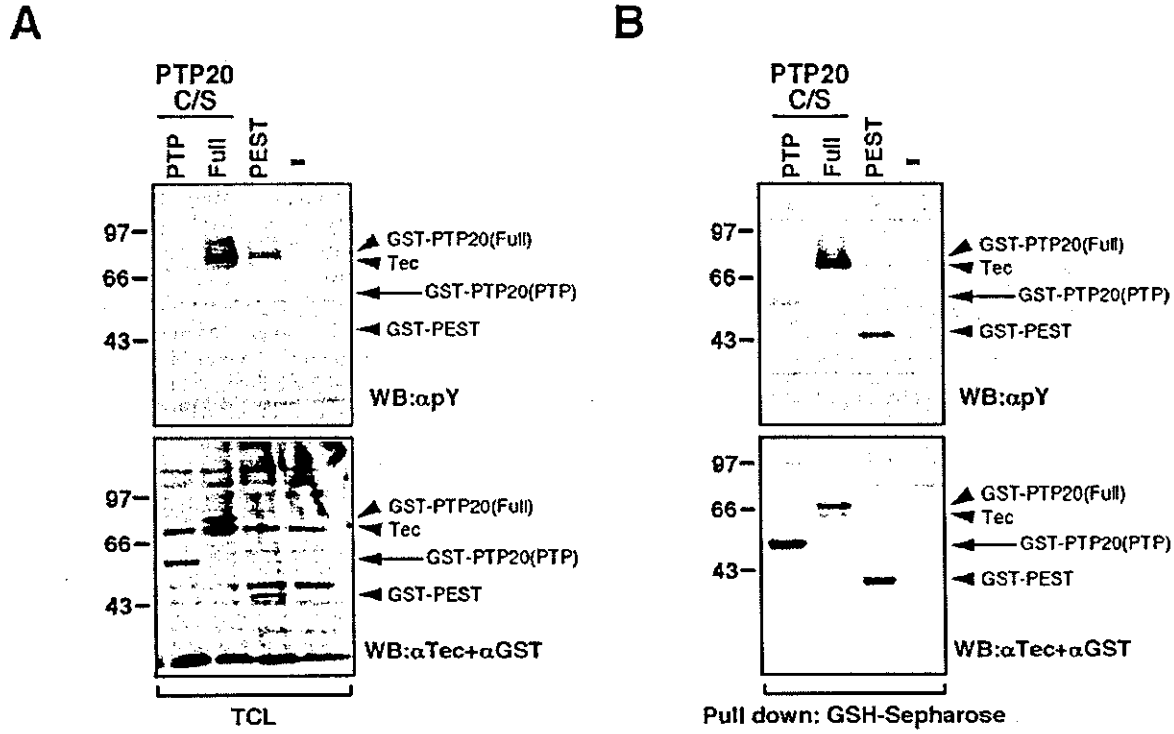

FIG. 6. **Both PTP catalytic and PEST domains of PTP20 are involved in maximal phosphorylation of Tec and association with Tec.** Tec was co-transfected with either empty pEBG vector (−) or that bearing the PTP20 catalytic domain (*PTP*), full-length PTP20 (*Full*), or the non-catalytic PEST domain of PTP20 (*PEST*). *A*, aliquots of total cell lysates (*TCL*) were subjected to immunoblotting with anti-phosphotyrosine antibody (*αpY, upper panel*). The same membrane was reprobed with a mixture of anti-Tec and anti-GST antibodies. *B*, remaining cell lysates were precipitated with GSH-Sepharose beads and processed as mentioned above. The bands corresponding to individual products are indicated by *arrows*.

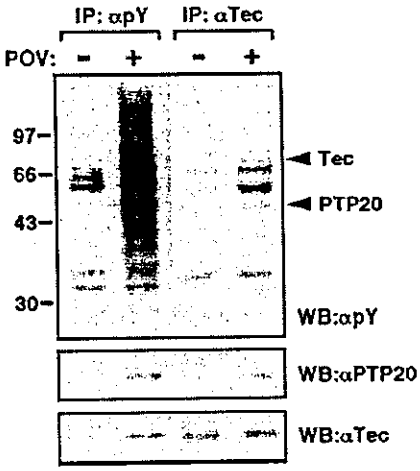

FIG. 7. **Tyrosine phosphorylation-dependent interaction of endogenous PTP20 with endogenous Tec in Ramos B cells.** Ramos cells were treated with 0.1 mM POV for 15 min at 37 °C, lysed, and subjected to immunoprecipitation with either anti-phosphotyrosine (*αpY*) or anti-Tec antibody. The immunoprecipitates (*IP*) were immunoblotted (*WB*) by anti-phosphotyrosine antibody. The same membranes were sequentially reprobed with anti-PTP20 and -Tec antibodies. The bands corresponding to Tec and PTP20 are indicated by *arrowheads*.

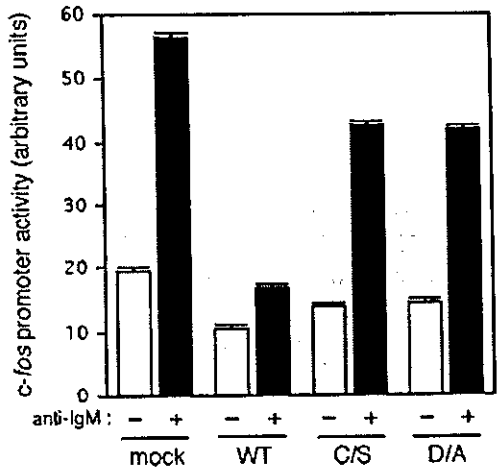

FIG. 8. **Negative role of PTP20 in BCR signaling.** Ramos cells (1×10^7) were subjected to electroporation with 2 μg of the pfos/luc reporter plasmid together with 10 μg of pcDNA3 vector (mock) or bearing PTP20 WT, C/S, or D/A mutant. Five hours after transfection cells were incubated for an additional 5 h in the absence (*open bars*) or presence (*closed bars*) of anti-IgM (ab') (10 μg/ml). Cells lysates were then assayed for luciferase activity. Data are expressed as mean ± S.D. of triplicate determinations.

Ramos B cells (Fig. 9*C*), suggesting that other direct or indirect mechanisms to regulate PTP20 activity are involved in dephosphorylation and deactivation of Tec in the cells. However, we cannot exclude the possibility that phosphorylation on serine and threonine residues rich in the C-terminal region of PTP20 might affect catalytic activity of PTP20, as PTP20 can be regulated under the control of follicle-stimulating hormone in rat ovarian granulosa cells, where no tyrosine phosphorylation on PTP20 was observed (14).

It has been reported that constitutively active Lck phosphorylates tyrosine residues 354 and 381 on PTP20, which are in turn recognized by the Csk SH2 domain (11). In that report it was also documented that mutation of both the tyrosine resi-

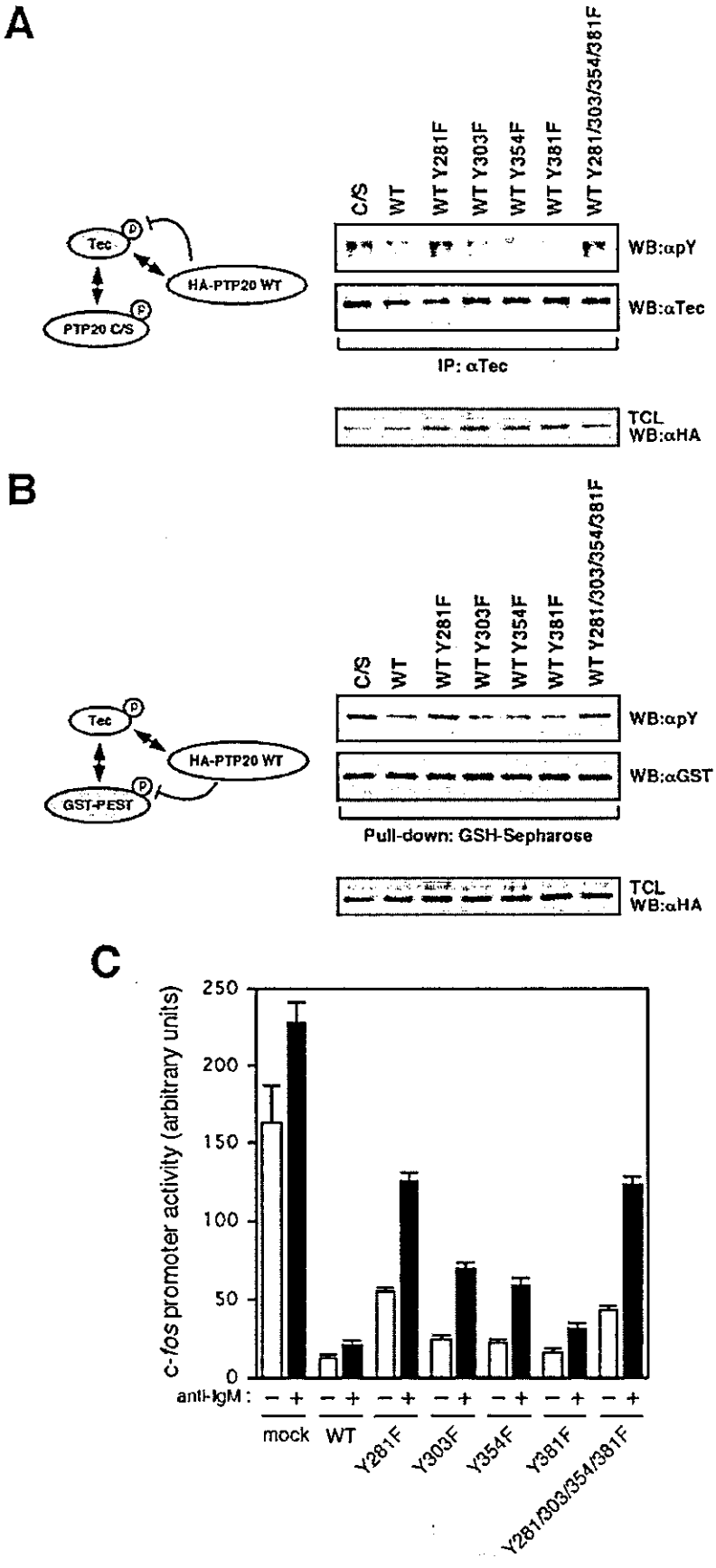

FIG. 9. **Tyrosine 281 is critical for *in vivo* phosphatase activity of PTP20.** *A*, COS7 cells were co-transfected with Tec, PTP20 C/S, and HA-PTP20 WT or its YF mutants. HA-PTP20 C/S was also included as a negative control. Cells were lysed, and Tec was immunoprecipitated with anti-Tec antibody. The immunoprecipitates (*IP*) were separated by SDS-PAGE followed by immunoblotting (*WB*) with indicated antibodies. Expression of HA-PTP20 was confirmed using aliquots of total cell lysates (*TCL*) with anti-HA antibody. *αpY*, anti-phosphotyrosine antibody. *B*, COS7 cells were transfected as above, but PEST-encoding GST-PTP20 PEST domain (GST-PEST) in place of PTP20 C/S was included. Cell lysates were subjected to precipitation with GSH-Sepharose beads and immunoblotted with the indicated antibodies. Expression of HA-PTP20 was confirmed using aliquots of total cell lysates (*TCL*) with anti-HA antibody. *C*, Ramos cells were transfected by electroporation with 2 μg of the pfos/luc reporter plasmid together with 10 μg of pcDNA3 vector (mock) or bearing PTP20 WT or its YF mutant and processed as described in legend to Fig. 8.

