. Shibamori, M. Yamashita, A. Numata, K. Takase, S. Kobayashi, S. Shibata, Y. Asano, H. Gondo, K. Sekiguchi, K. Nakayama, T. Nakayama, T. Okamura, S. Okamura, Y. Niho, Tyk2 plays a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function, Immunity 13 (2000) 561–571.
[21] K. Shimoda, K. Kamesaki, A. Numata, K. Aoki, T. Matsuda, K. Oritani, S. Tamiya, K. Kato, K. Takase, R. Imamura, T. Yamamoto, T. Miyamoto, K. Nagafuji, H. Gondo, S. Nagafuchi, K. Nakayama, M. Harada, Cutting edge: tyk2 is required for the induction and nuclear translocation of Daxx which regulates IFN-alpha-induced suppression of B lymphocyte formation, J. Immunol. 169 (2002) 4707–4711.
[22] R. Gongora, R.P. Stephan, R.D. Schreiber, M.D. Cooper, Stat-1 is not essential for inhibition of B lymphopoiesis by type I IFNs, J. Immunol. 165 (2000) 2362–2366.
[23] M. Karaghiosoff, H. Neubauer, C. Lassnig, P. Kovarik, H. Schindler, H. Pircher, B. McCoy, C. Bogdan, T. Decker, G. Brem, K. Pfeffer, M. Muller, Partial impairment of cytokine responses in Tyk2-deficient mice, Immunity 13 (2000) 549–560.
[24] T. Haro, K. Shimoda, H. Kakumitsu, K. Kamezaki, A. Numata, F. Ishikawa, Y. Sekine, R. Muromoto, T. Matsuda, M. Harada, Tyrosine kinase 2 interacts with and phosphorylates receptor for activated C kinase-1, a WD motif-containing protein, J. Immunol. 173 (2004) 1151–1157.
[25] K. Shide, K. Shimoda, K. Kamezaki, H. Kakumitsu, T. Kumano, A. Numata, F. Ishikawa, K. Takenaka, K. Yamamoto, T. Matsuda, M. Harada, Tyk2 mutation homologous to V617F Jak2 is not found in essential thrombocythemia, although it induces constitutive signaling and growth factor independence, Leuk. Res. 31 (2007) 1085–1092.
[26] I. Firmbach-Kraft, M. Byers, T. Shows, R. Dalla-Favera, J.J. Krolewski, Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes, Oncogene 5 (1990) 1329–1336.

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ORIGINAL ARTICLE

Absence of gain-of-function JAK1 and JAK3 mutations in adult T cell leukemia/lymphoma

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Abstract Janus kinase 1 (JAK1) and JAK3 plays a critical role in lymphocyte proliferation and differentiation. Somatic JAK1 mutations are found in 18% of adult precursor T acute lymphoblastic leukemias and somatic JAK3 mutations are found in 3.3% of cutaneous T cell lymphomas. Some of the mutations are confirmed as a gain-of-function mutation and are assumed to be involved in leukemogenesis. Adult T cell leukemia/lymphoma (ATLL) is a type of T cell neoplasm, and activation of JAK/STAT pathways is sometimes observed in them. We investigated JAK1 and JAK3 mutations in 20 ATLL patients. No JAK1 mutations were found, and five types of single nucleotide polymorphisms were observed in 12 cases, whose frequencies almost match those in Asian populations. As for JAK3, a synonymous mutation was found in one case. JAK1 and JAK3 mutations are unlikely involved in the leukemogenesis of ATLL.

Keywords Adult T cell leukemia/lymphoma · JAK1 · JAK3 · Single nucleotide polymorphism

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1 Introduction

Adult T cell leukemia/lymphoma (ATLL) is an aggressive T cell neoplasm caused by human T cell leukemia virus type 1 (HTLV-1). Retroviral HTLV-1 is transmitted to neonatal $CD4^+$ T cells through breast-feeding by cell-to-cell transmission. Reverse transcriptase generates proviral DNA from genomic viral RNA, and the provirus is randomly integrated into the host genome by viral integrase. Viral proteins, such as p40tax (Tax), p27rex (Rex), or HTLV-1 bZip factor (HBZ) induce viral replication, DNA instability, and the acquisition of proliferation advantage or apoptotic resistance in HTLV-1-infected T cells [1, 2]. ATLL occurs after a 40–60 year period of latency in about 3–5% of HTLV-1-infected individuals [1]. The biological characteristics of ATLL cells include the following features: overexpression of cell membrane receptors and cytokines, such as IL-2R/IL-2 [3–5], aberrant activation of transcription factors, such as nuclear factor-κB (NF-κB) or signal transducer and activator of transcription (STAT) [6, 7] and genetic or epigenetic changes of cell cycle regulator genes, such as p53, p15, p14, and p16 [8, 9]. However, the mechanism underlying the development of ATLL is not yet fully understood.

Janus kinase (JAK) is a non-receptor tyrosine kinase that transduces cytokine signals for cellular proliferation, survival, and differentiation [10]. There are four members of JAKs (JAK1, JAK2, JAK3, and TYK2), and each JAK is specifically activated by a particular cytokine. JAK associates constitutively with a variety of cytokine receptors lacking intrinsic kinase activity, and propagates signal flow by phosphorylating tyrosyl residues of activated receptors to trigger the recruitment and activation of STATs [11]. The JAK/STAT signaling cascade is essential for cytokine signaling and blood cell development and activation.

JAK1-deficient mice are defective in T and B cells as a result of an impaired response to IL-7 [12], JAK2-deficient mice are defective in definitive erythropoiesis and die in utero due to an impaired response to erythropoietin, IL-3, GM-CSF, or SCF [13], and JAK3-deficient mice are defective in T, B, and NK-cells from an impaired response to IL-2, 7, 9, or 15 [14, 15]. These observations suggest that dysregulation of the JAK/STAT signaling pathway can lead to autonomous cell growth. In fact, somatically acquired genetic alterations of JAK family members and consequent aberrant activation of JAK/STAT pathways have been reported in some hematological malignancies. The TEL–JAK2 fusion gene is detected both in chronic myeloproliferative diseases and in acute lymphoblastic leukemias (ALL) [16, 17]. JAK1 mutations are detected in 18% of adult precursor T acute lymphoblastic leukemias (T-ALL) and a small fraction of acute myelogenous leukemias (AML) [18–20], JAK2 mutations are detected in 40–90% of myeloproliferative diseases [21, 22] and in 18% of Down's syndrome-associated ALL [23], and JAK3 mutations are detected in 10% of acute megakaryoblastic leukemias [24] and 3.3% of cutaneous T cell lymphomas [25]. The mutation-induced activation of JAK/STAT pathways and the acquisition of proliferation advantage are thought to play roles in the leukemogenesis of these diseases. Activation of JAK1 and JAK3 is observed in HTLV-1-infected T cell lines [26] or in uncultured leukemic cells from ATLL patients [7], we therefore examined the mutation of JAK1 and JAK3 in ATLL cells.

