capping に関わるタンパクであり，細胞骨格のダイナミックな変化に重要な働きを担っている。したがって、MDS の特徴である血球形態異常、好中球遊走能の低下は CapG の異常による可能性がある。ちなみに CapG ノックアウトマウスでは好中球遊走能の低下が認められている。また、CapG はホスファチジルイノシトール 4,5-二リン酸と結合し、イノシトールリン脂質代謝の制御に関わっており、クロマチン再構築、転写機構、アポトーシスシグナル、小胞輸送などさまざまな細胞機能に影響を与え、血球の増殖、分化を制御している可能性がある。以上より CapG の異常は MDS の病因、病態に深く関与していることが示唆される。

MDS に高発現していた蛋白である TSA はペルオキシレドキシンに属する蛋白であり，活性酸素種(ROS)消去に重要な役割を担っている。MDS の血球はさまざまなサイトカイン(TNF α など)により酸化ストレスを受け,ROS が増加し、それが無効造血を来たす一因となっていると可能性がある。したがって，それらの酸化ストレスに対するフィードバック機構として TSA の発現が増加している可能性が考えられ、このような ROS 消去因子を増強することにより、本症の血球減少を改善できる可能性があると思われる。

E. 結論

MDS 患者好中球において特異的に異常発現している蛋白を同定した。これらの蛋白はMDSの病因あるいは病態に深く関与していると推測され、その解析は MDS に対する分子標的療法の開発につながる可能性があると考える。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

CGHアレイを用いたMDSの原因遺伝子の探索

分担研究者　小川誠司　東京大学大学院医学系研究科　21世紀COEプログラム　特任助教授

研究要旨

本分担研究では、骨髄異形成症候群(MDS)におけるゲノム変異のゲノムワイドな解析を通じてMDSの分子病態の解明と新規治療標的分子の同定を目指している。本年度の研究では、高密度SNPアレイを用いた従来のコピー数解析に加えて、新たに開発したアレイデータ解析アルゴリズムにより、MDSゲノムにおけるコピー数の異常をアレル特異的に解析することにより、従来MDSでは検出が不可能であったコピー数異常を伴わないアレルのホモ接合(UPD)を鋭敏に解析することにより、MDSにおける病型特異的なUPDの集積領域を同定し、これらの領域のからMDS特異的に活性化変異を生ずる遺伝子を複数同定した。さらに、MDSの近縁疾患である骨髄増殖性疾患(MPD)についてSNPアレイを用いた同様の解析を行うことにより、1pにおけるUPDがcMPLの活性化型変異と関連して、MPDおよびMDSの独立した病型を規定していることを明らかにするとともに、cMPLを介したシグナル伝達系が、MPDに対する新たな分子標的薬剤のターゲットとなりうることを示した。

A. 研究目的

骨髄異形成症候群(MDS)に対しては、現在、造血幹細胞移植以外には根治的治療法がなく、今後、副作用の少ない有効な治療法を確立するためには、本疾患の原因分子の同定とこれを標的とした分子標的薬の開発が重要である。MDSは造血前駆細胞におけるゲノム異常の蓄積によって発症すると考えられるが、RASやAML1など一部の例外を除いて、未だこうしたゲノム異常の標的となっている遺伝子に関する知見は限られている。本分担研究では、昨年度に確立した高密度SNPアレイを用いたmolecular allelokaryotypingにより、ゲノムに生ずるコピー数の異常およびアレル不均衡を網羅的に探索することにより、MDSの発症に関わる標的遺伝子の同定を試みる。

B. 研究方法

(1) 高密度SNPアレイによるMDS症例のMolecular allelokaryotyping

SNPアレイを用いた解析では、ゲノムコピー数の変化がアレル特異的に測定されている。そこで自己正常DNAが得られる場合には、正常組織のアレル組成をコントロールとして腫瘍組織におけるアレル特異的なコピー数の定量が可能であるが、自己正常試料の得にくいMDSのような疾患では、従来アレル特異的なコピー数の解析が不可能であった。そこで我々は、腫瘍組織に混入する正常組織からのシグナルを利用して、腫瘍DNAのSNP特異的シグナルにおける微細なひずみを検出することにより、自己正常試料の得られない腫瘍試料で鋭敏にアレルの不均衡を検出する方法(AsCNAR)を開発し、これを用いて153検体のMDS試料における従来のゲノムコピー数の解析に加えて、アレル不均衡のゲノム網羅的な解析を行った(Molecular allelokaryotyping)。

(2) SNPアレイを用いたMPDにおけるMolecular allelokaryotyping

MPDは一部のMDSとの病態が類似しており、MDSの近縁疾患であると考えられる。そこで、種々のMPDの病型、すなわち、真性多血症(PV)、特発性骨髄線維症(IMF)および本態性血小板血症(ET)を含む、53例のMDSについて、同様に高密度SNPアレイを用いたMolecular allelokaryotypingを行うことにより、MPDにおけるゲノムワイドなコピー数異常ならびにアレル不均衡の解析を行うとともに、MDSにおけるゲノム変異との比較解析を行った。

(3) MDSおよびMPDにおけるUPDのゲノムワイドな分布の解析と新規分子標的の探索

MDSおよびMPDにおいてUPDが集積する領域については、UPDの標的となる候補の遺伝子について、HPLCを用いたhetero-duplex法および直接塩基配列決定法により、腫瘍特異的な変異のスクリーニングを行った。

(倫理面への配慮)

検討に用いた検体は、当該患者からインフォームドコンセントを得たのちに連結可能匿名化を施して検討に用いた。東京大学医学部の設置する倫理委員会の承認済みである。

C. 結果

MDSにおけるMolecular alllokaryotypingにより、従来の解析技術では検出が不可能であった多数のアレル不均衡、とくにゲノムコピー数変化を伴わないヘテロ接合性の

消失(LOH)、いわゆる UPD が多数同定された。すなわち、このような UPD は MDS の約 30%の症例で認められ、またこれらの UPD は 1p, 1q, 4q, 7q, 9p, 11q, 17q, 21q などの特定の染色体領域に集積する傾向が認められた。Knudson による two hit theory によれば、UPD は癌抑制遺伝子の二つのアレルが不活化される重要なメカニズムの一つとされているが、実際、今回の MDS においても 17qUPD および 21qUPD においては、高率に p53 および AML1 遺伝子のホモ変異が同定され、これらの遺伝子が 17q および 21q の UPD の標的遺伝子であることが示されたが、興味深いことに、1q および 14q の UPD においては、N-Ras および FLT3 遺伝子の活性化型変異がホモ接合を示していること、また、9p の UPD においては、近年 MPD の原因遺伝子変異として同定された JAK2 変異がホモ接合を生じていることがしめされ、UPD が癌抑制遺伝子のみならず癌遺伝子のさらなる活性化のメカニズムになっていることが示唆された。

さらに、我々は MDS において高度に UPD が集積する領域の解析から、従来報告されていなかった新たな癌遺伝子の変異を同定した。当該変異は、同染色体部位の UPD に完全に一致して検出され、MDS の約 5%の症例に認められた。また、これらの症例の多くは、巨視的な染色体変化の乏しい CMMoL の病型に属しており、当該遺伝子変異は、MDS における新たな亜系を規定する遺伝子と考えられた。また、これまでの解析によりば、同遺伝子変異は、機能的には、チロシンキナーゼ pathway の増強に働くと推定され、また、変異は特定のドメインに集中していることから、今後本分子を標的として新たな低分子阻害剤の開発が期待される。