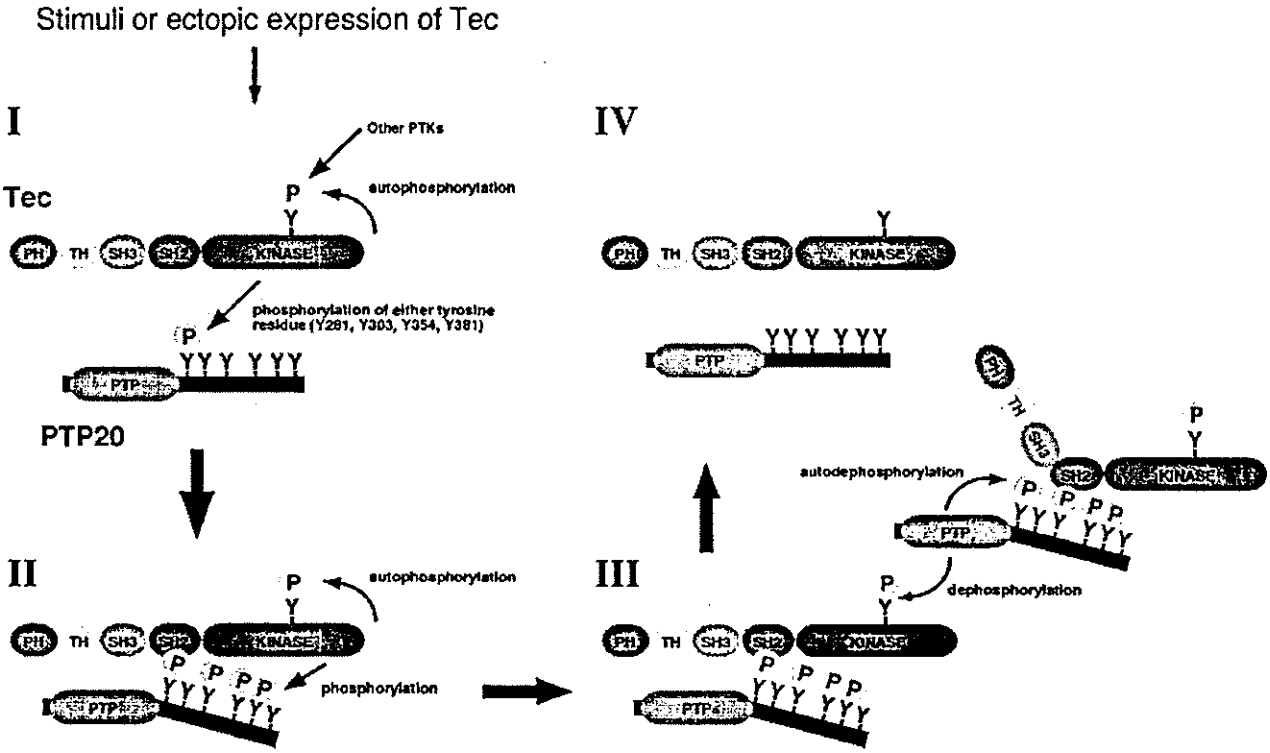

FIG. 10. **Working hypothesis for interaction of PTP20 with Tec.** *I*, upon stimuli or ectopic expression of Tec, Tec becomes tyrosine-phosphorylated and activated through autophosphorylation and other PTK catalytic activity. In turn, Tec phosphorylates tyrosine residues (Tyr-281, Tyr-303, Tyr-354, Tyr-381) on PTP20. *II*, phosphorylated PTP20 associates with Tec SH2 domain of remaining inactive Tec, thereby activating the Tec kinases. Interaction of Tec with PTP20 increases a pool of activated Tec and PTP20. *III*, activated PTP20 by phosphorylation then dephosphorylates Tec as well as PTP20 itself. Note that free of phosphorylated tyrosine 281 from association with Tec SH2 domain might be necessary for expression of PTP20 dephosphorylation activity. *IV*, finally, both Tec and PTP20 return to basal and inactive states.

dues on PTP20 caused no changes in catalytic activity by *in vitro* phosphatase assay. We have also showed that PTP20 was tyrosine-phosphorylated by Lck and Src and was associated with the PTKs (Fig. 2). However, neither the SH2 nor the SH3 domain of Lck was shown to be involved in the association with PTP20 (data not shown). Recently, another cytosolic protein-tyrosine kinase c-Abl also was shown to phosphorylate PTP20 and in turn to be dephosphorylated by PTP20 (10). Although PTP20-Tec and PTP20-cAbl interactions seem to be analogous, association between PTP20 and c-Abl is indirect, and PSTPIP, which is also a substrate of PTP20, instead serves as an adapter by bridging PTP20 to c-Abl. In contrast, association between PTP20 and Tec kinase seems to be direct, and involvement of adaptor molecules such as PSTPIPs is unlikely because the Tec SH2 domain alone could capture tyrosine-phosphorylated PTP20 (Fig. 3*D*) and, consistently, substitution of tyrosine residues on PTP20 dramatically reduced the mutual binding (Fig. 5*B*). These imply that PTP20 might be differentially tyrosine-phosphorylated by Lck, Tec, and c-Abl kinases depending on cellular context.

The Tec kinase was initially isolated from mouse liver (40) and was subsequently shown to be expressed in many tissues, including spleen, lung, brain, and kidney (41). Four Tec-related PTKs, including Btk (42, 43), Itk (also known as Emt or Tsk) (44–46), Bmx (47), and Txk (or Rlk) (48, 49), have also been molecularly cloned. Tec and the related kinases can be activated by cytokine receptors, lymphocyte surface antigens, G protein-coupled receptors, receptor type PTKs, or integrins (13, 20, 22–26). However, little is known about how the inactivation of Tec kinase is achieved. In this study, we have showed that PTP20 is a potential negative regulator in Tec-mediated signaling pathway and that the Tec SH2 domain is essential for the negative regulation by PTP20. Itk, another member of Tec family, might also be regulated by PTP20 in T cells in a similar fashion,[2] whereas Btk and Bmx seem not to interact with PTP20 (Fig. 1). Recently, the Tec SH2 domain has been shown to bind to Dok-1, which is tyrosine-phosphorylated by Tec, causing inhibition of BCR-mediated c-*fos* promoter activation (18). Another publication has demonstrated that a docking protein, BRDG1, binds to the Tec SH2 domain and acts downstream of Tec in a positive fashion in BCR signaling (50). Thus, the Tec SH2 domain might differentially participate in BCR signaling in a positive or negative way.

PTP D1, which comprises another subfamily of cytosolic PTPs, is shown to be a potential regulator and effector for not only Bmx/Etk kinase but also Tec kinase (51). The PH but not SH2 domain of Bmx/Etk is involved in the interaction with the central portion (residues 726–848) of PTP D1, and such binding is phosphotyrosine-independent, unlike PTP20-Tec interaction. Interaction between Bmx/Etk and PTP D1 stimulates the kinase activity of Bmx/Etk, resulting in an increased phosphotyrosine content in both proteins. Although it is obvious that PTP D1 is a substrate of Bmx/Etk and Tec, PTP D1 appears not to dephosphorylate the kinases. Rather, PTP D1 is a positive regulator in Bmx/Etk- and Tec-mediated signaling pathway leading to STAT3 activation. By co-transfection experiments, we observed that PTP36, which belongs to the same PTP subfamily as PTPD1, was tyrosine-phosphorylated by Tec kinase (data not shown). Thus, Tec-mediated signaling could be negatively or positively regulated by interacting with PTPs.

In conclusion, PTP20 appears to play a negative role in the

[2] S. Yamasaki and N. Aoki, unpublished data.

Tec-mediated, in particular in BCR, signaling pathways and the tyrosine phosphorylation-dependent interaction between Tec and PTP20 might form a negative feedback loop. To our knowledge this is the first report demonstrating that tyrosine phosphorylation-dependent interaction between PTK and PTP is relevant for their mutual state in some cellular context.

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〈抄録〉第25回 日本臨床薬理学会年会 2004年9月17～18日 静岡
シンポジウム2（安全性分野）：トキシコゲノミクス―現状と臨床薬理学への応用―

4．日本人組織を用いたトキシコゲノミクス研究

大島康雄* 藤村昭夫*

我々の研究室では日本人由来の組織を用いたトキシコゲノミクス研究を行っている。現状では日本人組織を商業ベースで合法的に入手することはできないために、我々は自治医科大学附属病院で手術を受ける患者さんにご協力いただき、手術時に病変部位と同時にやむを得ず切除される正常組織を研究に利用することとした。動物実験で得られた情報だけではヒトへの外挿が必ずしも十分ではなく、ヒトで最終的に確認できればより好ましい。一方、臨床検体の入手には困難な点が多く、研究計画の自由度は低い。動物実験・細胞株を用いた研究と我々の様にヒト組織を用いた研究は相補的な位置づけとされ、それぞれの役割を担うことが期待される。

1．倫理評価ワーキンググループ：
治療を目的として我々の病院を訪れる患者さんから、研究のための組織を提供していただくにあたり、我々は倫理評価ワーキンググループを立ち上げ、研究計画の審査・インフォームドコンセント取得の手順・検体採取後の病理組織の評価・匿名化の手順・関係書類の保管などにつき詳細な検討を行った。倫理評価ワーキンググループの中には、宗教家・法律家などの学外委員も含まれている。彼らとの討論の中で、医療関係者以外の第三者から誤解を受けやすい点が一つ浮かび上がった。今回我々が研究に用いる検体は、従来の手術方法で切除されてしまう非病変部組織であり、通常廃棄処分される組織部分を研究へ利用させていただく計画であった。しかし、非医療関係者は、病変の治療のためには必要もないのに研究目的のためだけに正常組織を切除するものと誤解されるようであった。このような誤解を受けやすい部分を今後も啓発することによって、日本人組織を研究・開発に利用しやすい社会環境を形成してゆく必要がある。

2．臨床検体取得の現場：
患者さんよりインフォームドコンセントをいただき、附属病院の手術室から臨床検体を取得し、プライマリーカルチャーを作成することができた。我々が試みた範囲ではディスパーゼとトリプシンを併用する方法が安定した良好な結果をもたらすものと思われた[1]。得られた細胞は、上皮性の細胞として矛盾のない形態を示し、腎臓皮質由来の細胞の多くはGlut-2抗原及びγGTP活性を示し、尿細管由来であることが示された。また、肝臓由来の細胞はアルブミン産生能及びCyp3A4抗原の存在から、主に肝細胞であると判断した。

3．臨床検体を用いた遺伝子発現解析の問題点：
出版された論文とともに公開されたデータベース(http://www.ncbi.nlm.nih.gov/geo/)をレビューした結果、171の臨床検体を用いたGeneChipデータのうち29 Chip (17%)ではRNAの質が不良であると判定された。一方で63の非臨床検体を用いたGeneChipデータではRNAの質がすべて良と判定された。このようにすでに公開・出版されているデータですら臨床検体のデータには問題点があることが示された。これは、臨床検体の取り扱いの難しさを示している。同時に、臨床検体を用いた網羅的遺伝子発現解析の実行・データの解釈にはRNAの質に注意すべきであることも示している。
網羅的遺伝子発現解析のもう一つの問題点は、個別の遺伝子発現全てにつきそのデータの信頼性とそれが意味するところを研究者自身が検証しながら研究を進めていくことが困難であることがあげられる。このためチップデータ全体の質を管理することが必要と考えられる。我々が使用している

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Affymetrix 社の GeneChip システムでは、こうした実験の質の管理を行うために用いることのできる様々なパラメータを実験結果の一部として得ることができる。このようなパラメータにはバックグラウンドノイズ・ハウスキーピングジーンの 3'/5'比・パーセントプレゼントなどがある。こうしたパラメータを活用し、研究の質をコントロールしつつ臨床検体の処理・プライマリーカルチャーの作成を行うことによって、我々はより良質のプライマリーカルチャーを作成することができたと考えている。また、既知濃度のスパイク RNA を用いて検出系の定量性・ダイナミックレンジの検討も行った。その結果、1.5 pM~100 pM での範囲での RNA 濃度の読みとりの直線性は良好であった。

4. 日本人組織を用いる必要性～人種差の克服：

海外で調整済みのプライマリーカルチャーを用いることに比較して、日本人組織を用いた遺伝子発現解析の利点として、理論的には研究結果について人種差を懸念する必要がないこと、海外調整済みのプライマリーカルチャーの利用にかかわる資金や場合によっては知的所有権などが海外へ流出する心配がないこと等がある。もちろんヒト組織であるから、種差も存在しない。さらに、自前でヒト組織を調整するため、組織の質を制御することが可能である。