2 Materials and methods

2.1 Patients

ATLL patients whose percentage of peripheral abnormal lymphocytes was greater than 30% total cell count at the time of blood examination were sequentially enrolled into the study from 2000 to 2007. Profiles and clinical data for each case are summarized in Table 1. Diagnosis of ATLL was based on clinical features, cytologically proven mature T cell malignancy, the presence of anti-HTLV-1 antibody, and monoclonal integration of HTLV-1 proviral DNA into tumor cells confirmed by Southern blotting. This study was approved by the Institutional Review Board at Miyazaki University, and informed consent was obtained in accordance with the Helsinki Declaration.

Table 1 Profiles and clinical data for each ATLL case

Case no.	Age, years	Sex	WBC count, $\times 10^9$/L (5.0–9.0)	Lymphocyte count, $\times 10^9$/L	Abnormal lymphocyte (%)	LDH, IU/L (119–229)	Ca^{2+}, mg/dL (8.5–9.8)	sIL-2R, U/mL (145–519)	Phenotype of leukemic cells				Clinical type
									CD3 (%)	CD4 (%)	CD8 (%)	CD25 (%)	
1	27	F	2.8	2.8	35	1112	9	180000	84.3	84.5	0.8	81.5	Acute
2	35	F	13.8	13.4	59	801	10.4	45000	95.6	93.4	2	91.6	Acute
3	36	M	52.3	49.7	72	134	16.9	13100	81.4	72.8	19.3	30.3	Acute
4	37	M	24.7	22	47	375	13.4	76200	53.9	97.8	0.7	93.3	Acute
5	44	M	40.5	31	74	1120	9.6	46500	97.8	94.9	3.5	68.9	Acute
6	47	F	47.6	39.5	64	485	10.9	5540	89.4	95.3	1.1	55.5	Acute
7	51	F	68	42.8	53	827	9.7	16200	26.9	95.3	2.2	91.3	Acute
8	54	M	3.9	2.5	40	168	11.5	95900	70.3	53.4	21.3	41.5	Acute
9	55	M	16.7	13.7	30	457	11.2	71100	86.1	84.7	2.3	81.4	Acute
10	55	F	40.1	38.5	90	541	9.3	33300	24.9	92.7	2.9	66.3	Acute
11	57	F	12.3	8.6	47	249	9.2	5380	96.5	88.8	9.9	80.2	Acute
12	57	M	174	165.3	30	724	8.8	ND	99.7	98.7	2.4	79.1	Acute
13	63	M	9.5	6.3	39	183	9.7	3940	90.4	99.5	3.4	85.1	Chronic
14	69	M	31.3	26.6	68	700	9.2	34500	88.5	5.2	12.1	58.9	Acute
15	70	F	33.2	19.6	51	756	15.8	155000	95.3	97	0.9	91.2	Acute
16	80	M	85.6	68.1	56	1364	10.3	47700	96.3	93.5	0.6	70	Acute
17	83	M	117	88.9	57	616	12.9	111000	37.3	97.1	0.9	86.2	Acute
18	83	M	1.7	1.7	67	218	19.5	30700	97.2	98.9	1.4	96.2	Acute
19	84	F	27.9	19.8	55	369	11.1	ND	33.9	91.5	1.4	93.6	Acute
20	91	M	7.4	7.1	73	978	9.7	14400	96.5	97.1	1.3	96.7	Acute

Values in brackets refer to normal range

ND not done

2.2 Laboratory analysis of ATLL cells

Peripheral blood mononuclear cells (PBMCs) that contained more than 30% ATLL cells were isolated by density gradient centrifugation over Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden). Cells were frozen within 3 h, and cryopreserved at −80°C. To explore possible contributions of somatically acquired JAK1 and JAK3 mutations in ATLL, frozen samples of PBMCs collected from ATLL patients were thawed and genomic DNA was isolated using standard protocol. The coding sequence of the JAK1 gene (exons 2 through 25) and JAK3 gene (exons 2 through 24) was amplified by the polymerase chain reaction (PCR) method with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined by fluorescent dye chemistry sequencing with an ABI PRISM3000 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and analyzed with Sequencing Analysis software (Applied Biosystems, Foster City, CA, USA). The sequence of PCR primers for JAK1 was kindly provided by Dr. Marco Tartaglia (Istituto Superiore di Sanità, Roma, PhD) [18]. The sequence of PCR primers for JAK3 was designed as described in the previous report [27]. These primers were purchased from Hokkaido System Science Co., Ltd. By referencing the assembled sequence in the Ensembl genome database, the presence of mutations or single nucleotide polymorphisms (SNPs) was checked. To determine the sensitivity for the detection of JAK mutations, the calibrating cDNA samples mixture with murine wild-type (WT) JAK1 and mutant JAK1 (1970G>T in exon 14) cDNA were prepared. The mixing ratio of WT and mutant JAK1 was 5:5, 6:4, 7:3, 8:2, 8.5:1.5, 9:1, and 9.5:0.5; which represented the percentage of cells harboring heterozygous mutation 100, 80, 60, 40, 30, 20, and 10%, respectively. Each mixture cDNA sample was amplified by PCR and directly sequenced. As the mutant T peak was clearly detected in samples which mixing ratio of WT and mutant JAK1 cDNA was above 8.5:1.5, we determined that the sensitivity for the detection of mutation in this analysis was 30%.

3 Results

Twenty patients were enrolled in this study. The mean value of WBC and lymphocyte number was 40.5 × 10^9/L and 33.4 × 10^9/L, respectively. The percentage of abnormal lymphocytes in peripheral blood ranged from 30 to 90%, and the mean value was 55.4%. The mean value of LDH, Ca^{2+}, or soluble interleukin-2 receptor (sIL-2R) was 609 IU/L, 11.4 mg/dL, or 54748 U/mL, respectively. According to Shimoyama criteria [28], 19 cases were diagnosed as acute-type ATLL, with more than one of the following observations: LDH is greater than twice the normal upper limit, Ca^{2+} >11.0 mg/dL, or multiple organ ATLL involvement. One case, without elevation of LDH and Ca^{2+} and without multiple organ ATLL involvement, was diagnosed as chronic-type ATLL (Case 13). The surface markers of all but one abnormal PBMC were $CD3^+CD4^+CD8^-CD25^+$. In that one exception (Case 14), loss of CD4 expression was observed.