一方、MPD における SNP アレイ解析では、20q における欠失をのぞき、ゲノムコピー数の変化が集積する領域は殆ど検出されなかった。これは、5q および 7q の欠失に代表される不均衡型の変化が主体をなす MDS と顕著に異なる点である。一方、UPD については、従来報告のある 9p への高度の集積と 1p への集積が認められた。注目すべきことに、9p における UPD は JAK2 のホモ変異の頻度から推定されるより遙かに高い頻度で認められたことで、これは、既存の解析では JAK2 変異の多くがリンパ球などの混入によりヘテロ接合と判定されていたことを強く示唆する。我々の解析では、80%におよぶ正常細胞の混入下においても、正確に UPD を検出することが可能で、今回の高い解析精度により、JAK2 変異を有する MPD 症例の多くで、実際には 9 番染色体短腕の mitotic recombination により、JAK2 変異をホモに有する腫瘍集団が生じていることが明らかとなった。また、JAK2 のホモ変異は PV および IMF のほぼ全例に生じているのに対して、ET における JAK2 変異は殆どがヘテロ接合となっていることが明らかとなった。他方、1p の UPD については、その全例で cMPL 遺伝子におけるホモ変異が同定された。変異は 2 カ所に生じており、また、4 症例の全例が IMF の病型を示していた。

D. 考察

本年度の研究で我々が開発したアレル特異的なコピー数の定量法(AsCNAR)は、自己正常 DNA に依存することなく、LOH が解析可能であるという点において、画期的な方法である。これはまた、高密度 SNP アレイと我々の開発したプログラムを用いることにより、今や分子レベルで、腫瘍細胞ゲノムの量的・質的異常がゲノムワイドに解析可能となったことを意味している(Molecular allelokaryotyping)。

さらに、高度の正常細胞の混入した腫瘍検体においても高感度にアレルの不均衡を検出することが可能である。殆どの腫瘍試料では正常細胞の混入が生じていること、また一般には正常組織を採取することが容易ではないことから、腫瘍ゲノムの解析において極めて広範な応用が可能である。

本法を用いて、我々は本研究課題である MDS の網羅的なゲノム解析を行い、UPD という観点から、MDS の特定の病型を定義することが可能であることを示した。さらに、これらの一つについては、その分子=遺伝子標的を同定することに成功した。これらは MDS の 5%の症例に見いだされる活性化型の変異で、当該分子の特定のドメインに生じていることから、これらの変異を標的とした新規分子標的治療薬の開発が強く期待されるところであり、現在のマウスモデルを用いた機能的な解析とともに、同変異分子を阻害する低分子薬剤の開発を視野にいれたさらなる解析を進めている。

一方、MPD における UPD の解析からは、その発症メカニズムに関する興味深い知見が得られた。MPD における JAK2 のホモ変異は従来報告されているよりも遙かに高頻度に生じており、また、いくつかの症例では、異なる切断点を有する UPD クローンが検出されていることが明らかとなった。近年、多数の BFU コロニーの解析により、我々と同様の観察結果が得られて

いるが、これらの観察結果は、JAK2 変異が MPD のクローン選択において極めて強力に作用していること、また腫瘍の発症において、ある変異が次ぎの変異の腫瘍集団への固定をどのように促進するかを示している。JAK2 のホモ変異は PV および IMF では実質的にほぼ全例に認められるが、ET では変異は殆どヘテロ接合である。このことは、JAK2 変異のアレルの状態が病型を規定している可能性を示唆しており、今後の解析が興味深い。

9pUPD と関連した JAK2 のホモ接合型変異は、MPD における顕著な特徴となっているが、MPD の他の subset では、同様の現象が 1pUPD における cMPL の変異を伴って生じていることが今回の解析で初めて明らかとなった。これらの症例は全例 IMF であり、1pUPD/cMPL 変異は、MPD の明確な一病型を規定している可能性が類推される。さらに、今後 cMPL を標的とした新たな分子標的治療薬の開発が期待されるところである。

E. 結論

我々が独自に開発した高密度 SNP アレイを用いた Molecular allelokaryotyping の手法を用いて、MDS および MPD の分子病態を明らかにするとともに、新たな分子標的治療薬開発のターゲットとなる新規分子の同定に成功した。本研究の成果を踏まえて、今後民間製薬企業との連携を含めた、MDS に対する画期的な治療法の開発が期待される。

F. 健康危険情報

なし

G. 研究発表

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H．知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

III.　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表（論文）

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TEL/ETV6 induces apoptosis in 32D cells through p53-dependent pathways

Tetsuya Yamagata, Kazuhiro Maki, Kazuo Waga, Kinuko Mitani *

Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi 321-0293, Japan

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Abstract

TEL is an ETS family transcription factor that is critical for maintaining hematopoietic stem cells in adult bone marrow. To investigate the roles of TEL in myeloid proliferation and differentiation, we introduced *TEL* cDNA into mouse myeloid 32Dcl3 cells. Overexpression of TEL repressed interleukin-3-dependent proliferation through blocking cell cycle progression. Also, the presence of TEL triggered apoptosis through the mitochondrial intrinsic pathway on exposure to granulocyte colony-stimulating factor. We found an increase in p53 protein and its DNA binding in the TEL-overexpressing cells. Forced expression of TEL stimulated transcription via the p53-responsive element and increased the expression of cellular target genes for p53 such as cell cycle regulator *p21* and apoptosis inducer *Puma*. Consistently, induction of apoptosis was delayed by pifithrin-α treatment and completely blocked by increased expression of Bcl-2 in the TEL-overexpressing cells. These data collectively suggest that TEL exerts a tumor suppressive function through augmenting the p53 pathway and facilitates normal development of myelopoiesis.

Keywords: Apoptosis; 32D; ETS; Granulocyte colony-stimulating factor; Mitochondria; p21; p53; Pifithrin-α; Puma; TEL; Transcription factor

The *TEL* gene, mapped at 12p13, was originally cloned as a fusion partner for the *platelet-derived growth factor receptor β* gene in the t(5;12)(q33;p13) translocation that was found in chronic myelomonocytic leukemia [1]. TEL is a member of the ETS family of transcription factors that acts as a transcriptional repressor [2,3]. The helix–loop–helix (HLH) domain at the N-terminus is necessary for homodimerization [4] and heterodimerization with other ETS family members such as FLI-1 [5] and TEL2 [6]. The ETS domain at the C-terminus is responsible for DNA binding to the ETS-binding consensus site (EBS) that contains a purine-rich GGAA/T core motif [2]. TEL is known to associate with mSin3A and N-CoR through the HLH and central repression (amino acids 268–303) domains, respectively [7]. TEL is reported to bind directly to histone deacetylase (HDAC)-3 through its internal domain, which is located between the HLH and ETS domains. Through interacting with HDAC, directly or indirectly, TEL is believed to mediate transcriptional repression of genes such as *FLI-1* [2], *Id1* [8], *stromelysin-1* [9], and *Bcl-X_L* [10]. TEL's transcriptional modulator capacity is negatively regulated through phosphorylation on specific serine residues by mitogen-activated protein kinase family member p38 and ERK [11,12].