我々の研究室ではこれまでのところ 11 名分の組織を研究に使用している。この 11 名につきそれぞれ 3 回、薬物等の刺激に未曝露の状態で発現解析を行ったところ、約 44,000 のトランスクリプトの内、統計学的に有意に個人差がある（t-test p 値が 0.01 未満）トランスクリプトは 100 に満たなかった。大多数の遺伝子発現は、薬物未曝露の状態では有意な個人差が見られないと判断された。

5. 現状と限界：

これまで 20 以上の薬物をプライマリーカルチャーへ曝露し、遺伝子発現解析を行った。腎障害をしばしば起こすことによって臨床的に問題になる薬物を用いて、有意に誘導や抑制される遺伝子を同定した。現在その遺伝子発現の確認を進めるとともに、その細胞内での働きを解析している。また、クラスタリングされた遺伝子群に有意に高頻度に出現する転写因子（DNA binding protein）認識配列を検索するシステムを構築した。[2]

本稿で記載した、個人差の研究により得られた情報は、重要な基礎的検討である。先行している実験動物や培養細胞株の研究に追従する形で曝露化合物の数を増加することよりも、データの質を管理するプロセスや、未刺激の日本人プライマリー腎細胞にどのような遺伝子に個人差があるのかを明らかにすることは、日本人の臨床検体を取り扱っている我々が取るべき、より優先順位の高い課題である。これらこそが限られたリソースと与えられた条件のなかで今後の日本人組織を用いた研究を活かし、示唆に富む情報を提供するもである。

[1] Yasuo Oshima, Shinsuke Kurokawa, Akihiko Tokue, Hiroyuki Mano, Ken Saito, Makoto Suzuki, Masashi Imai, and Akio Fujimura. Primary Cell Preparation of Human Renal Tubluar Cells for Transcriptome Analysis. *Toxicology Mechanisms and Methods*, 14:309-316, 2004

[2] Yasuo Oshima, Yusuke Ishida, Ayumi Shinohara, Hiroyuki Mano, Akio Fujimura. Expression Profiling of Gene with Upstream Aml1 Recognition Sequence in Hematopoietic Stem Cell-Like Fractions from Individuals with the M2 Subtype of Human Acute Myeloid Leukemia. Annual Meeting for International Society of Experimental Hematology, New Orleans, LA. USA. Jul 16-20, 2004

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Signal Transducers and Activators of Transcription 3 Augments the Transcriptional Activity of CCAAT/Enhancer-binding Protein α in Granulocyte Colony-stimulating Factor Signaling Pathway*

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The Janus kinase (Jak)-Stat pathway plays an essential role in cytokine signaling. Granulocyte colony-stimulating factor (G-CSF) promotes granulopoiesis and granulocytic differentiation, and Stat3 is the principle Stat protein activated by G-CSF. Upon treatment with G-CSF, the interleukin-3-dependent cell line 32D clone 3(32Dcl3) differentiates into neutrophils, and 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in G-CSF without differentiation. Gene expression profile and quantitative PCR analysis of G-CSF-stimulated cell lines revealed that the expression of *C/EBPα* was up-regulated by the activation of Stat3. In addition, activated Stat3 bound to CCAAT/enhancer-binding protein (C/EBP)α, leading to the enhancement of the transcription activity of C/EBPα. Conditional expression of C/EBPα in 32Dcl3/DNStat3 cells after G-CSF stimulation abolishes the G-CSF-dependent cell proliferation and induces granulocytic differentiation. Although granulocyte-specific genes, such as the G-CSF receptor, lysozyme M, and neutrophil gelatinase-associated lipocalin precursor (*NGAL*) are regulated by Stat3, only NGAL was induced by the restoration of C/EBPα after stimulation with G-CSF in 32Dcl3/DNStat3 cells. These results show that one of the major roles of Stat3 in the G-CSF signaling pathway is to augment the function of C/EBPα, which is essential for myeloid differentiation. Additionally, cooperation of C/EBPα with other Stat3-activated proteins are required for the induction of some G-CSF responsive genes including lysozyme M and the G-CSF receptor.

The proliferation and differentiation of hematopoietic progenitor cells are regulated by cytokines (1). Among these, granulocyte colony-stimulating factor (G-CSF)[1] specifically stimulates cells that are committed to the myeloid lineage (2). The importance of G-CSF to the regulation of granulopoiesis has been confirmed by the observation of severe neutropenia in mice carrying homozygous deletions of their G-CSF or G-CSF receptor genes (3, 4). Cytokines activate several intracellular signaling pathways, and the Janus kinase (Jak) signal transducers and activators of transcription (Stat) pathway is essential for cytokine function (5, 6). The binding of G-CSF to cell surface G-CSF receptors activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7–9). Stat3 is the principle protein activated by G-CSF (8, 10). Phosphorylated Stats translocate from the cytoplasm into the nucleus and induce transcription of their target genes within a short period of time. 32Dcl3 cells differentiate to neutrophils following treatment with G-CSF. In contrast to their parental cells, 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in the presence of G-CSF, but they maintain immature morphologic characteristics without evidence of differentiation (11). Additionally, transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). To clarify the role of Stat3 in the G-CSF signaling pathway, we wished to identify target genes of Stat3.

We found that the levels of CCAAT/enhancer-binding protein (C/EBP) α mRNA were up-regulated following G-CSF stimulation in 32Dcl3 but were unchanged in 32Dcl3/DNStat3. In addition, the activation of Stat3 augmented the function of C/EBPα, which is the essential transcriptional factor for myeloid differentiation. G-CSF-induced granulocytic differentiation was restored in 32Dcl3/DNStat3 cells by the conditional expression of C/EBPα. These results show that one of the major

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[1] The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; C/EBPα, CCAAT/enhancer-binding protein; NGAL, neutrophil gelatinase-associated lipocalin precursor; Jak, Janus kinase; Stat, signal transducers and activators of transcription; DNStat3, dominant-negative Stat3; IRES, internal ribosome entry site; GFP, green fluorescent protein; ER, endoplasmic reticulum; IFN, interferon; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; LUC, luciferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TK, thymidine kinase; 4-HT, 4-hydroxytamoxifen.

roles of Stat3 in the G-CSF signaling pathway is to enhance the function of C/EBPα.

MATERIALS AND METHODS

Cell Culture, Expression Plasmid, and Cytokines—32D clone 3 (32Dcl3) and 32Dcl3/DNStat3 cells (DNStat3 deletes the transactivation domain of Stat3) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ICN, Osaka, Japan), penicillin/streptomycin (Invitrogen), recombinant murine interleukin-3 (IL-3) (Kirin Brewery, Takasaki, Japan), and recombinant human G-CSF (Chugai Pharmaceutical, Tokyo, Japan). 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine.

For the construction of pTag2A-G-CSF receptor, the human G-CSF receptor cDNA (13) (pHQ3, kindly provided by S. Nagata and R. Fukunaga) was excited from the pBluescipt vector and inserted into the FLAG-tagged mammalian expression plasmid pCMV-Tag2A (Clontech). pcDNA3-rat C/EBPα was described before (14). Stat3 cDNA was amplified by PCR and inserted into pCMV-HA vector (Clontech). Stat3c cDNA was elicited from RCMV-Stat3c (15), kindly given from Dr. Darnell, and inserted into pcDNA3.1 (Clontech). For the construction of pMY-IRES-GFP/C/EBPα-ER, full-length human C/EBPα cDNA was fused in-frame with ligand-binding domain (amino acids 281–599) of mouse estrogen receptor harboring a mutation (G525R) that confers selective responsiveness to 4-hydroxytamoxifen (4-HT). A reporter construct of a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK) was described previously (14).

Murine recombinant leukemia inhibitory factor, natural IFN-α, and recombinant IFN-γ were purchased from Sigma, HyCult Biotechnology (Uden, The Netherlands), and Peprotech (Rocky Hill, NJ), respectively. For Western blotting, 32Dcl3 cells or 32Dcl3/DNStat3 cells were deprived of IL-3 for 12 h. Then cells were stimulated with G-CSF (10 ng/ml), IL-3 (10 ng/ml), leukemia inhibitory factor (10 ng/ml), IFN-α (1,000 units/ml), or IFN-γ (1,000 units/ml) for 30 min.

Microarray Analysis—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine in RPMI 1640 containing 10% fetal bovine serum for 8 h and then stimulated with 10 ng/ml G-CSF. Total RNA was extracted, by the acid guanidinium method, from 32Dcl3 and 32Dcl3/DNStat3 cells before or after the stimulation for 2 h with G-CSF. Double-stranded cDNA synthesized from the total RNA (20 μg/sample) was then used to prepare biotin-labeled cRNA for the hybridization with GeneChip MGU74Avs2 microarrays (Affymetrix, Santa Clara, CA) harboring oligonucleotides corresponding to ~6000 known genes as well as ~6000 expressed sequence tag sequences. Hybridization, washing, and detection of signals on the arrays were performed with the GeneChip system (Affymetrix).

Quantitative Real-time Reverse Transcription-PCR Assay—32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3, were washed twice with PBS and starved of cytokines for 8 h and then stimulated with 10 ng/ml G-CSF. Cells were harvested at the indicated times, and total RNA was isolated using Isogen (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. One microgram of extracted RNA was transcribed in a 20-μl cDNA synthesis reaction using an RNA PCR kit (AMV) (Takara, Tokyo, Japan). Real-time PCR for C/EBPα, G-CSF receptor, lysozyme M, neutrophil gelatinase-associated lipocalin precursor (NGAL), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed by a TaqMan assay on an ABI 7000 system. PCR primers and probes were designed as follows: murine C/EBPα, sense, 5′-CCA TGT GGT AGG AGA CAG AGA CCT A-3′, and antisense, 5′-CTC TGG GAT GGA TCG ATT GTG-3′; probe FAM-5′-CGG CTG GCG ACA TAC AGT ACA CAC AAG-3′-TAMRA, and sense, 5′-CCA AGA AGT CGG TGG ACA AGA-3′, and antisense, 5′-CGG TCA TTG TCA CTG GTC AAC T-3′; probe FAM-5′-AGC ACC TTC TGT TGC GTC TCC ACG TT-3′-TAMRA; murine G-CSF receptor, sense, 5′-CTA AAC ATC TCC CTC CAT GAC TT-3′, and antisense, 5′-GGC CAT GAG GTA GAC ATG ATA CAA-3′; probe FAM-5′-CAT CTT CTC TGT CCC CAC CGA CCA A-3′-TAMRA; murine lysozyme M, sense, 5′-TGC CTG TGG GAT CAA TTG C-3′, and antisense, 5′-ATG CCA CCC ATG CTC GAA T-3′; probe 5′-FAM-CAG TGA TGT CAT CCT GCA GAC CA-TAMRA-3′; murine NGAL, sense, 5′-GGC CTC AAG GAC GAC AAC A-3′, and antisense, 5′-CAC CAC CCA TTC AGT TGT CAA T-3′; probe 5′-FAM-CAT CTT CTC TGT CCC CAC CGA CCA A-TAMRA-3′, and murine GAPDH sense, 5′-ACG GCA AAT TCA ACG GCA-3′, and antisense, 5′-AGA TGG TGA TGG GCT TCC-3′; probe 5′-FAM-AGG CCG AGA ATG GGA AGC TTG TCA TC-TAMRA-3′. PCR amplifications were performed in a 50-μl volume, containing 4 μl of cDNA template, 50 mM KCl, 10 mM Tris-HCl(pH 8.3), 10 mM EDTA, 60 nM, 200 μM dNTPs, 3 mM $MgCl_2$, 200 nM each primer, 0.625 units of AmpliTaqGold, and 0.25 units of AmpErase uracil *N*-glycosylase. Each amplification reaction also contained 100 nM appropriate detection probe. Each PCR amplification was performed in duplicate, using conditions of 50 °C for 2 min preceding 95 °C for 10 min followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). In each reaction, GAPDH was amplified as a housekeeping gene to calculate a standard curve and allow for the correction for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to GAPDH by the instrument software.