We examined the entire coding sequence of the JAK1 and JAK3 gene in 20 ATLL patients. As for JAK1, there were no missense or nonsense mutations in them. Five types of synonymous substitutions were found in 12 cases (Table 2). All synonymous substitutions were SNPs, as determined from referencing the base sequence in the Ensembl genome database (http://www.ensembl.org/Homo_sapiens/Transcript/Sequence_cDNA?db=core;g=ENSG00000162434;r=1:65071494-65204775;t=ENST00000342505). The genotype frequency of each SNP in the general Asian population is listed in Table 2 [29, 30]. In the ATLL patients examined, the genotype frequency (%) is c546 (rs11585932)-AA/AG/GG, 95/5/0; c1590 (rs2230586)-CC/CT/TT, 95/5/0; c2049 (rs2230587)-CC/CT/TT, 50/50/0; c2097 (rs3737139)-CC/CG/GG, 95/5/0; c2199 (rs2230588)-AA/AG/GG, 60/40/0 (Table 2). There is no statistical difference in genotype frequency pattern of these SNPs between the ATLL patients examined and the general Asian population on the Ensembl database. As for JAK3, there were no missense or nonsense mutations in them. We identified a synonymous substitution (c162C>T) in case 19.

4 Discussion

JAK/STAT pathways are crucial for signal transduction through the IL-2R family (IL-2R, 4R, 7R, 9R, 15R, 21R), which is primarily involved in growth and maturation of lymphoid cells. Upon binding of IL-2 to its receptor, Jak3 is recruited to IL-2R common γ-chain, while Jak1 is coupled to IL-2R β-chain [31]. In IL-2 signaling, these JAK kinases are activated and phosphorylate STAT3, STAT5A, and STAT5B. Phosphorylated STATs form homo- and hetero-dimers that translocate to the nucleus and bind to specific DNA sequences to initiate the transcription of IL-2 responsive genes, leading to T cell activation or proliferation [32]. JAK/STAT pathways work in a similar way in IL-7R signal transduction to promote T cell development [33]. Activation of JAK/STAT pathways is essential for lymphocyte proliferation, survival, and differentiation.

Constitutive activation of Jak1, Jak3, Stat1, Stat3, and Stat5 is observed in HTLV-1-infected T cell lines [26, 34] and in uncultured leukemic cells from ATLL patients [7].

Table 2 List of JAK1 polymorphisms identified in this study

Exon	cDNA position	Genotype	Case no.																			Frequency (%) in ATLL	Frequency (%) in Asian*
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
6	546	AA	○	○	○	○	○	○	○		○	○	○	○	○	○	○	○	○	○	○	○	95	100
		AG								○													5	0
		GG																					0	0
11	1590	CC	○	○	○	○	○	○	○		○	○	○	○	○	○	○	○	○	○	○	○	95	100
		CT								○													5	0
		TT																					0	0
15	2049	CC	○	○		○			○		○			○		○			○	○		○	50	49
		CT			○		○	○		○		○	○		○		○	○			○		50	36
		TT																					0	16
15	2097	CC	○	○	○	○	○	○	○		○	○	○	○	○	○	○	○	○	○	○	○	95	100
		CG								○													5	0
		GG																					0	0
16	2199	AA	○	○			○	○	○	○	○			○	○	○		○		○			60	38
		AG			○	○						○	○				○		○		○	○	40	50
		GG																					0	13

*Genotype frequency of c546, c2049 and c2097 is obtained from Japanese
Genotype frequency of c1590 and c2199 is obtained from Chinese

Migone et al. [26] demonstrated that constitutive activation of Jak1, Jak3, Stat3, and Stat5 correlated with IL-2-independence in HTLV-1-transformed T cell lines. Tomita et al. [35] also demonstrated that a JAK-specific inhibitor suppresses cell growth in HTLV-1-infected cell lines and primary ATLL cells. These reports suggest that activation of JAK/STAT pathways may play an important role in the leukemogenesis of ATLL. However, Zhang et al. [36] observed no detectable basal level phosphorylation of Jak3 and STAT5 in uncultured leukemic cells in ATLL patients. Cheng et al. [37] also demonstrated that constitutive activation of JAK/STAT pathways was not observed in freshly isolated $CD4^+$ T cells, but in HTLV-1-transformed T cell lines established 12 weeks post-infection. In HTLV-1-transformed T cell lines, Src homology 2 (SH2)-containing phosphatase 1 (SHP1), a negative regulator of the IL-2R signaling pathway, is downregulated, suggesting that constitutive activation of the JAK/STAT pathway might be a secondary phenomenon in vitro. Thus, a causal relationship between leukemogenesis and activation of JAK/STAT pathways in ATLL has not yet been established.

We investigated the acquisition of somatic mutations in JAK1 and JAK3 in ATLL. There were no missense or nonsense mutations in JAK1 and JAK3 in 20 ATLL patients. Recently, several somatically acquired JAK1 mutations have been reported in adult T-ALL. Flex et al. found six types of JAK1 mutation in 7 out of 38 T-ALL patients (18%) [18]. One mutation was located in the SH2 domain, two were in the pseudokinase domain, and three were in the kinase domain. All mutations were missense, and some of them were shown to induce the phosphorylation of JAK1 and STAT5 and lead to cytokine-independent proliferation. Somatically acquired JAK3 mutations have also been reported in hematological malignancies, such as Down syndrome (DS) and non-DS with acute megakaryoblastic leukemia (AMKL), DS-transient myeloproliferative disorder (DS-TMD) [24, 38, 39], and cutaneous T cell lymphoma (CTCL) [25]. The c1715C>T (A572V) in pseudokinase domain of JAK3 was identified in 1 out of 30 CTCL patients, and this mutation promoted lymphoproliferative disorders in murine bone marrow transplantation models [25]. These data suggest that dysregulation of JAK1 or JAK3 can be involved in the development or progression of T cell malignancy, such as T-ALL or CTCL. On the other hand, we did not find any gain-of-function JAK1 or JAK3 mutation in ATLL patients, although we did not have chance to examine the activation status of JAK/STAT signaling pathways in each case because of lack of fresh blood samples. JAK1 and JAK3 gain-of-function mutations might be unlikely to

be involved in the leukemogenesis of ATLL. Some SNPs in the regulatory region of TNFα or FAS (TNFRSF6/Apo-1/CD95), however, have been reported to associate with susceptibility to ATLL among HTLV-1 carriers [40, 41]. We only examined the coding region of JAK1 and JAK3 in this report, and the regulatory region of JAK1 and JAK3 remains to be investigated. Further investigation including downstream signaling molecules and inhibitory molecules in the JAK/STAT signaling pathway is necessary to clarify the mechanism contributing to the leukemogenesis of ATLL.