TEL is widely expressed in embryonic and adult tissues. TEL is dispensable for fetal hematopoiesis in the yolk sac and the liver, but is critical for post-natal hematopoiesis in the bone marrow [13,14]. Moreover, a recent conditional knock-out study demonstrated that TEL plays a role in maintaining hematopoietic stem cells in adult bone marrow [15]. For lineage-specific differentiation, TEL accelerates erythroid but represses megakaryocytic differentiation [16,17]. TEL's *in vitro* function in the myeloid lineage has not yet been reported. Besides its function as a hematopoietic regulator, TEL is reported to have a tumor suppressor function. Overexpression of TEL in Ras-transformed

* Corresponding author. Fax: +81 282 86 5630.
E-mail address: kinukom-tky@umin.ac.jp (K. Mitani).

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NIH3T3 cells inhibits cell growth in liquid and soft agar cultures, and suppresses tumor formation in nude mice [9,18].

To investigate whether TEL plays a role in proliferation and/or differentiation of myeloid progenitors, we used 32Dcl3 cells that proliferate in the presence of interleukin-3 (IL-3) and differentiate along the myeloid lineage on granulocyte colony-stimulating factor (G-CSF) treatment [19]. Overexpression of TEL in 32Dcl3 cells impaired IL-3-driven cell proliferation and caused apoptosis on G-CSF treatment. We observed strong induction of p53 protein and augmentation of its downstream targets. We also showed that inhibition of the p53 pathway blocks TEL-induced cell death. These data indicate that TEL works upstream of p53 and stimulates apoptosis of immature myeloid cells.

Materials and methods

Plasmid construction. The expression plasmids pME18S-FLAG-TEL, pCXN2-FLAG-TEL, and pCXN2-FLAG-ΔHLH-TEL [16], and the reporter plasmids WT-2xRGC-Luc and MT-2xRGC-Luc [20,21] have been described previously. TheΔETS-TEL fragment from pME18S-FLAG-ΔETS-TEL [16] was inserted into the *Eco*RI site of pCXN2 to generate pCXN2-FLAG-ΔETS-TEL. The Bcl-2 coding region was excised from pUC-CAGGS-human Bcl2 and introduced into the *Eco*RI site of pCAGIPuro.

Cell culture. Parental 32Dcl3 cells and derivative clones were maintained in RPMI1640 medium supplemented with 10% FCS and 0.25 ng/ml of murine recombinant IL-3 or 10 ng/ml of human recombinant G-CSF (Kirin, Gunma, Japan). Five micrograms per milliliter of cycloheximide (Sigma, St. Louis, MO) was added to the culture to inhibit protein synthesis. The specific p53 inhibitor pifithrin-α (Sigma) was used at a concentration of 0.25 or 1 μM.

Isolation of 32D stable transfectants. To establish stable transfectants overexpressing wild-type TEL, ΔHLH-TEL or ΔETS-TEL, 1×10^7 cells were electroporated with 20 μg cDNA cloned into a pCXN2 plasmid carrying the Neo^R gene at 380 V and 975 μF using a Gene Pulser (BIO-RAD, Hercules, CA). Transfected cells were then selected with 0.8 mg/ml of G418 (Sigma), and limiting dilution was performed to obtain stable clones. The resultant stable clone overexpressing wild-type TEL was further electroporated with 20 μg of pCAGIPuro-Bcl-2 carrying the $puromycin^R$ gene, and selected with 0.5 μg/ml of puromycin (Sigma), and limiting dilution was performed to isolate stable double transfectants.

Northern analysis. To obtain a *p53* probe, a mouse *p53* cDNA fragment of 404 bp was amplified by PCR using a primer set (5′-AACCGCC GACCTATCCTTAC-3′ and 5′-TATGGCGGGAAGTAGACTGG-3′). Northern analysis was performed as described previously [17].

Western analysis. Cells were harvested and lysed in RIPA buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 2.5 mM EDTA, 0.8 mM PMSF, and 2000 U/ml Ulinastatin). Western analysis was performed as described previously [12] with primary antibodies against FLAG-tag (Sigma), MDM2, p53, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected using the ProtoBlot AP system (Promega, Madison, WI).

Cell cycle analysis. At indicated time points, 5×10^5 cells were harvested, washed with PBS, fixed, and stained with 50 μg/ml of propidium iodide (PI) at room temperature for 10 min using Cycle TEST™ PLUS (Becton, Dickinson and Company, New Jersey). The DNA content of nuclei was determined with a flow cytometer. Percentages of apoptotic cells were evaluated as the sub-G1 fraction using Cell Quest (Becton–Dickinson and Company).

Annexin V/PI staining. At indicated time points, 5×10^5 cells were harvested, washed with PBS, and then subjected to annexin V/PI staining using an Annexin V/FITC Kit (Bender MedSystems, Vienna). Percentages of apoptotic cells were detected as the annexin V(+)/PI(−) fraction in the flow cytometer and analyzed with CellQuest software.

Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$). At indicated time points, 5×10^5 cells were harvested, washed with PBS, and incubated with 1 μl/ml of JC-1 and JC-9 dyes using JC-1 and JC-9 Mitochondrial Potential Sensor Kit (Molecular Probe, Eugene, Oregon) at 37 °C for 10 min in the dark. Fluorescence emission shift from red to green was evaluated with a flow cytometer.

EMSA. Electrophoretic mobility shift assay (EMSA) was performed as described previously [16] with [α-^{32}P]dCTP-labeled p53 oligonucleotide (5′-TACAGAACATGTCTAAGCATGCTG-3′). In competition studies, a 100-fold molar excess of unlabeled p53 oligonucleotides (a specific competitor) or mutant p53 oligonucleotides (a non-specific competitor; 5′-TACAGAATCGCTCTAAGCATGCTG–3′) was added to the reaction mixture. Anti-p53 antibody (Santa Cruz Biotechnology) was used for super-shift assay.

Luciferase assay. Exponentially growing parental 32Dcl3 cells were electroporated with 10 μg of the reporter plasmid alone or together with 10 μg of p53 expression plasmid [21], or 5, 10 or 15 μg of TEL expression plasmid (pME18S-FLAG-TEL) filled up with pME18S at 380 V and 970 μF using a Gene Pulser (BIO-RAD). Luciferase assay was performed as described previously [12].

Quantitative RT-PCR analysis. Cells were exposed to G-CSF and total RNA was isolated at times indicated. The genomic DNA was digested before reverse transcription. Specific mRNA was amplified using the SYBER Green amplification system and detected by the ABI Prism 7700 real-time PCR system (Applied Biosystems, CA). The primer sequences are: *p53*: 5′-GTAAACGCTTCGAGATGTTCC-3′ and 5′-GACTGGCC CTTCTTGGTCT-3′, *p21*: 5′-CTGTCTTGCACTCTGGTGTCT-3′ and 5′-GGCACTTCAGGGTTTTCTCT-3′, *Puma*: 5′-GTGACCACTGGCA TTCATTT-3′ and 5′-CCTGACTCCCCATCTTCCT-3′, *BAX*: 5′-ACAG GGGCCTTTTTGCTAC-3′ and 5′-GAGACACTCGCTCAGCTTCTT-3′, *BID*: 5′-GAGATGGACCACAACATCCA-3′ and 5′-AGGCTGTCTT CACCTCATCAA-3′, *BAK*: 5′-TACCTCCACCAGCAGGAAC-3′ and 5′-GACCCACCTGACCCAAGA-3′, and *HPRT*: 5′-CTCTCGAAGTG TTGGATACAG-3′ and 5′-ACAAACGTGATTCAAATCCC-3′.