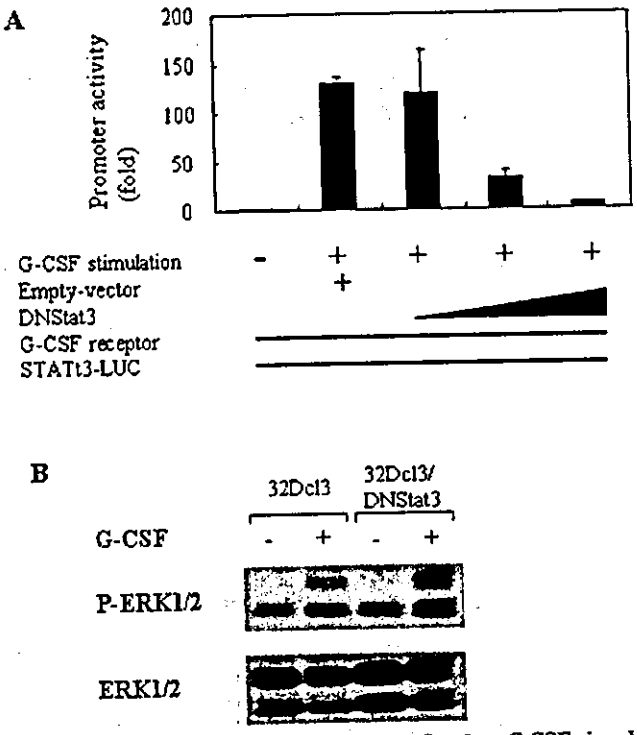

FIG. 1. **The effect of dominant-negative Stat3 on G-CSF signaling pathway.** *A*, transient transfection in 293T cells with a reporter construct with α2-macroglobulin promoter (*STATt3-LUC*), dominant-negative Stat3, and G-CSF receptor. Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The *vertical axis number* is the fold induction when compared with control. *B*, 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the G-CSF for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. *p-ERK1/2*, phosphorylated ERK1/2.

Conditional C/EBPα Expression—pMY-IRES-GFP/C/EBPα-ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells by electroporation. 5×10^6 cells were transfected with 20 μg of expression vector, and GFP-positive cells were sorted by FACS Vantage (BD Biosciences). Expression of C/EBPα was determined by Western blotting analysis (see below).

Luciferase Assay—293T cells were transfected by the calcium phosphate precipitation method in 6-well plates, and luciferase activity was assayed using a luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's protocol. Each expression plasmid amount was 50–100 ng/well, and the same amount of empty expression vector was used as control, respectively. Results of reporter assays represent the average values for relative luciferase activity generated from five independent experiments.

Flow Cytometry—1×10^7 cells were incubated with 5 μl of recombinant phosphatidylethanolamine-conjugated rabbit anti-murine Gr1 monoclonal antibody (BD Biosciences) for 30 min at 4 °C, washed twice in PBS, and analyzed on a FACS Calibur (BD Biosciences).

Immunoprecipitation and Immunoblotting—Cells were lysed with lysis buffer, and lysates were immunoprecipitated with anti C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8). Total cell lysates or the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes

TABLE I
Microarray analysis

32Dcl3 and 32Dcl3/DNStat3 cells were starved of cytokines for 8 h and then stimulated or left unstimulated with 10 ng/ml G-CSF. Total RNA was extracted from each fraction and was subjected to the hybridization with high-density oligonucleotide microarrays (MGU74Av2). Fold induction means a rate of increase in gene expression level by G-CSF stimulation. Candidate genes were identified as transcripts that were up-regulated in 32Dcl3 cells and down-regulated or unchanged in 32Dcl3/DNStat3 cells after G-CSF stimulation.

Gene product name	Abbreviation	Accession number	Fold induction	
			32Dcl3	32Dcl3/DNStat3
B-cell leukemia/lymphoma α	Bcl2	L31532	35.6	0.0629
CyclinE1	*Ccne1*	NM007633	29.7	0.690
Serotonin-gated ion channel	*5HT3*	M74425	27.2	0.592
KIF3B protein	*kif3b*	D26077	21.5	0.921
Protein kinase, serine/arginine-specific 1	*Srpk1*	AB012290	18.7	0.321
MAP kinase-interacting serine/threonine kinase 1	*Mknk1*	Y11091	15.7	0.845
Protein tyrosine phosphatase	*Ptpn13*	D83966	12.4	0.964
Transferrin receptor	*Trfr*	X57349	10.6	0.964
Lymphocyte antigen 57	*Ly57*	AF068182	9.62	0.968
Macrophage stimulating 1 receptor	*Mst1r*	X74736	8.83	0.762
Mitogen-activated protein kinase kinase 7	*MKK7*	AB005654	8.14	0.980
RAR-related orphan receptor alpha	*Rora*	U53228	7.94	0.861
Hemoglobin Y, β-like embryonic chain	*Hbb-y*	V00726	7.38	0.375
Runt related transcription factor 1	*Runx1*	NM009821	7.01	0.226
Microtubule-associated protein 6	*Mtap6*	Y14754	5.06	0.885
CCAAT/enhancer binding protein α	*C/EBPα*	M62362	2.05	0.840
Ecotoropic viral integration site 1	Evi1	M21829	1.55	0.239
Integrin alpha L	*Itgal*	M60778	1.35	0.567
Ninjurin 1	*Ninj1*	U91513	1.34	0.783
Interleukin 17 receptor	*IL17R*	U31993	1.24	0.449
Mucosal addressin	*MAdCAM*	D50434	1.14	0.527
Carbon catabolite repression 4 homolog	*Ccr4*	X16670	1.06	0.0768
Friend leukemia integration 1	*Fli1*	X59421	1.01	0.305

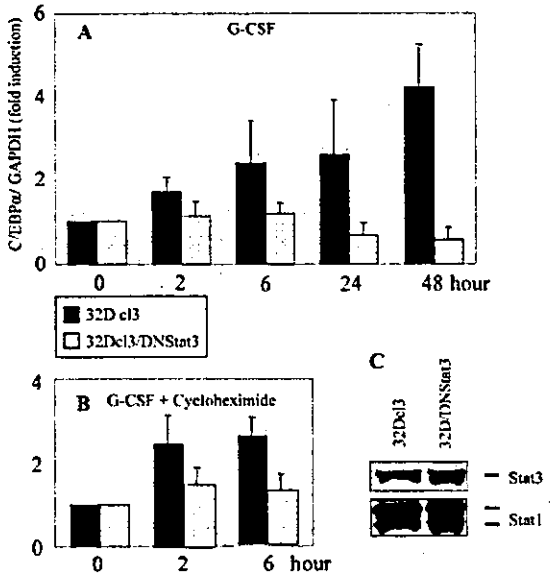

FIG. 2. **Expression of C/EBPα mRNA in G-CSF-stimulated 32Dcl3 and 32Dcl3/DNStat3 cells.** *A* and *B*, 32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF (*A*) or 10 ng/ml G-CSF and 10 μg/ml cycloheximide (*B*). Total RNA was isolated from both cell lines at the indicated times and transcribed to cDNA, which was subjected to real-time PCR for murine C/EBPα. The *numbers* given on the *vertical axis* represent the fold induction of the ratios of GAPDH-normalized expression values when compared with those before G-CSF stimulation. Results are expressed as mean fold of two independent experiments.

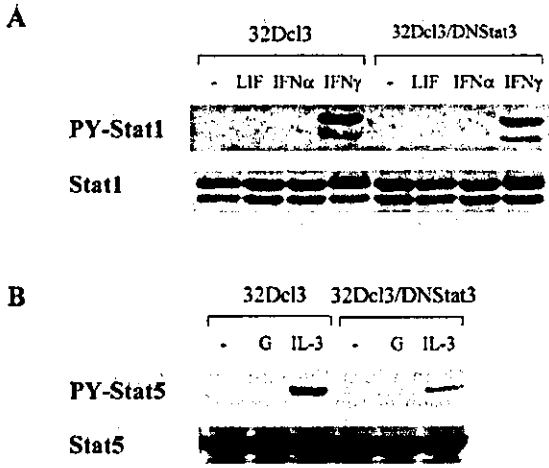

FIG. 3. **The effect of the abrogation of Stat3 on other cytokine signaling pathway.** 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the indicated cytokines for 30 min and lysed. Post-nuclear supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. *LIF*, leukemia inhibitory factor.

were probed using the indicated antibodies followed by an IgG-horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) and visualized with the ECL detection system (Amersham Biosciences). Anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). Anti-phospho-Stat1 and -Stat5 antibodies were obtained from New England Biolabs (Beverly, MA), and anti-Stat1, -Stat3, and -C/EBPα antibodies were purchased from Santa Cruz Biotechnology. Membranes were probed using and visualizes with the ECL detection system (Amersham Biosciences).

Proliferation Assay—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved of cytokine for 8 h and then stimulated with 10 ng/ml G-CSF. The number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. [^{3}H]Thymidine incorporation assays were also performed. Briefly, cells (1×10^5) in 100 μl of medium stimulated with murine IL-3 (1.0 ng/ml) or recombinant human G-CSF (10 ng/ml) were cultured for 48 h. During the final 4 h, [^{3}H]thymidine (1μCi/well) was added. Cells were then harvested by filtration, and radioactivity was counted by scintillation spectrophotometer.

RESULTS

G-CSF-induced Intracellular Signal Response in 32Dcl3/DNStat3 Cells—32Dcl3 cells differentiate into neutrophils following treatment with G-CSF, but 32Dcl3 cells expressing a

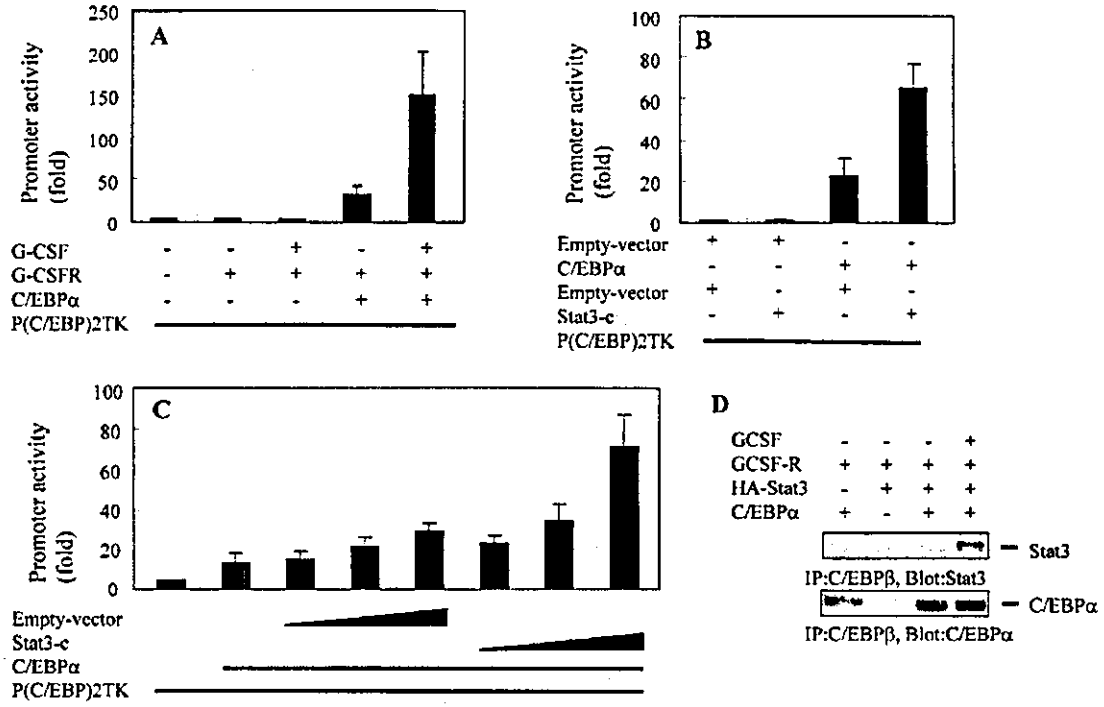

FIG. 4. **Activated Stat3 makes complex with C/EBPα, leading to the enhancement of C/EBPα-induced transcription.** *A*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with CEBP-binding sites (*p(C/EBP)2TK*), C/EBPα, and G-CSF receptor (*G-CSFR*). Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The *vertical axis number* is the fold induction when compared with control. *B* and *C*, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with CEBP-binding sites (*p(C/EBP)2TK*), C/EBPα, Stat3c, and control vectors. Promoter activity was measured as luciferase activity 24 h after transfection. The *vertical axis number* is the fold induction when compared with control. *D*, transient transfection in 293T cells with a construct of G-CSF receptor, HA-Stat3, and C/EBPα and control vectors. After 24 h, cells were lysed and immunoprecipitated (*IP*) with anti C/EBPβ. Cells were stimulated with G-CSF during the final 9 h in the culture. The immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Stat3 was detected by immunoblotting.