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Conflict of interest The authors declare no competing financial interests.

References

1. Mortreux F, Gabet AS, Wattel E. Molecular and cellular aspects of HTLV-1 associated leukemogenesis in vivo. Leukemia. 2003;17:26–38.
2. Yasunaga J, Matsuoka M. Leukaemogenic mechanism of human T-cell leukaemia virus type I. Rev Med Virol. 2007;17:301–11.
3. Maruyama M, Shibuya H, Harada H, Hatakeyama M, Seiki M, Fujita T, et al. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded p40x and T3/Ti complex triggering. Cell. 1987;48:343–50.
4. Siekevitz M, Feinberg MB, Holbrook N, Wong-Staal F, Greene WC. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. Proc Natl Acad Sci USA. 1987;84:5389–93.
5. Baba H, Yamada Y, Mori N, Hayashibara T, Harasawa H, Tsuruda K, et al. Multiple gammac-receptor expression in adult T-cell leukemia. Eur J Haematol. 2002;68:362–9.
6. Horie R. NF-kappaB in pathogenesis and treatment of adult T-cell leukemia/lymphoma. Int Rev Immunol. 2007;26:269–81.
7. Takemoto S, Mulloy JC, Cereseto A, Migone TS, Patel BK, Matsuoka M, et al. Proliferation of adult T cell leukemia/lymphoma cells is associated with the constitutive activation of JAK/STAT proteins. Proc Natl Acad Sci USA. 1997;94:13897–902.
8. Yamada Y, Kamihira S. Inactivation of tumor suppressor genes and the progression of adult T-cell leukemia-lymphoma. Leuk Lymphoma. 2005;46:1553–9.
9. Hofmann WK, Tsukasaki K, Takeuchi N, Takeuchi S, Koeffler HP. Methylation analysis of cell cycle control genes in adult T-cell leukemia/lymphoma. Leuk Lymphoma. 2001;42:1107–9.
10. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. Immunol Rev. 2009;228:273–87.
11. Murray PJ. The JAK-STAT signaling pathway: input and output integration. J Immunol. 2007;178:2623–9.
12. Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell. 1998;93:373–83.
13. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell. 1998;93:397–409.
14. Thomis DC, Gurniak CB, Tivol E, Sharpe AH, Berg LJ. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. Science. 1995;270:794–7.
15. Park SY, Saijo K, Takahashi T, Osawa M, Arase H, Hirayama N, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. Immunity. 1995;3:771–82.
16. Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science. 1997;278:1309–12.
17. Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood. 1997;90:2535–40.
18. Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, Knoops L, et al. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med. 2008;205:751–8.
19. Xiang Z, Zhao Y, Mitaksov V, Fremont DH, Kasai Y, Molitoris A, et al. Identification of somatic JAK1 mutations in patients with acute myeloid leukemia. Blood. 2008;111:4809–12.
20. Tomasson MH, Xiang Z, Walgren R, Zhao Y, Kasai Y, Miner T, et al. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. Blood. 2008;111:4797–808.
21. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7:387–97.
22. Levine RL, Pardanani A, Tefferi A, Gilliland DG. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nat Rev Cancer. 2007;7:673–83.
23. Bercovich D, Ganmore I, Scott LM, Wainreb G, Birger Y, Elimelech A, et al. Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome. Lancet. 2008;372:1484–92.
24. Walters DK, Mercher T, Gu TL, O'Hare T, Tyner JW, Loriaux M, et al. Activating alleles of JAK3 in acute megakaryoblastic leukemia. Cancer Cell. 2006;10:65–75.
25. Cornejo MG, Kharas MG, Werneck MB, Le Bras S, Moore SA, Ball B, et al. Constitutive JAK3 activation induces lymphoproliferative syndromes in murine bone marrow transplantation models. Blood. 2009;113:2746–54.
26. Migone TS, Lin JX, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G, et al. Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. Science. 1995;269:79–81.
27. De Vita S, Mulligan C, McElwaine S, Dagna-Bricarelli F, Spinelli M, Basso G, et al. Loss-of-function JAK3 mutations in TMD and AMKL of Down syndrome. Br J Haematol. 2007;137:337–41.
28. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). Br J Haematol. 1991;79:428–37.
29. Consortium TIH. The international HapMap project. Nature. 2003;426:789–96.
30. Consortium TIH. A haplotype map of the human genome. Nature. 2005;437:1299–320.
31. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science. 1994;266:1042–5.

32. Witthuhn BA, Silvennoinen O, Miura O, Lai KS, Cwik C, Liu ET, et al. Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature. 1994;370:153–7.
33. Zeng YX, Takahashi H, Shibata M, Hirokawa K. JAK3 Janus kinase is involved in interleukin 7 signal pathway. FEBS Lett. 1994;353:289–93.
34. Mulloy JC, Migone TS, Ross TM, Ton N, Green PL, Leonard WJ, et al. Human and simian T-cell leukemia viruses type 2 (HTLV-2 and STLV-2(pan-p)) transform T cells independently of Jak/STAT activation. J Virol. 1998;72:4408–12.
35. Tomita M, Kawakami H, Uchihara JN, Okudaira T, Masuda M, Matsuda T, et al. Inhibition of constitutively active Jak-Stat pathway suppresses cell growth of human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. Retrovirology. 2006;3:22.
36. Zhang Q, Lee B, Korecka M, Li G, Weyland C, Eck S, et al. Differences in phosphorylation of the IL-2R associated JAK/STAT proteins between HTLV-I(+), IL-2-independent and IL-2-dependent cell lines and uncultured leukemic cells from patients with adult T-cell lymphoma/leukemia. Leuk Res. 1999;23:373–84.
37. Cheng J, Zhang D, Zhou C, Marasco WA. Down-regulation of SHP1 and up-regulation of negative regulators of JAK/STAT signaling in HTLV-1 transformed cell lines and freshly transformed human peripheral blood CD4+ T-cells. Leuk Res. 2004;28:71–82.
38. Jeong EG, Kim MS, Nam HK, Min CK, Lee S, Chung YJ, et al. Somatic mutations of JAK1 and JAK3 in acute leukemias and solid cancers. Clin Cancer Res. 2008;14:3716–21.
39. Sato T, Toki T, Kanezaki R, Xu G, Terui K, Kanegane H, et al. Functional analysis of JAK3 mutations in transient myeloproliferative disorder and acute megakaryoblastic leukaemia accompanying Down syndrome. Br J Haematol. 2008;141:681–8.
40. Tsukasaki K, Miller CW, Kubota T, Takeuchi S, Fujimoto T, Ikeda S, et al. Tumor necrosis factor alpha polymorphism associated with increased susceptibility to development of adult T-cell leukemia/lymphoma in human T-lymphotropic virus type 1 carriers. Cancer Res. 2001;61:3770–4.
41. Farre L, Bittencourt AL, Silva-Santos G, Almeida A, Silva AC, Decanine D, et al. Fas 670 promoter polymorphism is associated to susceptibility, clinical presentation, and survival in adult T cell leukemia. J Leukoc Biol. 2008;83:220–2.