Results

Overexpression of wild-type TEL in 32Dcl3 cells suppresses cell growth in the presence of IL-3 and provokes rapid cell death on G-CSF treatment

We first examined whether TEL plays any role in myeloid cell proliferation and/or differentiation using the murine IL-3-dependent myeloid cell line 32Dcl3. We established several stable cell lines overexpressing wild-type TEL by transfecting FLAG-tagged TEL expression plasmid (pCXN2-FLAG-TEL). Western analysis with anti-FLAG antibody showed that clones T-1 and T-3 expressed high levels of TEL proteins (Fig. 1A). Two clones transfected with the empty plasmid were used as mock-transfected controls (M-2 and M-3). In parental cells, endogenous TEL proteins were not detected in the Western analysis (data not shown). In the presence of IL-3, the two mock clones showed almost the same rates of cell proliferation and viability as the parental 32Dcl3 cells (data not shown). Notably, the growth rates of the TEL-overexpressing cells were compromised compared with those of the mock cells (Fig. 1B). On withdrawal of IL-3, all clones started to deteriorate and completely lost their viability within 2 days, indicating

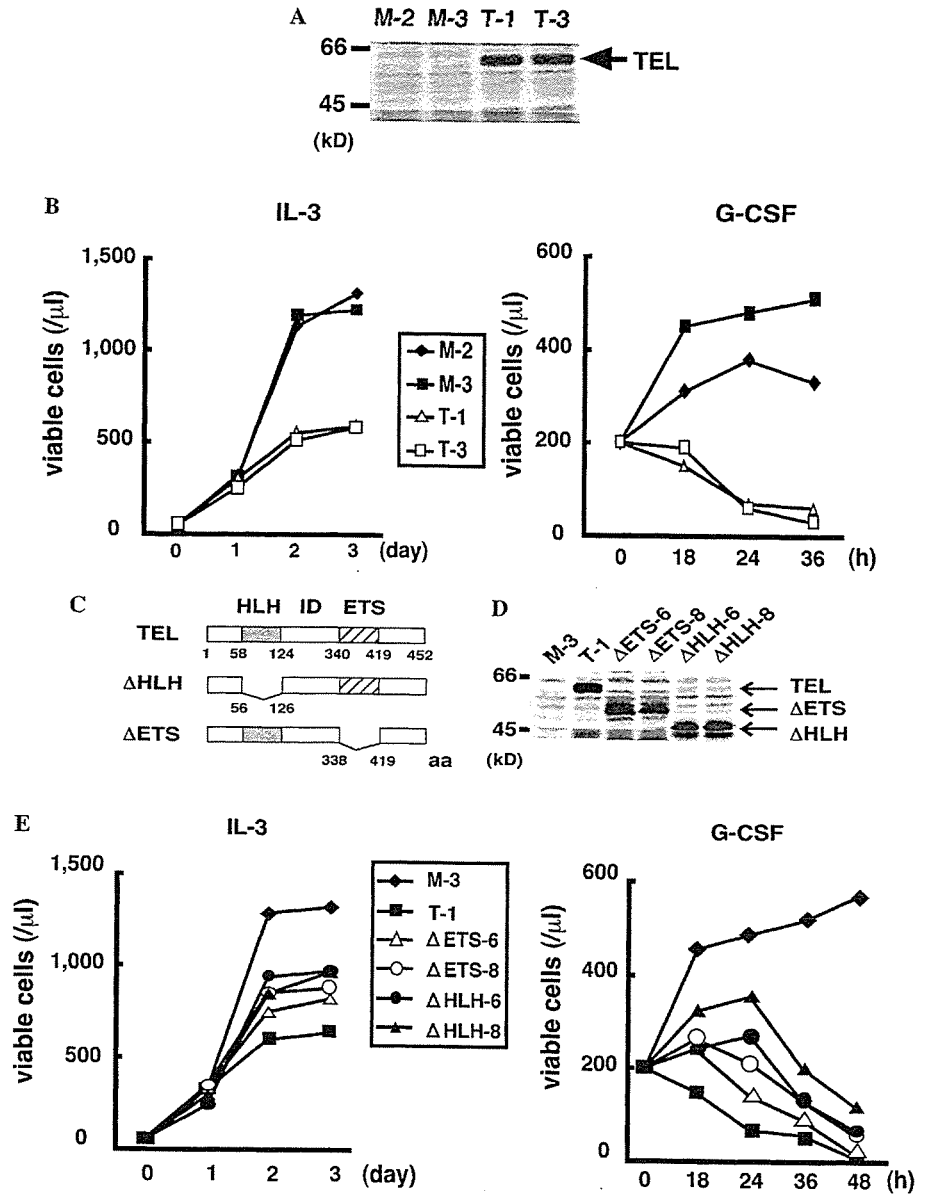

Fig. 1. Overexpression of wild-type TEL causes rapid cell death in 32D cells on G-CSF exposure. (A) Western analysis of 32D clones stably overexpressing wild-type TEL (T-1 and T-3) and mock controls (M-2 and M-3). Arrow indicates overexpressed wild-type TEL detected by anti-FLAG antibody. (B) Time course analysis of viable cell counts in the mock and TEL-overexpressing clones in the presence of IL-3 (in the left panel) or G-CSF (in the right panel). Experiments were repeated three times and reproducible results were obtained. Representative data are shown. (C) Structures of wild-type TEL and the two deletion mutants (ΔHLH-TEL and ΔETS-TEL). The HLH and ETS domains are shown by shaded and hatched boxes, respectively. Numbers indicate amino acids. (D) Western analysis of 32D clones stably overexpressing wild-type TEL (T-1), ΔETS-TEL (ΔETS-6 and ΔETS-8) or ΔHLH-TEL (ΔHLH-6 and ΔHLH-8) and a mock control (M-3) with anti-FLAG antibody. Arrows indicate overexpressed wild-type TEL, ΔETS- and ΔHLH-TEL. (E) Analysis of viable cell counts in M-3, T-1, ΔETS-6, ΔETS-8, ΔHLH-6, and ΔHLH-8 in the presence of IL-3 (in the left panel) or G-CSF (in the right panel). Experiments were repeated three times and similar results were obtained. Representative data are shown.

their dependence on continuous signaling from the IL-3 receptor (data not shown). When IL-3 was replaced with G-CSF, the mock clones underwent differentiation into mature granulocytes, similar to the parental 32Dcl3 cells. In contrast, the TEL-overexpressing clones showed progressive cell death and completely lost their viability within 2 days. We also introduced mutant forms of *TEL* cDNA, *ΔHLH-TEL* and *ΔETS-TEL*, into parental 32Dcl3 cells (Fig. 1C). These cells expressed amounts of the protein similar to those of T-1 (Fig. 1D). The growth rates of the mutant clones were lower than those of the mock cells but higher than those of the wild-type TEL-expressing cells (Fig. 1E). When the ΔHLH-TEL- or ΔETS-TEL-overexpressing cells were treated with G-CSF, their death kinetics fell between those of the mock and wild-type TEL-overexpressing cells. These data suggest that overexpressed TEL suppresses the growth of 32Dcl3 cells in the presence of IL-3 and causes rapid cell death on G-CSF exposure, which partially depend on both the HLH and the ETS domains.