dominant-negative Stat3 (32Dcl3/DNStat3) proliferate following G-CSF treatment. These cells maintain immature morphologic characteristics without evidence of differentiation (11). First, we examined the effect of dominant-negative Stat3, carboxyl-truncated Stat3 that lacked 55 amino acids including the transactivation domain. We transfected reporter construct of STATt3-LUC, in which the α2-macroglobulin promoter (16) drives expression of the luciferase (LUC) reporter gene and G-CSF receptor, together with empty vector (pcDNA3) or DNStat3 to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and luciferase assay was performed. As shown in Fig. 1*A*, G-CSF induced the transcriptional activity of Stat3 by 150-fold, and DNStat3 inhibited this G-CSF-induced Stat3 activation in a dose-dependent manner.

G-CSF mainly induces the phosphorylation of Stat3, but it also phosphorylates Stat1 and Stat5 in some cells among the Stats family (8) and induces the activation of MAP kinases. In both 32Dcl3 cells and 32Dcl3/DNStat3 cells, neither Stat1 nor Stat5 was phosphorylated in response to G-CSF (data not shown). As for the MAP kinase activation, the degree of the phosphorylation of ERK1/2 by G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1*B*).

Identification of Genes Regulated by Stat3 in the G-CSF Signaling Pathway by Oligonucleotide Array Analysis—To identify Stat3-regulated genes involved in granulocytic differentiation, we compared gene expression change in both cell lines using microarray analysis. 32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were washed twice with PBS and starved in RPMI 1640 containing 10% fetal bovine serum lacking cytokine for 8 h and then stimulated with 10 ng/ml G-CSF. Total RNA was isolated from 32Dcl3 cells and 32Dcl3/DNStat3 cells treated with G-CSF after 0 and 2 h, transcribed to biotin-labeled cRNA, and hybridized to GeneChip MGU74Av2 arrays to compare the expression profile of ~12,000 murine genes. The fold induction in the expression level of each gene was calculated as the ratio of GAPDH-normalized fluorescence intensity value of G-CSF-stimulated cells when compared with those before G-CSF stimulation. As shown in Table I, we could identify a set of candidate genes for Stat3 targets, expression of which was up-regulated in 32D cl3 cells but down-regulated or unchanged in 32Dcl3/DNStat3 cells. Such Stat3-dependent expression profiles were confirmed in triplicate experiments.

C/EBPα Is a Target Gene for Stat3 in G-CSF Signaling Pathway—Among the identified genes, it was decided to focus further efforts on *C/EBPα*. C/EBPα has been shown to be critical for early granulocytic differentiation (17–19), and the factors regulating its activity are unclear. The expression of C/EBPα was examined by real-time quantitative reverse transcription-PCR. C/EBPα mRNA levels are rapidly up-regulated in 32Dcl3 cells, being elevated 2.39-fold after 6 h and 4.20-fold after 48 h (Fig. 2*A*). In contrast to 32Dcl3 cells, the C/EBPα mRNA levels were not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2*A*). A similar expression pattern was seen in separate experiments with independently designed primers and probes (data not shown). Levels of C/EBPα mRNA were unaffected by cycloheximide treatment (Fig. 2*B*). The expression level of the sum of Stat3 plus dominant-negative Stat3 in 32Dcl3/DNStat3 cells is a little larger than that of Stat3 in 32Dcl3 cells (Fig. 2*C*).

Activated Stat3 Binds to C/EBPα and Enhances the Transcription Activity of C/EBPα—We next examined the effect of

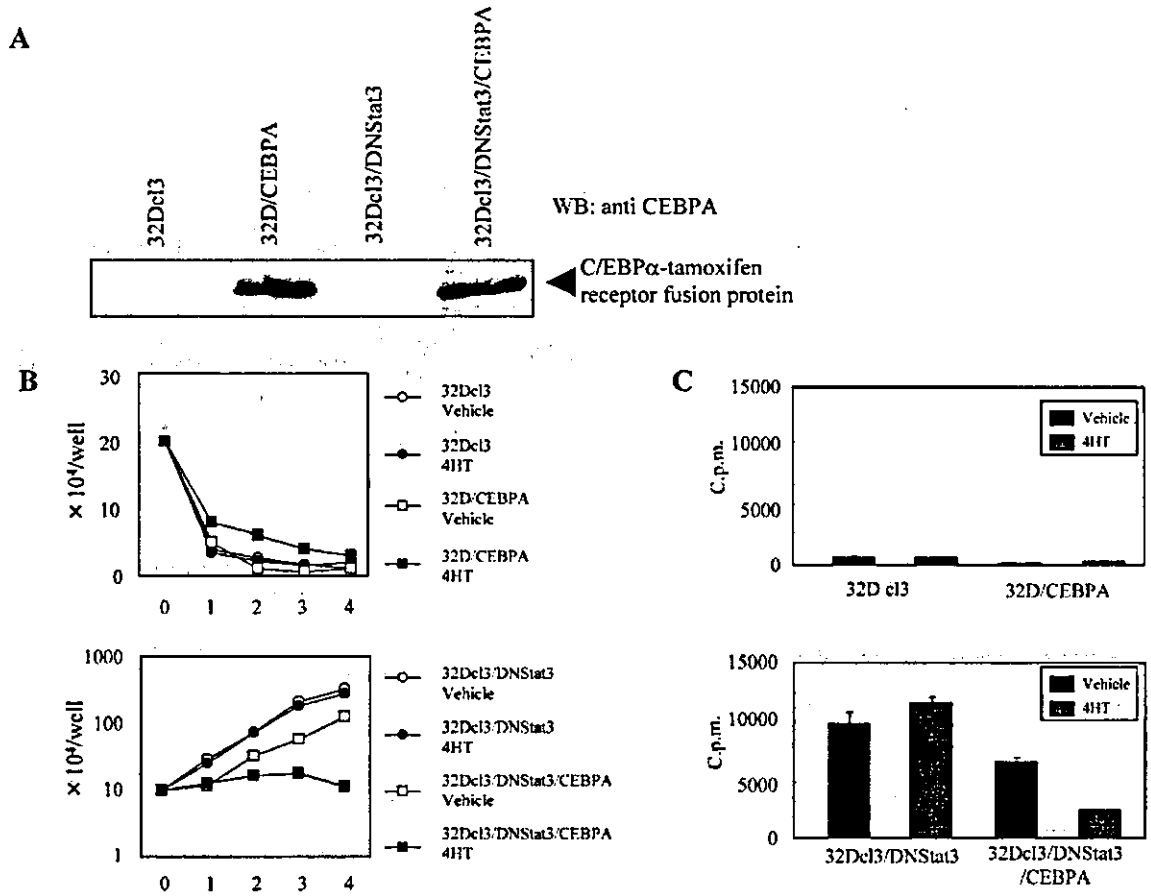

FIG. 5. **Proliferation of 32Dcl3 and 32Dcl3/DNStat3 by restoration of C/EBPα.** *A*, the expression vector pMY-IRES-GFP/C/EBPα-ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells. The expression of C/EBPα-ER was examined by Western blotting (*WB*) using anti-C/EBPα polyclonal antiserum. *Lane 1*, 32Dcl3; *lane 2*, 32D/CEBPA; *lane 3*, 32Dcl3/DNStat3; *lane 4*, 32Dcl3/DNStat3/CEBPA. *B*, growth curve of 32Dcl3, 32Dcl3/CEBPA cells (*upper panel*), and 32Dcl3/DNStat3, 32Dcl3/DNStat3/CEBPA cells (*lower panel*). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μM 4-HT or vehicle. Viable cells were counted daily by trypan blue dye exclusion method at the indicated times. The *numbers* given on the *vertical axis* represent the mean cell counts (×10⁴/well) of triplicate wells. Standard deviations (S.D.) were less than 15% of each mean. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results. *C*, ³H incorporation assays in 32Dcl3, 32Dcl3/CEBPA (*upper panel*) and 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells (*lower panel*). Cells maintained in IL-3 were washed twice with PBS and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μM 4-HT or vehicle for 48 h. During the final 4 h, 1 μCi of [³H]thymidine was added, cells were harvested by filtration, and radioactivity was counted by scintillation spectrophotometer. Results are expressed as mean cpm of triplicate wells ± S.D. Three independent experiments were performed, and similar results were obtained. Data shown are representative of these results.

Stat3 abrogation on the balance of intracellular signals in other cytokine pathways. Although Stat1 was not phosphorylated by leukemia inhibitory factor stimulation in neither 32Dcl3 cells nor 32Dcl3/DNStat3 cells, its activation in response to IFN-γ occurred at the same degree in both 32cl3 cells and 32Dcl3/DNStat3 cells (Fig. 3*A*). As for the Stat5 activation, the phosphorylation of Stat5 by IL-3 stimulation in 32Dcl3 cells was stronger than that in 32Dcl3/DNStat3 cells (Fig. 3*B*). These data indicated that there was the possibility that abrogation of Stat3 signaling can alter the balance of intracellular signals in other cytokine signaling pathways. The transcription of C/EBPα is regulated by C/EBPα itself (20, 21). Then we examined whether activated Stat3 in G-CSF signaling enhance C/EBPα activity or not.

We transfected a reporter construct of a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK), C/EBPα, and G-CSF receptor to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and a luciferase assay was performed. C/EBPα up-regulated the C/EBPα-dependent gene expression, and the G-CSF stimulation enhanced this C/EBPα-dependent gene expression (Fig. 4*A*). Next we examined the effect of constitutive active Stat3 (Stat3C) on the augmentation of C/EBPα transcriptional activity instead of the G-CSF stimulation. We transfected reporter construct p(C/EBP)2TK, C/EBPα, and Stat3C to 293T cells. After 24 h of transfection, luciferase assay was performed. Stat3C augmented the C/EBPα-dependent gene expression, although Stat3C alone had no influence on the luciferase activity (Fig. 4, *B* and *C*).

As p(C/EBP)2TK contains only a C/EBPα-binding site and does not contain a Stat3-binding sequence, the possibility that Stat3C makes a complex with C/EBPα and augments the function of C/EBPα is raised. Then we transfected C/EBPα, Stat3, and G-CSF receptor to 293T cells and stimulated cells with G-CSF for 6 h. There is no detectable level of endogenous C/EBPα or C/EBPβ protein in 293T cells. Cells were lysed and immunoprecipitated with C/EBPβ antibody (this antibody cross-reacts with C/EBPα). As shown in Fig. 4*D*, immunoprecipitants with anti-C/EBPβ contain Stat3. In addition, the complex formation between C/EBPα and Stat3 is augmented by G-CSF stimulation, indicating that activated Stat3 makes the complex with C/EBPα.