bjh research paper

JAK2 V617F uses distinct signalling pathways to induce cell proliferation and neutrophil activation

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Summary

The acquired *JAK2* V617F mutation is observed in the majority of patients with *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN). *BCR-ABL1* negative MPN displays myeloproliferation with an elevated leucocyte alkaline phosphatase (LAP) activity, a neutrophil activation marker. We tried to separate the downstream signalling of JAK2 V617F to stimulate myeloproliferation and LAP activity. NB4, a myeloid lineage cell line, was transduced with *Jak2* V617F mutation or wild-type *Jak2*. We found that *Jak2* V617F mutation, but not wild-type *Jak2* enhanced LAP expression in NB4-derived neutrophils and proliferation of NB4 cells. JAK2 V617F induces constitutive phosphorylation of STAT3 and STAT5, and uses signalling targets such as Ras/MEK/ERK and PI3K/Akt pathways. By using MEK1/2 inhibitor U0126, PI3K inhibitor LY294002, and STAT3 or STAT5 siRNAs, JAK2 V617F was found to specifically use the STAT3 pathway to enhance LAP expression, while STAT5, Ras/MEK/ERK and PI3K/Akt, but not STAT3 pathways, were able to stimulate cell proliferation. These data strongly suggest that JAK2 V617F uses distinct signalling pathways to induce typical pathological features of MPN, such as high LAP activity and enhanced cell proliferation.

Keywords: myeloproliferative neoplasm, JAK2 V617F, STAT3, STAT5, leucocyte alkaline phosphatase.

The *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN) include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Dameshek, 1951). The G-to-T point mutation at position 617 (V617F) in Janus kinase 2 (JAK2) that results in substitution of phenylalanine for valine has been reported in patients with *BCR-ABL1* negative MPN. This mutation is seen in approximately 95% of the patients with PV and in about 50% of the patients with ET or PMF (Baxter *et al*, 2005; James *et al*, 2005; Jones *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005). JAK2 is a member of the non-receptor tyrosine kinase family that signals between type I cytokine receptors and downstream pathways, such as STAT3 (signal transducers and activators of transcription 3) and STAT5 (Witthuhn *et al*, 1993; Parganas *et al*, 1998). These pathways are critical for cell growth and differentiation. The *JAK2* V617F mutation induces constitutive activation of the downstream signalling pathways such as STAT3/STAT5, Ras/MEK/ERK and PI3K/Akt: the expression of JAK2 V617F in cytokine-dependent cell lines confers cytokine-independent growth by constitutively activating STAT3, STAT5, Akt and ERK (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). The enforced expression of JAK2 V617F in murine bone marrow cells *in vivo* resulted in erythrocytosis and subsequent myelofibrosis in recipient mice (James *et al*, 2005; Lacout *et al*, 2006; Wernig *et al*, 2006; Zaleskas *et al*, 2006). Therefore, it is possible that the *JAK2* V617F mutation directly induces pathogenic features of *BCR-ABL1* negative MPN.

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Leucocyte alkaline phosphatase (LAP) is a monophosphoesterase that is the product of the gene encoding the liver/bone/kidney-type alkaline phosphatase (ALP) isoforms (Hayhoe & Quaglino, 1958; Weiss *et al*, 1988; Gianni *et al*, 1994; Sato *et al*, 1994). In neutrophils, LAP is stored in unique secretory vesicles, and is exported to the plasma membrane by chemotactic agents (Borregaard *et al*, 1987, 1992). Neutrophils play a critical role in host defence by phagocytozing and digesting microorganisms. This task is accomplished by mobilization of several types of neutrophil granules, including azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles. These granules contain potent antimicrobial compounds, proteolytic enzymes and also many membrane proteins. The azurophilic granules are formed during the promyelocytic stage, while specific granules and gelatinase granules are formed in more matured cells. Secretory vesicles appear in segmented neutrophils, and constitute a reservoir of membrane protein needed for neutrophil-mediated inflammatory response. Alkaline phosphatase is located at the luminal face of the membrane in secretory vesicles. Acquisition of LAP enzymatic activity is a very late event during myeloid maturation, and indicates that mature neutrophils acquire secretory vesicles. Thus, LAP is an indispensable marker of functionally mature neutrophils (Stewart, 1974; Garattini & Gianni, 1996; Borregaard & Cowland, 1997; Falanga *et al*, 2000; Faurschou & Borregaard, 2003). In clinics, the level of LAP in neutrophils is scored to evaluate neutrophil activation, which was classically designated as the neutrophil alkaline phosphatase (NAP) score (Kaplow, 1955, 1963; Shibata *et al*, 1985). Evaluation of the LAP score has been useful to distinguish chronic myeloid leukaemia (CML) from *BCR-ABL1* negative MPN, or from infection- or inflammation-associated leucocytosis: untreated CML patients usually have a low LAP score, whereas it is elevated in patients with PV, PMF and reactive leucocytosis.

In CML patients, the release of premature granulocytes from the bone marrow into the peripheral blood might result in low LAP scores (Dotti *et al*, 1999). It is, however, unclear why the LAP scores in patients with PV or PMF are elevated. Recent reports have shown that patients with *BCR-ABL1* negative MPN carrying the *JAK2* V617F mutation display higher LAP scores as compared to those carrying the wild-type *JAK2* alleles (Passamonti *et al*, 2006; Basquiera *et al*, 2007; Kondo *et al*, 2008). This finding suggests that an elevated LAP score in these diseases could be due to constitutive activation of the JAK2 signalling via the *JAK2* V617F mutation.