Overexpression of TEL in 32Dcl3 cells stimulates apoptosis on G-CSF treatment

To determine whether this negative effect of wild-type TEL on cell survival after G-CSF treatment is due to induction of apoptosis, we performed DNA content analysis after 12, 24, and 36 h incubation in medium containing G-CSF. Before G-CSF treatment (in the presence of IL-3 only), the G2/M fraction was lower in clones T-1 and T-3 than in clones M-2 and M3 (Fig. 2A), suggesting that TEL has some inhibitory role in cell cycle progression from G0/G1 to the S phase. The occurrence of apoptosis was unlikely in T-1 and T-3 in the presence of IL-3, since we did not observe any increase in the sub-G1 fraction in such conditions. After G-CSF exposure, however, the percentage of the sub-G1 fraction was higher in T-1 and T-3 than in M-2 and M-3 at all the time points examined. Double staining with annexin V and PI was performed to detect early apoptotic cells. Compared with the mock clones, T-1 and T-3 exhibited rapid onset and a higher percentage

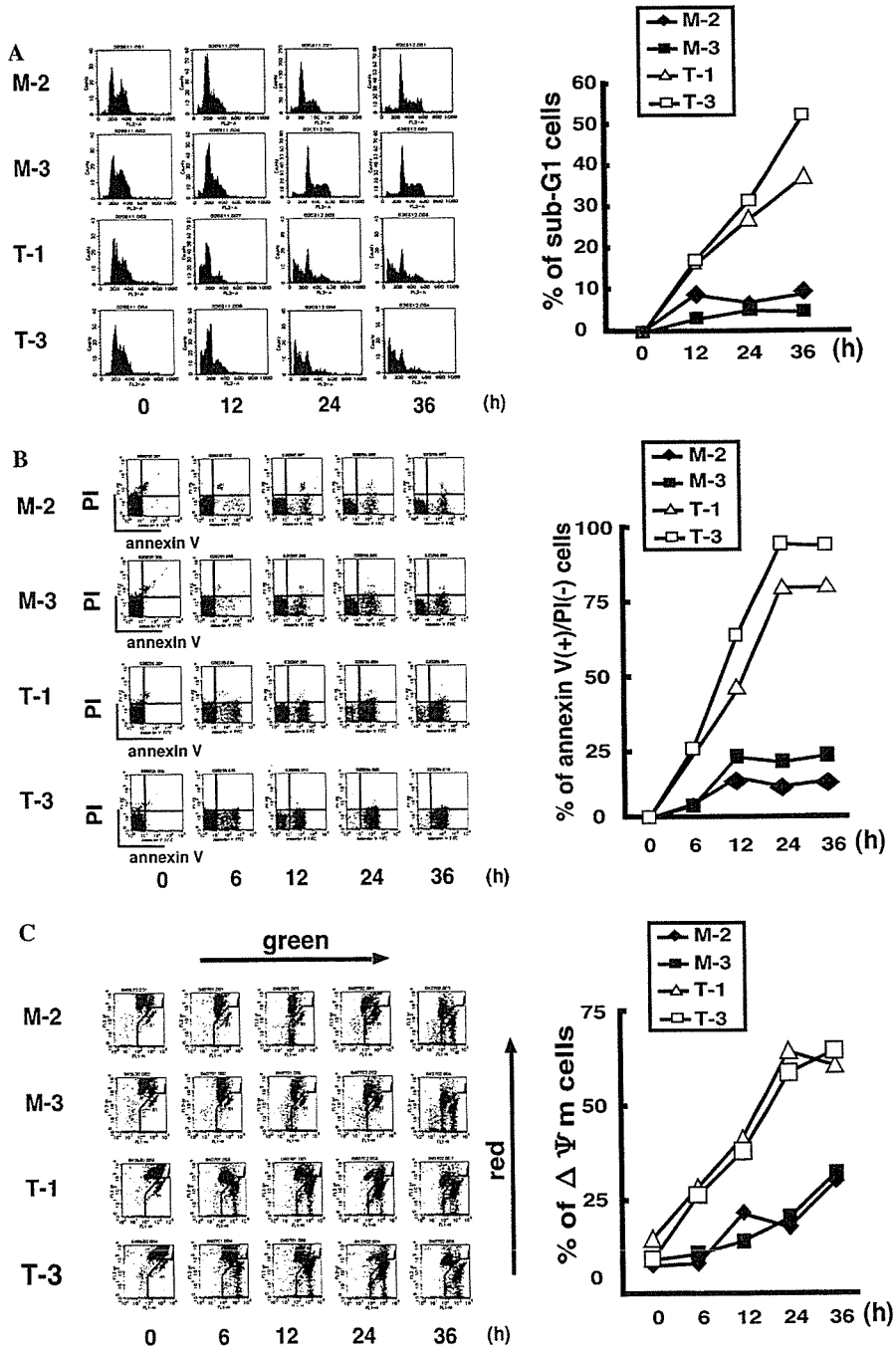

Fig. 2. TEL induces apoptosis in 32D cells under G-CSF treatment. The dead cell fraction was determined using DNA-content analysis (A) or annexin V(+)/PI(−) staining. (B) At indicated time points, cells were stained with PI alone or FITC-conjugated annexin V(+) PI and analyzed in a flow cytometer. (C) TEL-induced apoptosis is associated with loss of mitochondrial membrane potential. At indicated time points, cells were stained with JC-1. Percentages of cells in the lower right fraction were evaluated.

of the early apoptotic fraction (annexin V(+)/ PI(−) quadrant) during G-CSF treatment (Fig. 2B). Thus, TEL enhances apoptotic cell death in 32Dcl3 cells treated with G-CSF.

Wild-type TEL induces apoptosis through mitochondrial depolarization

JC-1 is a cell-permeable cationic dye that selectively accumulates in the polarized mitochondria of live cells but not in the depolarized mitochondria of apoptotic cells [22]. Accumulation of JC-1 in the mitochondria is detected by red fluorescence, which represents aggregation of the dye, while its decomposition, yielding a fluorescence shift from red to green, indicates loss of mitochondrial membrane potential and subsequent apoptosis. Thus, we performed JC-1 staining of the clones in the presence of G-CSF. The ratios of cells showing a shift from red to green after 6-h incubation were much higher in T-1 and T-3 than in the mock cells (Fig. 2C). Thus, overexpression of TEL elicits a drop in the mitochondrial transmembrane potential in G-CSF-treated 32Dcl3 cells. These data indicate that TEL induces apoptosis in 32Dcl3 cells through mitochondrial membrane depolarization.