C/EBPα Restores G-CSF-induced Granulocytic Differentiation in 32Dcl3/DNStat3 Cells—To analyze the role of Stat3-

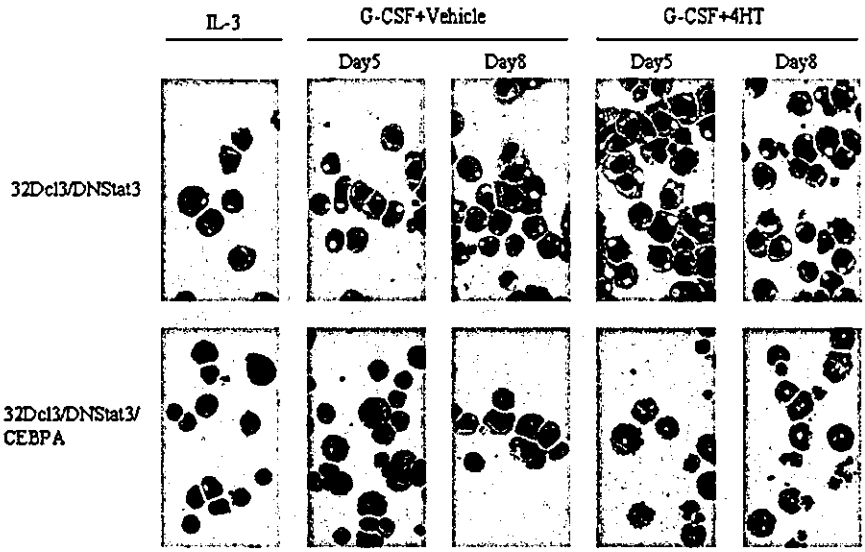

FIG. 6. **Morphologic features of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells.** Granulocytic differentiation of 32Dcl3/DNStat3 cells after induction of C/EBPα is shown. Cells were maintained in IL-3 and washed twice with PBS and then starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μM 4-HT or vehicle for 5 or 8 days. The cells were cytospun and stained with May-Grunwald and Giemsa stain (original magnification, ×400).

TABLE II
Differential count of 32Dcl3/DNStat3 and 32Dcl3/DNStat3/CEBPA cells

32Dcl3/DNStat3 and32Dcl3/DNStat3/CEBPA cells were maintained in IL-3 and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF plus 0.5 μM 4-HT or vehicle for 5 days. Differential count was performed by May-Grunwald and Giemsa stain. Values are the mean ± S.D. percent of cells from three independent experiments. Myelocyte includes promyelocytes, myelocytes, and metamyelocytes. Band(seg) includes band and segmented neutrophils.

Cells	G-CSF+Vehicle	G-CSF+4HT
32Dcl3/DNStat3		
Myeloblasts	98.0 ± 0	99.3 ± 0.47
Myelocytes	1.3 ± 0.94	0.67 ± 0.47
Band(seg)	0.67 ± 0.94	0 ± 0
32Dcl3/DNStat3/CEBPA		
Myeloblasts	90.7 ± 3.3	3.0 ± 2.8
Myelocytes	5.0 ± 0.82	54.3 ± 2.4
Band(seg)	4.3 ± 2.6	42.7 ± 0.47

regulated C/EBPα function in the G-CSF signaling pathway, we transfected a C/EBPα-tamoxifen receptor fusion protein (C/EBPα-ER) into 32Dcl3 and 32Dcl3/DNStat3 cells (32Dcl3/CEBPA cells, 32Dcl3/DNStat3/CEBPA cells, respectively). The expression of C/EBPα-ER in these cells was verified by Western blotting (Fig. 5*A*). C/EBPα-ER localizes to the cytoplasm and is in an inactive form in the absence of tamoxifen. Upon treatment with tamoxifen, it translocates from cytoplasm to nucleus and becomes active. 32Dcl3, 32Dcl3/CEBPA, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells were cultured with G-CSF in the presence or absence of tamoxifen, and cell proliferation was examined by both counting viable cells and [^{3}H]thymidine incorporation. 32Dcl3/DNStat3 proliferated in response to G-CSF, and proliferation was not affected by the presence of tamoxifen. Conversely, G-CSF-induced proliferation of 32Dcl3/DNStat3/CEBPA cells in the presence of tamoxifen was dramatically reduced (Fig. 5, *B* and *C*).

32Dcl3/DNStat3 cells maintain morphologically immature characteristics and proliferate without granulocytic differentiation after G-CSF stimulation. We examined the morphological changes in 32Dcl3 and 32Dcl3/DNStat3 cells induced by G-CSF after translocation of C/EBPα from the cytoplasm to the nucleus. When tamoxifen was added to medium containing G-CSF, 32Dcl3/DNStat3/CEBPA cells rapidly began to differentiate into granulocytes, and 5 days later, about 40% of the cells were morphologically similar to mature neutrophils. In contrast, 32Dcl3/DNStat3/CEBPA cells cultured in G-CSF-containing medium without tamoxifen appeared immature with blast-like morphologic features (Fig. 6, Table II). To quantitatively analyze the difference in granulocyte maturation in 32Dcl3/DNStat3/CEBPA cells stimulated by G-CSF in the presence of tamoxifen, the mature granulocyte marker Gr-1 was monitored by FACS analysis. 32Dcl3 cells differentiate into Gr-1-positive neutrophils in response to G-CSF (Fig. 7*A*). As shown in Fig. 7*D*, Gr-1-positive cells were increased by the addition of tamoxifen in 32Dcl3/DNStat3/CEBPA cells treated with G-CSF, although low levels were detected in the absence of tamoxifen.

C/EBPα Up-regulates Genes That Are Related to Granulocytic Differentiation—In a conditional expression system, induction of C/EBPα leads to expression of granulocyte-specific genes, such as neutrophil primary granule genes (lysozyme M, *NGAL*) and the G-CSF receptor gene (17). In 32Dcl3/DNStat3 cells, the expression of these genes following G-CSF stimulation was inhibited (Fig. 8, *A*, *C*, and *E*). Interestingly, only *NGAL* was up-regulated by G-CSF in 32Dcl3/DNStat3/CEBPA cells following the restoration of C/EBPα (Fig. 8*B*). Conversely, the expression of lysozyme M and the G-CSF receptor were not changed by the restoration of C/EBPα (Fig. 8, *D* and *F*). These data suggest that regulatory factors in addition to C/EBPα may be involved in the induction of expression of granulocyte-specific genes by G-CSF.

DISCUSSION

G-CSF plays a pivotal role in granulopoiesis and granulocytic differentiation. The binding of G-CSF to its receptor leads to the activation of the Jak-Stat pathway, phosphatidylinositol-3 kinase pathway, and Ras-MAP kinase cascade (22). In the Jak-Stat pathway, G-CSF activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7, 8).

Dominant-negative Stat3 inhibits G-CSF-induced transcriptional activity of Stat3 (Fig. 1*A*), as does G-CSF-induced granulocytic differentiation *in vitro* (11). Also, more transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). Consequently, Stat3 is thought to play an essential role in G-CSF-induced granulocytic differentiation.

32Dcl3 cells differentiate into neutrophils after treatment with G-CSF, and 32Dcl3/DNStat3 cells (32Dcl3 cells expressing dominant-negative Stat3) proliferate in G-CSF without differentiation. The degree of the phosphorylation of ERK1/2 by

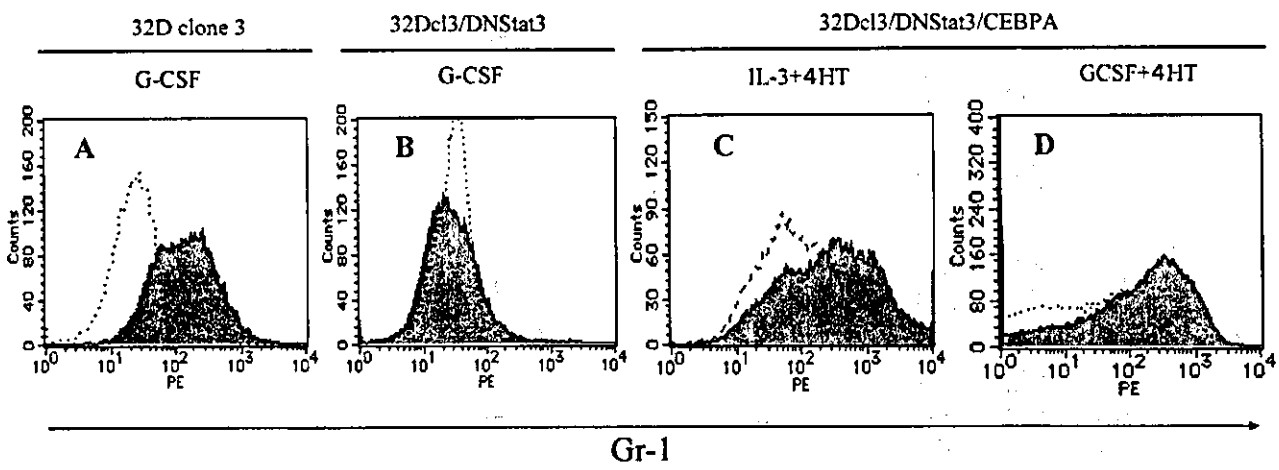

FIG. 7. **The expression of Gr-1 on 32Dcl3, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/CEBPA cells.** 32Dcl3 (*A*) and 32Dcl3/DNStat3 cells (*B*) maintained in IL-3 (*broken line*) were starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF for 5 days (*solid line*). 32Dcl3/DNStat3/CEBPA (*C*) cells maintained in IL-3 were starved of cytokine for 8 h and stimulated with 1.0 ng/ml IL-3 (*C*) or 10 ng/ml G-CSF (*D*) plus 0.5 μM 4-HT (*solid line*) or vehicle (*broken line*) for 5 days.

G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1*B*). We reported that Stat3 null bone marrow cells displayed a significant activation of ERK1/2 after G-CSF stimulation than wild-type bone marrow cells did using Stat3 conditional deficient mice (23). Then the augmented phosphorylation of ERK1/2 in response to G-CSF in 32Dcl3/DNStat3 cells might be caused by the functional abrogation of Stat3 in 32Dcl3/DNStat3 cells.

We compared gene profiles between two cell lines, 32Dcl3 and 32Dcl3/DNStat3 cells, to identify target genes of Stat3 in G-CSF signaling. We found that C/EBPα mRNA levels are rapidly up-regulated in 32Dcl3 cells following G-CSF treatment; these levels are increased 2.39-fold after 6 h and 4.20-fold after 48 h of treatment. In contrast to 32Dcl3 cells, C/EBPα mRNA levels are not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2*A*). The observation that cycloheximide does not inhibit G-CSF-induced increases in C/EBPα transcript levels (Fig. 2*B*) suggests that C/EBPα is induced by G-CSF directly downstream of Stat3. Dahl *et al.* (24) also reported that G-CSF induced the expression of C/EBPα in IL-3-dependent progenitors. *SOCS3* is one of the major target genes of Stat3. We previously reported that the expression level of SOCS3 protein in Stat3-deficient bone marrow cells is a trace, and it is not augmented by G-CSF stimulation (23). Contrary to this suppression of SOCS3 in Stat3-deficient cells, the induction of SOCS3 by G-CSF is not abolished in 32Dcl3/DNStat3 cells (data not shown).

The phosphorylation of ERK1/2 by G-CSF is stronger and the phosphorylation of Stat5 by IL-3 is weaker in 32Dcl3/DNStat3 cells when compared with those in 32D/Cl3 cells, although Stat1 phosphorylation by IFN-γ was not changed between these two cells (Figs. 1*B* and 3). Then there is the possibility that the transfection of dominant-negative Stat3 affects other signaling pathways in 32Dcl3/DNStat3 cells, resulting in the change of C/EBPα regulation. To clarify whether Stat3 directly up-regulates C/EBPα in the G-CSF signaling pathway in 32Dcl3 cells or not, we examined the effect of Stat3C on the transcription of C/EBPα. C/EBPα up-regulated the C/EBPα-dependent gene expression, and the G-CSF stimulation enhanced this C/EBPα-dependent gene expression (Fig. 4*A*). Strikingly, Stat3C augmented the C/EBPα-dependent gene expression as G-CSF stimulation did (Fig. 4, *B* and *C*). This means that G-CSF-induced up-regulation of C/EBPα-dependent gene expression is, at least partly, due to the activation of Stat3.