Neutrophil activation is the key feature of MPN as well as myeloproliferation. To understand the developmental mechanisms of MPN, we tried to separate the downstream signalling of JAK2 V617F in each pathway, and we used LAP expression as a neutrophil maturation/activation marker. The present study showed that the JAK2 V617F signalling stimulates LAP expression in neutrophils through specifically the activation of STAT3-dependent signalling pathway, whereas its stimulation of cell proliferation is dependent upon STAT5 activation. Thus, the acquisition of *JAK2* V617F at the haematopoietic stem cell (HSC) stage might induce at least two independent signals to alter myelopoiesis, including myeloproliferation and in elevation of the LAP score at the neutrophil stage, both of which are the pathological features specific to MPN.

Materials and methods

Cell lines and culture conditions

A human acute promyelocytic leukaemia (APL) cell line NB4, which retains t(15;17), was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). NB4 cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (ICN, Osaka, Japan). NB4 cells were differentiated into neutrophil-like cells after they were cultured with *all-trans* retinoic acid (ATRA) (10 μmol/l) (Sigma-Aldrich, St. Louis, MO, USA) and granulocyte colony-stimulating factor (G-CSF) (10 ng/ml) (Sigma-Aldrich) for 4 d (Gianni *et al*, 1994). Cells were counted by trypan blue dye exclusion staining.

Preparation of neutrophils from patients and healthy donors

This study was approved by Institutional Review Board of Kyushu University and all patients gave informed consent. Heparinized peripheral blood samples were collected from 15 healthy volunteer donors, 15 patients with PV, 21 patients with ET, and nine patients with PMF according to the World Health Organization criteria. Neutrophils were isolated by dextran sedimentation, hypotonic lysis of contaminating erythrocytes, and centrifugation with lymphocyte separation medium (LSM) (MP Biomedicals, Irvine, CA, USA) as described previously (Sullivan *et al*, 1984).

Mutation analysis and DNA sequencing of JAK2

DNA from patient's neutrophils was extracted using the Blood and Cell Culture DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To examine the putative *JAK2* V617F mutation, exon 14 of JAK2 was amplified using 5′-TATAGTCATGCTGAAAGTAGG-3′ and 5′-TAACTGAATAGTCCTACAGTG-3′ primers as described previously (Shide *et al*, 2007). The polymerase chain reaction (PCR) products were sequenced directly using an ABI3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Furthermore, when the *JAK2* V617F mutation was not detected by direct sequencing, we used the three-primer allele-specific PCR assay as described previously (Baxter *et al*, 2005).

Evaluation of LAP expression

Leucocyte alkaline phosphatase expression was evaluated by LAP score, flow cytometric analysis, or ALP enzyme assay.

Leucocyte alkaline phosphatase scores were measured by the LAP staining kit (Muto, Tokyo, Japan) according to the manufacturer's instructions (Sakamoto, 1966; Shibata *et al*, 1985).

Flow cytometric analysis was used for the analysis of cell surface expression of LAP (sLAP). Isolated neutrophils were washed once in phosphate-buffered saline (PBS) (Wako) and stained with phycoerythrin (PE)-conjugated anti-human LAP (BD PharMingen, San Diego, CA, USA) or PE-conjugated mouse $IgG_1\kappa$ (isotype-matched negative control) (BD PharMingen). The mean fluorescent intensity (MFI) of sLAP was used as the LAP expression value as described previously (Shibano *et al*, 1999).

In the ALP enzyme assay, LAP expression in 5×10^5 NB4-derived neutrophils or neutrophils was measured by the SensoLyte™ p-Nitrophenyl phosphate (pNPP) alkaline phosphatase assay kit (Ana Spec, San Jose, CA, USA) according to the manufacturer's instructions.

Jak2 lentiviral infection

Murine *Jak2* cDNA was kindly provided by Dr J. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA). Oligonucleotide-directed mutagenesis was performed to substitute phenylalanine for valine at residue 617 of *Jak2* as described previously (Shide *et al*, 2007). For the wild-type *Jak2* or the *Jak2* V617F construct, each of the cDNAs were cloned downstream of an E1Fα promoter in a third-generation lentiviral vector backbone consisting of a reporter gene encoding GFP driven by the promoter of the human gene that encodes phosphoglycerate kinase. The control vector CEP contained only the gene encoding GFP. Vector construction was confirmed by sequencing. Viruses 'pseudotyped' with the vesicular stomatitis virus G protein were produced by transient transfection of 293T cells as described previously (Takenaka *et al*, 2007) and were concentrated by ultracentrifugation. Functional titres of virus producing wild-type JAK2 or JAK2 V617F, as determined by infection of HeLa cells, were 1×10^7 or 2×10^7 infectious particles/ml, respectively. Virus, at a multiplicity of infection of 40–50, was added to 24-well tissue culture plates of NB4 cells. Infected cells were collected on day 3. The efficiency of gene transfer into cells was 20–45%, as assessed by GFP fluorescence performed using a FACSCalibur flow cytometer (Becton Dickinson [BD] Biosciences, San Jose, CA, USA). GFP^+ cells were sorted by a FACS Aria cell sorter (BD Biosciences) and then the GFP^+ NB4 cells were cloned at one cell per well into 96-well tissue culture plates.

Inhibitions of JAK2, Ras/MEK/ERK and PI3K/Akt pathways by protein kinase inhibitors

We investigated the effects of protein kinase inhibitors on LAP expression in neutrophils/NB4-derived neutrophils, and on proliferation of NB4 cells. Protein kinase inhibitors used to suppress JAK2 and its downstream pathways were as follows: AG490 (Calbiochem, San Diego, CA, USA) was used as a JAK2 inhibitor at a concentration of 5–50 μmol/l, U0126 (Calbiochem) as a MEK1/2 inhibitor at a concentration of 1–20 μmol/l, and LY294002 (Calbiochem) as a PI3K inhibitor at a concentration of 5–50 μmol/l.

Leucocyte alkaline phosphatase expression was evaluated by flow cytometric analysis after neutrophils were cultured with/without protein kinase inhibitors in the presence of G-CSF (50 ng/ml) for 24 h.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml), and protein kinase inhibitors for 4 d.

The proliferation of NB4 cells with medium alone or protein kinase inhibitors was evaluated for 4 d.