Overexpression of TEL increases expression of p53

To obtain a molecular insight into the induction of apoptosis, we analyzed the protein levels of Bcl-2 family members. However, expression levels of Bcl-2, Bcl-xL, Bax, Bad, and Noxa were comparable between the mock and TEL-overexpressing cells (data not shown). Surprisingly, we noticed up-regulation of p53 protein [23] in the TEL-overexpressing cells in the presence of either IL-3 or G-CSF (Fig. 3A). The rise in p53 protein level was associated with a small but apparent increase in its mRNA level in both Northern and quantitative PCR analyses (Figs. 3B and 4), suggesting that up-regulation of p53 protein is partly due to an increase of its transcription in TEL-overexpressing cells. This is supported by a rapid decline in

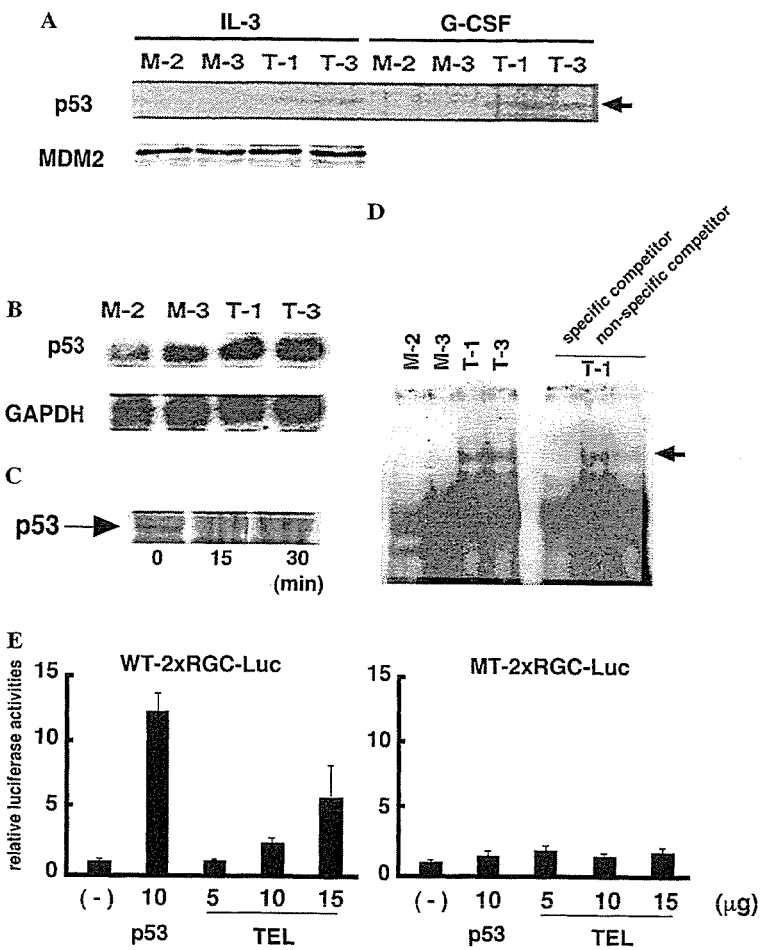

Fig. 3. p53 is up-regulated in TEL-overexpressing cells. (A) Analysis of p53 protein in the mock (M-2 and M-3) and TEL-overexpressing clones (T-1 and T-3) cultured in the presence of IL-3 or G-CSF. Cell lysates were subjected to Western analysis with anti-p53 or anti-MDM2 antibody. (B) Total RNA was subjected to Northern analysis using mouse *p53* cDNA probe. (C) The cells were cultured in the presence of IL-3 with or without 5 μg/ml of cycloheximide for 15 or 30 min. p53 protein was monitored using anti-p53 antibody. (D) EMSA using a specific p53-binding sequence. Arrow indicates specific p53/DNA complex. (E) Parental 32D cells were electroporated with 10 μg of wild-type (WT) or mutated (MT) 2xRGC-Luc reporter plasmid with or without 10 μg of p53 expression plasmid, or 5, 10 or 15 μg of TEL expression plasmid. The results are presented as relative luciferase activity.

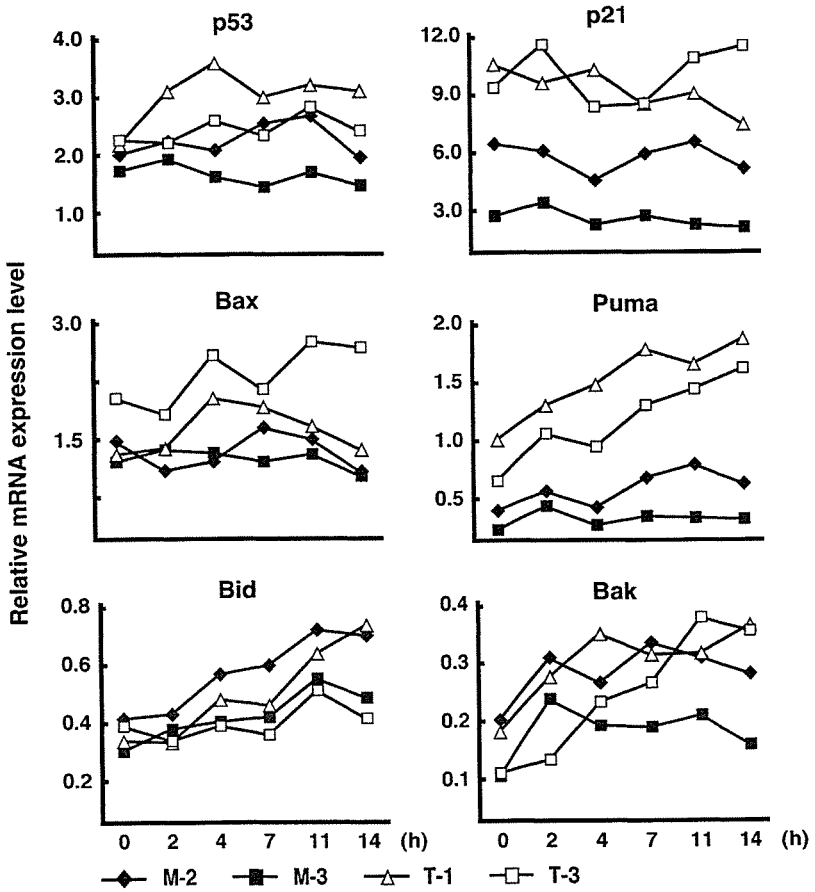

Fig. 4. Genes downstream of p53 are induced in TEL-overexpressing cells. The mock (M-2 and M-3) and TEL-overexpressing (T-1 and T-3) clones were exposed to G-CSF for indicated times and total RNA was isolated. The mRNA of the indicated genes was analyzed by quantitative real-time PCR and normalized against the HPRT mRNA level. BAK is not reported as a p53 target gene.

p53 protein levels after cycloheximide treatment ($T_{1/2}$: less than 7.5 min) (Fig. 3C), which indicates that most of the existing p53 protein is newly synthesized from its mRNA. We also looked at MDM2 protein, which causes ubiquitination and subsequent degradation of p53. Expression of MDM2 protein in the TEL-overexpressing cells was similar to that in the mock transfectants (Fig. 3A). These data suggest that the rise in p53 protein in TEL-overexpressing cells is likely to be due to translation from the increased mRNA rather than stabilization of the protein.