Two possibilities arise for the mechanism of the induction of C/EBPα transcription by activated Stat3 in the G-CSF signaling pathway. One is that activated Stat3 binds to the promoter region of *C/EBPα* and induces the transcription of C/EBPα. Analysis of the reported murine *C/EBPα* promoter sequence (20) identified no Stat-responsive elements (TTN5AA) (25, 26), but we found six Stat-responsive elements between 6 and 4 kb upstream of the *C/EBPα* transcription initiation site. Activated Stat3 might bind these Stat-responsive elements between 6 and 4 kb upstream of the C/EBPα transcription initiation site. The other possibility is that activated Stat3 might form the complex with C/EBPα and augment the transcriptional activity of C/EBPα because C/EBPα itself is the only protein reported to activate the murine *C/EBPα* promoter (20, 21). When a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK) together with C/EBPα was transfected to 293T cells, C/EBPα up-regulated C/EBPα-dependent gene expression. Activated Stat3 (Stat3C) enhanced this C/EBPα-dependent gene expression in collaboration with C/EBPα, although only Stat3C has no transcriptional activity on p(C/EBP)2TK (Fig. 4, *B* and *C*). In addition, the stimulation of G-CSF allows Stat3 to make the complex with C/EBPα (Fig. 4*D*). Then activated Stat3 by G-CSF makes the complex with C/EBPα and augments the transcriptional activity of C/EBPα. This is one of the reasons why induction of C/EBPα transcript through Stat3 activation by G-CSF occurred in 32Dcl3 cells. Several reports have described factors that repress *C/EBPα* promoter activity, such as SP1 (27), AP2A (28), or MYC (29). We show here for the first time that Stat3 augments the *C/EBPα* promoter activity.

Intracellular transcript levels of several genes were changed following G-CSF treatment downstream of Stat3 activation (Table I). To better identify the role of C/EBPα in Stat3-mediated signaling in G-CSF-induced granulocyte differentiation, C/EBPα-ER (C/EBPα-tamoxifen receptor fusion protein) was stably expressed in 32Dcl3 and 32Dcl3/DNStat3 cells. C/EBPα-ER translocates from the cytoplasm to the nucleus and becomes activated upon treatment with tamoxifen. Strikingly, transfection of C/EBPα-ER into 32Dcl3/DNStat3 cells abolished proliferation and induced myeloid differentiation by G-CSF without Stat3 activation (Figs. 5, *B* and *C*, and 6). These data indicate that C/EBPα activation induced by G-CSF through Stat3 plays an essential role in stopping the cell proliferation and inducing the differentiation to the myeloid lineage.

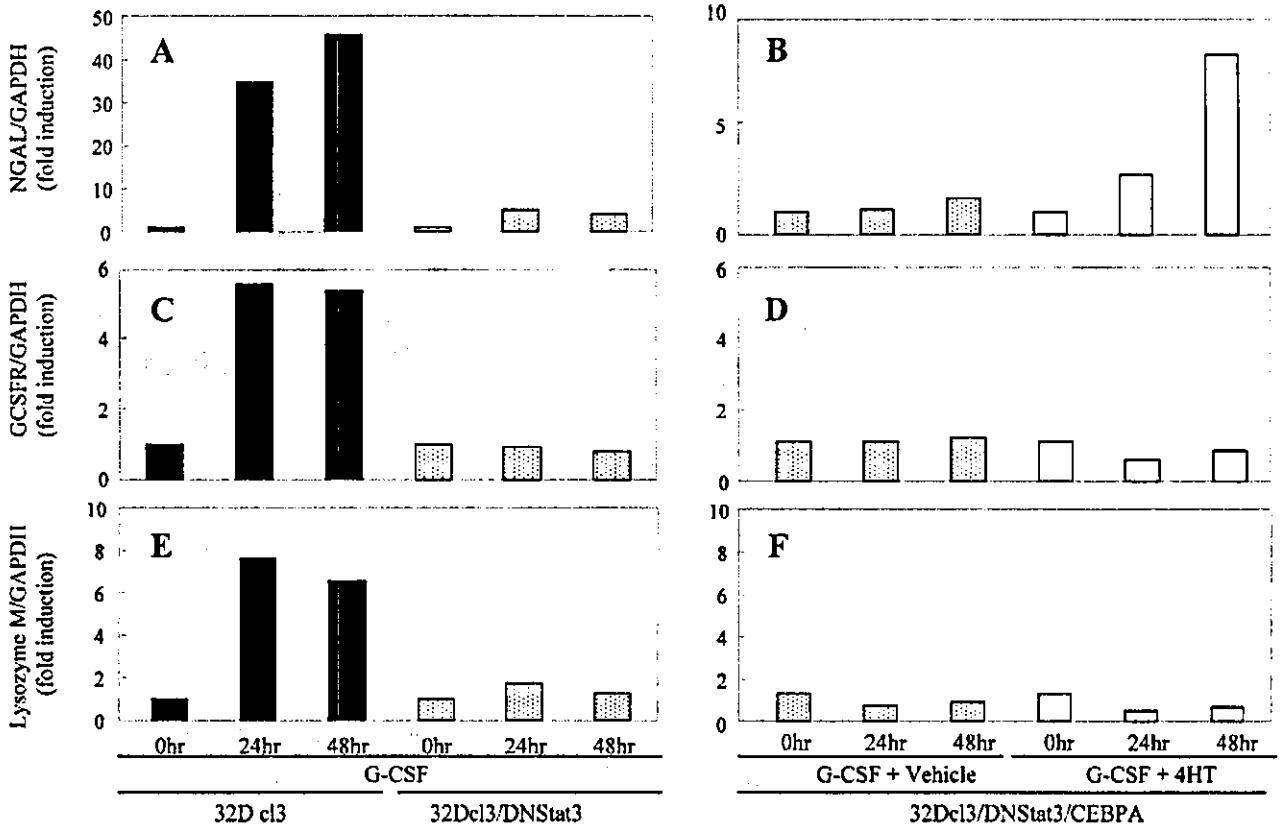

FIG. 8. **Granulocyte-specific gene expressions after C/EBPα induction.** The time course of NGAL (*A* and *B*), G-CSF receptor (*G-CSFR*) (*C* and *D*), and lysozyme M (*E* and *F*) mRNA expression following G-CSF stimulation in 32Dcl3 and 32Dcl3/DNStat3 cells (*A*, *C*, and *E*) or by G-CSF stimulation with 4-HT or vehicle in 32Dcl3/DNStat3/CEBPA cells (*B*, *D*, and *F*) is shown. Cells maintained in IL-3 were starved of cytokines for 8 h and stimulated with G-CSF, G-CSF, plus 4-HT and G-CSF plus vehicle. Total RNA was isolated at the indicated times after the stimulation and transcribed to cDNA, which was subjected to real-time PCR. The *numbers* given on the *vertical axis* represent the fold induction of ratios of average GAPDH-normalized expression values when compared with those before stimulation. Three independent experiments were performed, and similar results were obtained and shown data are the representative of them.

The CEBP family of transcription factor is expressed in multiple cell types, including hepatocytes, adipocytes, keratinocytes, enterocytes, and cells of the lung (30, 31). C/EBPα transactivates the promoters of hepatocyte- and adipocyte-specific genes, which are important for energy homeostasis (32, 33), and *C/EBPα*-deficient mice lack hepatic glycogen stores and die from hypoglycemia within 8 h of birth (34). In the hematopoietic system, C/EBPα is exclusively expressed in myelomonocytic cells (35, 36). C/EBPα expression is prominent in mature myeloid cells, and previous investigations found that C/EBPα is critical for early granulocytic differentiation. Mice with a targeted disruption of the *C/EBPα* gene demonstrate an early block in granulocytic differentiation, but they develop normal monocytes (19). Conditional expression of C/EBPα is sufficient to induce granulocytic differentiation (17). In contrast to the essential role of C/EBPα in granulocytic differentiation, the role of Stat in granulopoiesis is controversial. Stat3 is the principle Stat protein activated by G-CSF, with Stat5 and Stat1 also activated to a lesser degree (8, 10). In mice lacking *Stat5a* and *Stat5b*, the number of colonies produced in response to G-CSF was reduced 2-fold despite normal circulating numbers of neutrophils (9). Myeloid cell lines expressing dominant-negative forms of Stat3 (11, 37, 38) and transgenic mice with a targeted mutation of the G-CSF receptor that abolishes G-CSF-dependent Stat3 activation (12) demonstrate that Stat3-activation is required for G-CSF-dependent granulocytic proliferation and differentiation.

In the present study, we clearly demonstrate that the expression of C/EBPα mRNA is up-regulated through the activation of Stat3 in response to G-CSF, and the Stat3-C/EBPα signaling cascade plays an important role in G-CSF-induced differentiation. Contrary to these data, however, we and others showed that mice conditionally lacking Stat3 in their hematopoietic progenitors developed neutrophilia, and bone marrow cells were hyper-responsive to G-CSF stimulation (23, 39). Additionally, mice with tissue-specific disruption of *Stat3* in bone marrow cells die within 4–6 weeks after birth with Crohn's disease-like pathogenesis (40). These mice exhibit phenotypes with dramatic expansion of myeloid cells, leading to massive infiltration of the intestine with neutrophils, macrophages, and eosinophils. Cells of the myeloid lineage also demonstrate autonomous proliferation. These apparently disparate results may be explained by the need for molecules in addition to Stat3 to regulate C/EBPα expression *in vivo*, the *in vivo* functional redundancy among C/EBPα regulators, or the absence of the abrogation of SOCS3 induction by G-CSF in 32Dcl3/DNStat3 cells. In 32Dcl3 cells, the Stat3-C/EBPα pathway might be favored, and other pathways may contribute little to granulocytic differentiation in response to G-CSF.

Among C/EBP family, C/EBPε is important for late phase of granulocytic differentiation, and its expression is up-regulated by G-CSF independent of Stat3 (11). A previous report showed that C/EBPε is a transcriptional target of C/EBPα in 32Dcl3 cells (41). From these reports and our results, we speculated that a small amount of C/EBPα is enough for the induction of the transcription of C/EBPε by G-CSF or that there are multiple signaling steps except for Stat3-C/EBPα to induce the transcription of C/EBPε by G-CSF.

Induction of C/EBPα led to not only morphologic differentiation but also expression of granulocyte-specific genes (17). In 32Dcl3/DNStat3 cells, the induction of the G-CSF receptor, lysozyme M, and NGAL in response to G-CSF was abrogated (Fig. 8). Restoration of C/EBPα in these cells led to expression of only the *NGAL* gene, and thus, 32Dcl3/DNStat3 cells differentiated by the induction of C/EBPα may not be functional as mature neutrophils. In these cells, therefore, activation of C/EBPα is not sufficient for the induction of lysozyme M or G-CSF receptor genes, and the presence of other molecules appears to be required for their expression.

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Potent receptor-mediated cytotoxicity of granulocyte colony-stimulating factor-*Pseudomonas* exotoxin, a fusion protein against myeloid leukemia cells☆

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Abstract

A chimeric toxin in which the cell-surface binding domain of *Pseudomonas* exotoxin A was replaced with mature human granulocyte colony-stimulating factor (G-CSF) was produced in *Escherichia coli*, purified and tested for its biological activity on the human G-CSF-responsive myeloid leukemia cell line, UT7/GR. This fusion protein, termed G-CSF-PE40, showed potent cytotoxicity in the cell line in a dose-dependent manner. G-CSF-PE40 displaced binding of biotinylated G-CSF to its receptor, and the cytotoxicity of G-CSF-PE40 was neutralized by an excess of wild-type G-CSF, indicating the receptor-mediated effects of this chimeric toxin. When G-CSF-PE40 was injected into normal mice, they showed transient neutropenia but no significant changes in the numbers of red blood cells or platelets. Furthermore, G-CSF-PE40 prolonged the survival of mice transplanted with syngeneic myeloid leukemia cells. These observations suggest that G-CSF-PE40 may be useful in targeted therapy of myeloid leukemia cells expressing G-CSF receptors.