Inhibitions of STAT3/STAT5 pathways by small interfering RNAs (siRNAs)

To investigate the role of the STAT3 and STAT5 pathways in LAP expression in NB4-derived neutrophils and in proliferation of NB4 cells, STAT3 or STAT5 expression in NB4 cells was knocked down using double-stranded siRNAs. The siRNAs to knock down human STAT3, STAT5a and STAT5b were obtained from Invitrogen (Carlsbad, CA, USA). NB4 cells were transiently transfected with siRNAs using Nucleofector technology (Amaxa Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Non-specific siRNA (Invitrogen) was used as a negative control.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d.

The proliferation of NB4 cells with medium alone was evaluated for 5 d.

Western blotting

Total cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride nitrocellulose membranes (Amersham, Uppsala, Sweden) as described previously (Shide *et al*, 2008). Membranes were probed using the appropriate antibodies and visualized by enhanced chemiluminescence (Amersham). Anti-phospho Tyr^{705} STAT3, anti-phospho Ser^{727} STAT3, anti-phospho Tyr^{694} STAT5, anti-phospho Thr^{202}/Tyr^{204} ERK1/2, anti-ERK1/2, anti-phospho-Ser^{473} Akt, anti-Akt and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-JAK2, anti-STAT3, anti-STAT5a and anti-STAT5b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Real-time quantitative PCR

Total RNA was extracted from NB4 cells. Reverse transcription was performed using a high-capacity cDNA

reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Power SYBR Green Master Mix (Applied Biosystems) and subsequent primers were used for real-time quantitative PCR amplification of the murine *Jak2* cDNA, using 5′-AAGTGGAGCTTCGGGAC-CACTCT-3′ and 5′-GCTTATCTTCATAGAACTGCAGC-3′ primers on an ABI 7000 sequence detection system (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SAS or JMP software (SAS Institute Inc., Cary, NC, USA). LAP expressions among the groups were compared by Tukey's test or Dunnett's test. For inhibitor experiments, LAP expressions were compared using the least-squares mean test or paired *t*-test. For siRNAs experiments, LAP expressions were compared by paired *t*-test. For cell proliferation experiments, data were plotted on a single logarithmic graph and approximated by the straight line from the origin for each group. The gradients of the straight lines among the groups were compared by Tukey's test. Differences were considered statistically significant when the *P*-value was <0·05.

Results

Neutrophils with JAK2 V617F mutation express high levels of LAP in all types of MPN

The presence of the *JAK2* V617F mutation was documented by direct sequencing, or by allele-specific PCR of isolated neutrophils' DNA from *BCR-ABL1* negative MPN patients ($n = 45$). Patients were judged to be homozygous for the *JAK2* V617F mutation if their mutational load exceeded 50%. *JAK2* V617F mutation was identified in 13 of the 15 PV patients, 11 of the 21 ET patients and five of the nine PMF patients. Among them, five PV patients, eight ET patients and five PMF patients carried heterozygous mutations.

We analysed LAP expression of isolated neutrophils in *BCR-ABL1* negative MPN patients ($n = 45$) and healthy volunteer donors ($n = 15$) by flow cytometric analysis (Fig 1). *JAK2* V617F positive MPN patients ($n = 29$) had a higher LAP expression than *JAK2* V617F negative MPN patients ($n = 16$) or than healthy controls ($n = 15$) ($P < 0·01$). Among *JAK2* V617F positive MPN patients, those carrying homozygous mutation ($n = 11$) had higher levels of neutrophil LAP than those carrying heterozygous mutation ($n = 18$) ($P < 0·01$). Patients carrying heterozygous mutation also displayed higher LAP expression than healthy controls ($P < 0·05$), although the difference between *JAK2* V617F negative patients and patients carrying heterozygous mutation was not statistically significant. These data suggest that the expression level of JAK2 V617F correlates with the LAP levels in MPN neutrophils.

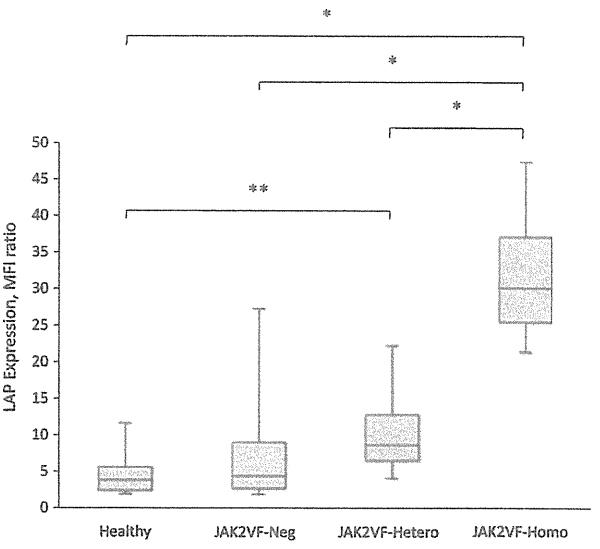

Fig 1. LAP expression of neutrophils in healthy volunteer donors ($n = 15$), *JAK2* V617F negative MPN (JAK2VF-neg, $n = 16$), *JAK2* V617F heterozygous MPN (JAK2VF-hetero, $n = 18$), and *JAK2* V617F homozygous MPN (JAK2VF-homo, $n = 11$). LAP levels were evaluated by flow cytometric analysis. LAP expression (ratio of mean fluorescence intensity, MFI) is shown in a box plot. JAK2VF-homo patients had a significantly higher level of LAP than those in the other three groups. *$P < 0·01$; **$P < 0·05$.

AG490, a JAK2 inhibitor, suppresses LAP expression in neutrophils with JAK2 V617F

To test whether the high level of LAP in *JAK2* V617F positive neutrophils is dependent upon JAK2 activation, we evaluated the effect of AG490, a JAK2 inhibitor, on LAP activity in *JAK2* V617F positive neutrophils. Neutrophils were purified from MPN patients with homozygous *JAK2* V617F, and were cultured for 24 h in the presence of AG490. AG490 significantly suppressed the LAP expression (Fig 2), confirming that the expression of LAP is dependent upon JAK2 activation in *JAK2* V617 positive neutrophils.