To compare the DNA-binding properties of p53 in the mock and TEL-overexpressing cells, nuclear extracts were prepared from each population and subjected to EMSA using radioactive p53-specific oligonucleotide probe. Consistent with the results of Western analysis, binding of p53 to the specific probe was markedly enhanced in the TEL-overexpressing cells (Fig. 3D). This binding was specific, since the protein–DNA complex was completely canceled by addition of specific cold competitors but not by non-specific competitors. Also, the binding of p53 to the specific probe was canceled by addition of anti-p53 antibody. Thus, overexpression of wild-type TEL results in increased protein levels and subsequent DNA binding of p53. We next investigated whether the increased level of p53 protein would enhance the activity of the p53-dependent reporter plasmid. For this purpose, we chose the WT-2xRGC-Luc reporter, which contains two binding sites for p53, from the ribosomal gene cluster [21]. We transfected WT-2xRGC-Luc together with p53 or wild-type TEL expression plasmid into 32Dcl3 cells and evaluated luciferase activity. Co-transfection of p53 expression plasmid resulted in an approximately 12-fold increase in luciferase activity (Fig. 3E). Interestingly, co-transfection of TEL also activated the reporter in a dose-dependent manner. This activity was completely blocked in reporter plasmid mutated at the p53-binding sites (MT-2xRGC-Luc). This result suggests that potential p53 target genes are activated in wild-type TEL-overexpressing cells.

The p53 target genes are activated in wild-type TEL transfectants

The above results suggest that overexpression of wild-type TEL increases the level of p53 protein, which in turn enhances expression of various p53 target genes involved in cell cycle arrest or apoptosis. To test this hypothesis, we looked at expression levels of p53 target genes in the TEL-overexpressing cells and mock transfectants after

G-CSF treatment. We examined the kinetics of p53 target gene expression after G-CSF exposure, reasoning that the increased level of p53 would elicit a more sensitive response to p53 upstream stimuli, so that the transcription of p53 target genes would increase. Thus, we treated cells with G-CSF and isolated their RNAs at different time points. The expression levels of representative p53 target genes were quantified using real-time PCR. The level of p53 mRNA was higher in the TEL transfectants than in the mock-transfected cells before G-CSF induction (0 h), and this tendency remained throughout the time course (Fig. 4). Thus, the *p53* gene is not induced on G-CSF treatment. Interestingly, the mRNA of p21, the major cell cycle inhibitor downstream of p53, was consistently higher in the TEL-overexpressing cells than in the controls. Also, the mRNA of *Puma*, a pro-apoptotic member of the Bcl-2 family critical for initiation of apoptosis [24], was dramatically induced after G-CSF treatment in the TEL-overexpressing cells. The basal level of *Puma* mRNA was high in T-1 and T-3, but the induction was more significant in these cells than in the controls. Other p53 target genes such as *Bid* and *Bax* showed no significant difference between the TEL-overexpressing and mock-transfected cells. Also, the expression of Bak, a pro-apoptotic gene that is independent of p53, remained the same in the two populations. Thus, induction of *Puma* is likely to be responsible for the death of TEL-expressing cells after G-CSF treatment.

Both transcription-dependent and -independent pathways of p53 contribute to cell death in TEL-expressing cells

The up-regulation of *p21* and *Puma* mRNA in TEL-expressing cells suggests that a transcription-mediated effect of p53 plays a role in G-CSF-triggered apoptosis and growth arrest. To evaluate this further, we treated cells with G-CSF in the presence or absence of pifithrin-α, a chemical compound known to suppress p53-mediated gene transcription [25]. In this experiment, suppression of the p53 target genes would attenuate G-CSF-induced apoptosis in the TEL-expressing cells. In the mock cells, addition of pifithrin-α had no effect on cell survival after G-CSF treatment (Fig. 5A, M-2 and M-3). In the TEL-overexpressing cells, however, pifithrin-α significantly delayed the onset of apoptosis; the cell counts increased at 12 h after G-CSF treatment (T-1: 0 μM vs 0.25 μM, $p = 0.264$; 0 μM vs 1.0 μM, $p = 0.002$; T-3: 0 μM vs 0.25 μM, $p = 0.049$; 0 μM vs 1.0 μM, $p = 0.005$. Student's t, two-tailed, unpaired) followed by a rapid decline after 24–36 h (Fig. 5A, T-1 and T-3). The protective effect of pifithrin-α was dose dependent, with more cells surviving at 12 h at a concentration of 1.0 μM than at 0.25 μM (T-1: 400/μl vs 296/μl; T-3: 383/μl vs 283/μl). Again, no such dosage effect was observed in the mock-transfected cells. These results indicate that induction of the p53 target genes in the TEL-overexpressing cells is at least partly responsible for the induction of apoptosis after G-CSF treatment.

Despite clear inhibition of apoptosis in its early phase by pifithrin-α, cell death still occurs after 24–36 h. It is known that p53 triggers cell death through transcription-independent mechanisms [26]. Physical interaction of mitochondrially translocated p53 with anti-apoptotic Bcl-xL and Bcl-2 is believed to liberate pro-apoptotic BAK from these inhibitors and induce outer membrane permeabilization [27]. Thus, we aimed to block this pathway by overexpression of Bcl-2 to sequester increased p53 protein in the TEL-overexpressing cells and prevent subsequent activation of BAK downstream. Fig. 5B shows the introduction of the Bcl-2 expression plasmid in the TEL-expressing clone T-1 (T-1/B2 and T-1/B5). We also made two stable lines of 32Dcl3 expressing Bcl-2 protein (B-1 and B-2). These