The enforced expression of JAK2 V617F stimulates the expression of LAP

To verify that JAK2 V617F signalling activates the expression of LAP in myeloid cells, we used NB4, a myeloid lineage cell line. NB4 was infected with lentiviral vectors harbouring wild-type *Jak2* or *Jak2* V617F mutation with GFP reporters. Infected cells were purified by positive expression of GFP by FACS. NB4 cells transfected with wild-type *Jak2* and *Jak2* V617F mutation showed an increased expression of JAK2 protein as compared to control by Western blotting, but their levels did not differ between these *Jak2*- or *Jak2* V617F-transduced NB4 clones (Fig 3A).

NB4 cells differentiated into neutrophil-like cells in the presence of ATRA and G-CSF for 4 d (Gianni *et al*, 1994). The NB4-derived neutrophils were then evaluated for LAP

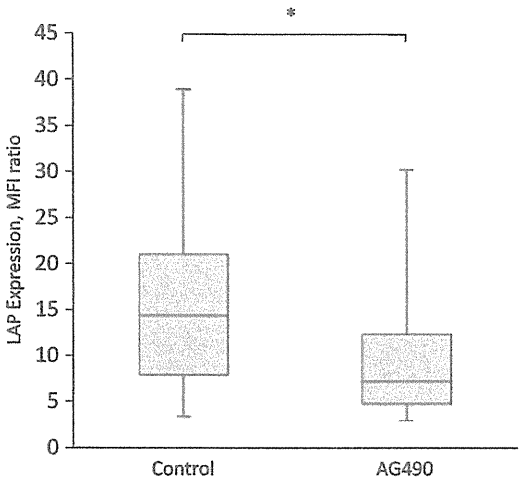

Fig 2. Effect of the AG490, a JAK2 inhibitor, on LAP expression in *JAK2* V617F positive neutrophils. Neutrophils from *JAK2* V617F positive MPN patients (n = 16) were cultured in medium in the presence of G-CSF (50 ng/ml), and the effect of addition of AG490 (50 μmol/l) on LAP expression was evaluated 24 h after culture on a flow cytometry. LAP expression (ratio of MFI) is shown in a box plot. AG490 suppressed LAP expression. *P < 0·01.

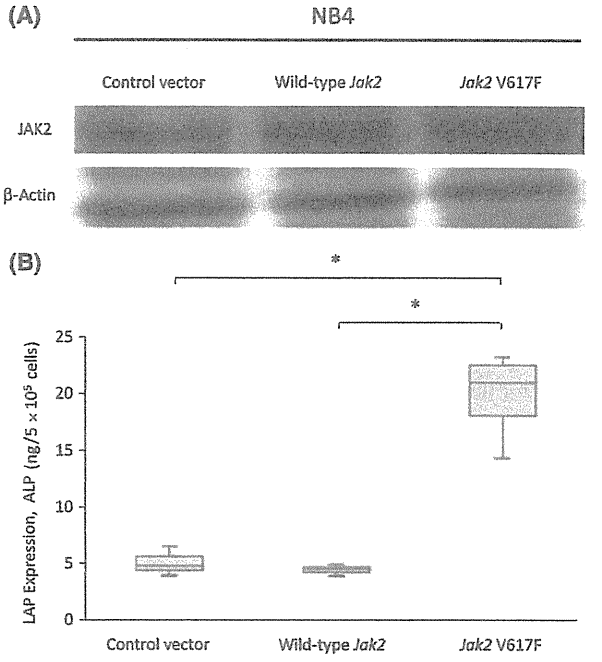

Fig 3. (A) JAK2 protein expression in NB4 cells by Western blotting. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were lysed and analysed by Western blotting with the indicated antibodies. Expression of JAK2 protein in wild-type *Jak2* and *Jak2* V617F-transduced NB4 cells was equivalent, but higher than that in the control vector-transduced cells. (B) Effect of the *Jak2* V617F mutation on LAP expression in NB4-derived neutrophils. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d and differentiated into neutrophil-like cells. LAP expression was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. *Jak2* V617F-transduced NB4-derived neutrophils had a higher LAP expression than the other two groups. *P < 0·01.

expression. We measured LAP levels in neutrophils differentiated from *Jak2* V617F clones, from wild-type *Jak2* clones, and from control clones with an empty vector by ALP enzyme assays. Representative data are shown in Fig 3A, B. Neutrophils from *Jak2* V617F clones expressed LAP at a much higher level, as compared to those from wild-type *Jak2* clones, whereas the expression levels of JAK2 in these clones were equivalent (P < 0·01) (Fig 3A, B). Thus, JAK2 V617F signalling is able to stimulate LAP expression in neutrophils.

STAT3 but not STAT5 signalling is required for JAK2 V617F-dependent LAP expression

JAK2 V617F mutation constitutively activates multiple JAK2 downstream signalling molecules, including STAT3/STAT5, Ras/MEK/ERK and PI3K/AKt (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). We therefore tried to identify the signalling pathway responsible for LAP expression. We first tested the requirement of STAT3 or STAT5 for LAP expression stimulated by JAK2 V617F in *Jak2* V617F-transduced NB4 cells after inducing neutrophil lineage differentiation by ATRA and G-CSF. STAT3 and STAT5 expression were inhibited by STAT3 and STAT5 siRNAs respectively, in NB4-derived neutrophils: *Jak2* V617F-transduced NB4 cells were transduced with STAT3 or STAT5 siRNA, and cultured with ATRA and G-CSF for 4 d. As shown in Fig 4A, C, STAT3 siRNA, but not STAT5 siRNA, inhibited LAP expression (P < 0·05). Western blot analysis showed that STAT3 and STAT5 siRNAs specifically inhibited the expression of STAT3 and STAT5 respectively, but did not inhibit the signalling molecules on Ras/MEK/ERK and PI3K/Akt pathways (Fig 4B, D). These data suggest that STAT3 is indispensable for LAP expression in JAK2 V617F signalling.

The LAP expression is dependent upon the MEK, but not the PI3K signalling pathway

We then tested the effects of MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) on LAP expression in *Jak2* V617F-transduced NB4 cells. LAP expression was evaluated by ALP enzyme assay. U0126 but not LY294002 significantly suppressed LAP expression in *Jak2* V617F-transduced NB4-derived neutrophils (Fig 5A, C). Further analysis of signalling molecules, such as ERK and Akt, downstream of MEK and PI3K respectively, revealed that U0126 and LY294002 efficiently inhibited phosphorylation of ERK and Akt respectively (Fig 5B, D).

Activated JAK2 transduces the signal cascade through phosphorylation of both the receptors and the major substrates, STATs. STAT3 is phosphorylated by JAK2 on a single tyrosine at position 705. This phosphorylated STAT3 is