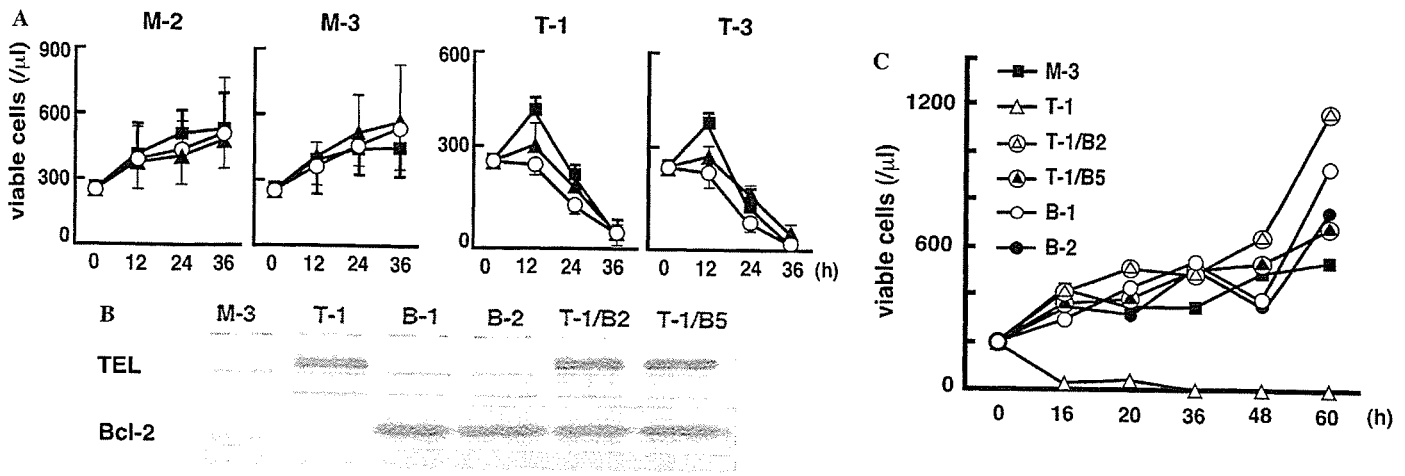

Fig. 5. TEL-induced apoptosis caused by p53 transcription-dependent and -independent mechanisms. (A) The p53 inhibitor pifithrin-α delays the onset of G-CSF-triggered apoptosis in the TEL-expressing cells. The mock (M-2 and M-3) and TEL-overexpressing (T-1 and T-3) clones were cultured in the presence of G-CSF with 0, 0.25 or 1.0 μM of pifithrin-α (shown as ○, ▲, and ■, respectively). Viable cells were counted at the times indicated. The results are shown as averages of three independent experiments, with bars representing standard deviations. (B) Western analysis of 32D clones stably overexpressing wild-type TEL (T-1), Bcl-2 (B-1 and B-2), and both wild-type TEL and Bcl-2 (T-1/B2 and T-1/B5) with anti-FLAG (in the upper panel) or anti-Bcl-2 (in the lower panel) antibody. Bcl-2 expression vector was introduced into parental cells or the TEL-overexpressing clone T-1. (C) Co-expression of Bcl-2 in TEL-overexpressing cells completely suppresses apoptosis. The time courses of viable cells were analyzed after G-CSF treatment.

clones, as well as M-3 and T-1, were treated with G-CSF and the viable cells were counted (Fig. 5C). The Bcl-2 and TEL double transfectants (T-1/B2 and T-1/B5) showed complete resistance to G-CSF-induced apoptosis, indistinguishable from that of Bcl-2 transfectants (B-1 and B-2). Thus, these data indicate that both transcription-dependent and -independent pathways of p53 contribute to cell death in the TEL-expressing cells.

Discussion

We demonstrated in this study that TEL has negative effects on proliferation and survival of 32Dcl3 cells. Wild-type TEL slows the growth of exponentially growing 32Dcl3 cells in the presence of IL-3, apparently as a result of cell cycle arrest. This observation is consistent with its tumor suppressive functions reported in Ras-transformed NIH3T3 cells [9]. In addition, we have found in this study that wild-type TEL rapidly induces apoptosis in 32Dcl3 cells exposed to G-CSF. Since the parental and mock cells differentiate into mature granulocytes on exposure to G-CSF, it is possible that the wild-type TEL-overexpressing cells lose their response to G-CSF stimulation. We examined expression of the G-CSF receptor in the TEL-overexpressing cells; both the mock and the TEL-overexpressing cells expressed similar levels of *G-CSF receptor* transcript, suggesting that loss of cell surface G-CSF receptors is not an underlying mechanism of TEL-induced apoptosis. We also examined the domains necessary for TEL-mediated apoptosis. Deletion of either the HLH or the ETS domain of TEL resulted in partial loss of its apoptosis-induction capacity. This is in contrast with the results of reporter analyses in which deletion of such domains caused complete loss of its function as a transcriptional repressor [16,28]. Thus, at this point it is unclear whether the G-CSF-induced apoptosis is related to TEL's transcriptional function or due to some transcription-independent mechanism. Nevertheless, our observation that TEL-induced apoptosis in the presence of G-CSF suggests its specific role in hematopoietic cells, which may act in synergy with co-existing hematopoietic cytokines.

Apoptosis is initiated following activation of either intrinsic or extrinsic signals. The former are produced by cellular stresses such as exposure to radiation, chemicals, growth factor deprivation, and oxidative stress, while the latter are mediated by crosslinking of the TNF receptor families. In general, intrinsic signals converge on mitochondria, where integration of the cell death machinery takes place [29]. The ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins determines how much cellular stress is necessary to initiate apoptosis [30]. Loss of mitochondrial membrane potential detected in the TEL-overexpressing cells suggests that TEL induces apoptosis through the intrinsic pathway. However, we were unable to detect changes in the protein levels of the Bcl-2 family members Bcl-2, Bcl-xL, Bax, Bad, and Noxa, which regulate apoptotic induction through the mitochondria.

Importantly, we observed increased levels of p53 protein in the TEL-overexpressing cells. It has been demonstrated that overexpression of p53 is sufficient to cause apoptosis in several experimental systems [31]. Thus, the higher expression of p53 could cause increased sensitivity to apoptosis once the minimum level of intrinsic death signals is provided. This observation led us to analyze the underlying mechanisms in the increased expression of p53 by wild-type TEL. Cycloheximide treatment for 15 min results in complete disappearance of p53 proteins, indicating that wild-type TEL assists up-regulation of p53 protein primarily through protein synthesis but not through protein stabilization. The finding that MDM2 proteins are expressed at similar levels in mock and TEL-overexpressing cells also supports this idea. Our Northern analysis and real-time PCR assay show that *p53* mRNAs are up-regulated in the TEL-overexpressing cells. Therefore, TEL could contribute in stimulating transcription of the *p53* gene and thereby causing an increase in p53 protein synthesis. The *p53* promoter region contains multiple EBS within 0.5 kb upstream of the translational initiation site [32], though we have not yet obtained evidence that TEL can transactivate the promoter.

The molecular mechanism of TEL's tumor suppressive function has hitherto been a mystery. Our study defined the p53 pathway as one such mechanism. Overexpression of TEL results in increased levels of p53 protein, which are reflected in its increased DNA binding and activation of the p53-responsive reporter. TEL-overexpressing clones show higher expression of p53 target genes such as *p21* and *Puma*, and the induction of apoptosis is blunted by the p53-specific transcription inhibitor pifithrin-α. Overexpression of Bcl-2 completely blocks apoptosis in the TEL-overexpressing clones, indicating that the intrinsic pathway, presumably activated by p53, is responsible for the apoptosis. Collectively, our paper proposes p53 as one of the downstream targets for the tumor suppressive function of TEL.

In human leukemia, the *TEL* gene is frequently translocated to form various fusion genes that generate multiple abnormal chimeric proteins. A proposed mechanism of leukemogenesis by such TEL-fusion proteins is that the HLH domain of TEL assists dimerization of the fusion proteins, thereby eliciting kinase activity intrinsic to the fused partners [1,33–35]. Conversely, generation of the fusion protein could act dominant-negatively to the fused partner, as seen in the case of TEL/AML1 generated as a consequence of t(12;21)(p13;q22) translocation in pre-B cell-type acute lymphoblastic leukemia [36,37]. Some studies suggest that TEL has a tumor suppressive function and that its inactivation could result in formation of the tumor [9]. Our study shows that the anti-oncogenic effect of TEL could be partly attributed to activation of a p53-dependent pathway. Some of the TEL-related fusion proteins have the HLH domain that is necessary for heterodimerization with wild-type TEL. This interaction might inhibit the normal function of wild-type TEL, including induction of p53 pathways,

and thereby assist the development of leukemia. A typical example would be TEL/AML1. We previously reported that TEL/AML1 imposes a dominant-negative effect over wild-type TEL through heterodimerization with the HLH domain [38]. Thus, fusion proteins of TEL that have the TEL–HLH domain may have a similar function to TEL/AML1 and could cause transformation of myeloid progenitors. Such candidate fusion molecules include TEL/ARG in t(1;12)(q25;p13) [39], TEL/ARNT in t(1;12)(q21;p13) [40], and TEL/PTPRR in inv(12)(p13q13) [41]. These proteins may impede the TEL–p53 pathway, which could constitute another mechanism of leukemia development caused by 12p13-related translocations.

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