Keywords: Targeting therapy; Granulocyte colony-stimulating factor; G-CSF receptor; *Pseudomonas* exotoxin A; Acute myeloid leukemia

Bacterial and plant toxins have been attached chemically or genetically to monoclonal antibodies and polypeptide hormones to target their cytotoxicity toward specific eukaryotic cells [1,2]. One such toxin, *Pseudomonas* exotoxin A (PE), is composed of three structural domains [3]: domain Ia is essential for binding to the cell-surface receptor, domain II for translocation across the cell membrane into the cytoplasm, and domain III for ADP-ribosylation of elongation factor 2, the process essential for cell death [2,4]. The role of domain Ib has yet to be determined [5]. A 40-kDa mutant of PE lacking domain Ia (PE40) was shown previously to retain full enzymatic activity but showed no cytotoxicity due to the lack of endocytosis [4]. The cytotoxic activity of PE40 can be restored by fusing the gene segment encoding PE40 to cDNAs encoding several growth factors, including transforming growth factor-α [4,6], interleukin-2 [7], interleukin-4 [8], interleukin-6 [9], and interleukin-13 [10], and expressing these chimeric genes in *Escherichia coli*.

Granulocyte colony-stimulating factor (G-CSF) is a 20-kDa glycoprotein, which is produced by activated macrophages, endothelial cells, and fibroblasts [11–13]. G-CSF primarily regulates production of neutrophilic granulocytes, enhances their maturation, and activates their function [13]. In fact, injection of pharmacological doses of G-CSF into normal mice results in marked increases in the numbers of granulocytes and their

☆ *Abbreviations:* G-CSF, granulocyte colony-stimulating factor; PE, *Pseudomonas* exotoxin A; AML, acute myeloid leukemia; SCT, stem cell transplantation.

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committed progenitors without significant effects on other types of hematopoietic cells [12]. Furthermore, mice lacking either G-CSF or its receptor have chronic neutropenia, myeloid progenitor cell deficiency, and impaired neutrophil mobilization [14,15]. G-CSF also stimulates clonogenic growth of myeloid leukemia cells in vitro [16,17]. The numbers of G-CSF receptors and responses to G-CSF in leukemia are controversial [12]. G-CSF specifically binds to its cell-surface receptor, which can be detected mainly in myeloid cells, including mature neutrophils and their progenitors, as well as the majority of myeloid leukemia cells. Thus, G-CSF appears to be an ideal vehicle for delivering certain biological substances into not only normal myeloid cells but also myeloid leukemia cells. Conjugation of a cytotoxic agent, such as PE40, to G-CSF would allow the development of novel lethal weapons against a subtype of acute myeloid leukemia (AML) cells in which surface expression of G-CSF receptors is abnormally up-regulated [18].

Here, we report the construction, expression, and purification of G-CSF-PE40. As expected, this toxin was active on myeloid leukemia cell line, which is dependent on G-CSF for growth and survival. Furthermore, injection of the G-CSF toxin resulted in not only transient neutropenia in normal mice but also prolonged survival of myeloid leukemic mice. The possibility of clinical application of G-CSF-PE40 is also discussed.

Materials and methods

Materials. Enzymes and chemicals were purchased from TaKaRa Bio (Ohtsu, Japan), New England Biolabs (Beverly, MA), Sigma–Aldrich (St. Louis, MO), or Amersham Biosciences (Piscataway, NJ), and used under the conditions recommended by the suppliers. Human recombinant G-CSF and a goat anti-human G-CSF antibody were provided by Chugai Pharmaceutical (Tokyo, Japan). A goat anti-PE antibody was obtained from Invitrogen (Carlsbad, CA). [^{3}H]Leucine was purchased from Amersham Biosciences.

Plasmids, bacterial strains, and cell lines. E. coli strain DH5α (Invitrogen) was used for transformation and amplification of plasmids. BL21(λDE3) (Invitrogen), which carries a T7 RNA polymerase gene in lysogenic and inducible form, was used for the synthesis of G-CSF-PE40. Plasmid pVC38H, which carries the human transforming growth factor-α cDNA under control of the phage T7 late promoter, was a kind gift from Dr. Pastan (National Cancer Institute, Bethesda, MD) [6]. Human G-CSF cDNA was a gift from Dr. Nataga (Osaka University, Osaka, Japan). The human myeloid leukemia cell line UT-7 (referred to as UT-7/EPO in the original report [19]) and its derivative, UT-7/GR (originally referred to as UT-7/EPO G-full), transformed with the human G-CSF receptor, were kind gifts from Dr. Komatsu (Jichi Medical School, Tochigi, Japan), and were maintained in Iscove's modified Dulbecco's medium with 10% fetal calf serum and 1 U/ml human erythropoietin (Chugai Pharmaceutical, Tokyo, Japan) as described previously [19].

Plasmid construction. The chimeric gene encoding G-CSF-PE40 was constructed as summarized in Fig. 1A. First, we created an *Nde*I site at the 5′ end and a *Hin*dIII site at the 3′ end of the G-CSF coding sequence by polymerase chain reaction using a pair of primers: sense, 5′-T GGA ATT CAT ATG ACa CCa tTa GGC CCT GCC AGC-3′;

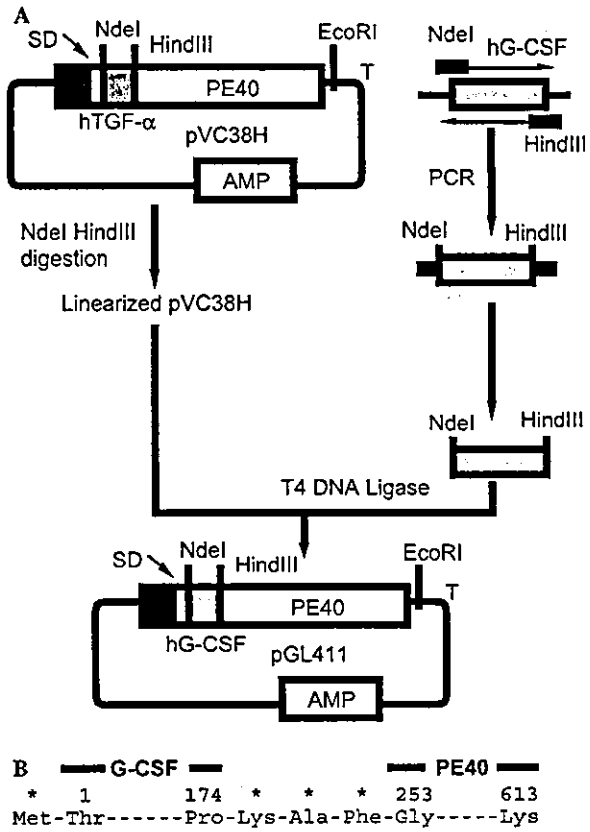

Fig. 1. (A) Scheme for construction of expression plasmid pGL411, encoding G-CSF-PE40. (B) Part of the amino acid sequence of G-CSF-PE40. One amino acid (*) was added at the amino-terminus of G-CSF to create an initiation codon. Three amino acids (*) were added between G-CSF and PE40 to create a *Hin*dIII site. *Abbreviations:* SD, Shine–Dalgarno sequence; hTGF-α, cDNA encoding human tumor growth factor α; AMP, ampicillin resistance gene.

antisense, 5′-CC TAA GCT TTG GGC TGG GCA AGG TGG CG-3′. (Restriction sites are underlined, and the mutated codons in the sense primer are indicated by lowercase letters, while these changes are silent for amino acid sequences of G-CSF.) After 30 cycles of polymerase chain reaction using linearized human G-CSF cDNA as a template, amplified DNA fragments were cut with *Nde*I and *Hin*dIII. After separation on a low-melting-point agarose gel, the 0.5 kb DNA fragment was eluted and subcloned into the corresponding sites of pVC38H, which carries the human transforming growth factor-α cDNA under control of the phage T7 late promoter. The resulting plasmid (pGL411) encoded an additional three amino acids between G-CSF and PE40 (Fig. 1B).

Expression and purification of G-CSF-PE40. We induced, refolded, and purified G-CSF-PE40 by techniques similar to those described previously [10,20–22]. BL21(λDE3) cells were transformed with the plasmid pGL411, cultured in 1000 ml LB broth (BioWittaker, Walkersville, MD) supplemented with carbenicillin (50 μg/ml) for 3 h, at which time optical density at 595 nm (OD_{595}) was 1.272, and then expression of the recombinant protein was induced by incubation with 1 mM isopropyl β-D-thiogalactoside for 150 min. The following procedures were performed at 4 °C unless otherwise stated. Cells were collected by centrifugation at 6000*g* for 30 min. The cell pellet was treated with 75 ml of ice-cold sucrose buffer (20% sucrose, 30 mM Tris, pH 8.0, and 1 mM EDTA) for 10 min and centrifuged at 8000*g* for 20 min. The cell pellet was resuspended in 150 ml of ice-cold water,

incubated for 10 min, and centrifuged at 15,000*g* for 20 min. Finally, the pellet was suspended in 180 ml of 50/20 TE buffer (50 mM Tris, pH 8.0, and 20 mM EDTA) and treated with 200 μg/ml lysozyme for 60 min at room temperature with intermittent shaking. Then, the suspension was homogenized after inactivation of lysozyme by addition of 4 ml of 5 M sodium chloride and centrifuged at 50,000*g* for 30 min. The resulting pellet was resuspended with 180 ml of 50/20 TE buffer supplemented with 0.1% Triton X-100 and collected by centrifugation at 50,000*g* for 30 min. This washing procedure was repeated five times. The resulting pellet was the inclusion body and was used as the source of G-CSF-PE40 fusion protein.

Solubilization and renaturation of the protein. The inclusion body was denatured in 15 ml of solubilization buffer (7 M guanidine hydrochloride, 0.1 M Tris, pH 8.0, and 2 mM EDTA). The solubilized inclusion body supernatant including approximately 100 mg protein was reduced by adding 100 mg of dry dithioerythritol and incubated at room temperature for 24 h. The reduced protein was diluted in 1000 ml of refolding buffer (1.5 M urea, 75 mM Tris, pH 8.0, 500 mM L-arginine, 1 mM oxidized glutathione, and 1.5 mM EDTA) and incubated at 10 °C for 48 h. The solution including refolded protein was dialyzed against 12 L of dialysis buffer (20 mM Tris, pH 8.0, and 100 mM urea) with three buffer changes in 24 h. The dialysate was centrifuged at 2500*g* for 30 min and filtered through a 0.45-μm filter.

Ion exchange chromatography on Q-Sepharose and MonoQ. The refolded and dialyzed protein solution was loaded onto an 8 ml Q-SepharoseFF column (Amersham Biosciences) attached to a fast protein liquid chromatograph (Amersham Biosciences) and eluted with a linear gradient of sodium chloride (0–1.0 M NaCl in 20 mM Tris, pH 8.0) in 8 column volumes. Half-column volume fractions were collected at a flow rate of 4.0 ml/min, and the eluted proteins were concentrated in two fractions, dialyzed against 20 mM Tris, pH 8.0, and then loaded onto a MonoQ column (Amersham Biosciences). The samples were eluted with a similar linear gradient of sodium chloride in 15 column volumes. One-column volume fractions were collected at a flow rate 0.5 ml/min. The purified fusion protein was analyzed for the presence of G-CSF-PE40 by SDS–PAGE and immunoblotting. The fractions containing the fusion protein were dialyzed against phosphate-buffered saline and filter sterilized for further biological assays.

Quantification of G-CSF-PE40. Protein concentration was measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

Gel electrophoresis and immunoblotting. SDS–PAGE on 10–20% gradient Tricine gels (Invitrogen) was performed according to the manufacturer's instructions. The gels were stained with Coomassie blue. For immunoblotting, the electrophoresed samples were transferred onto PVDF membranes (Millipore) and processed using antibodies to native PE or to human G-CSF. Detection was performed with ECL Western blotting detection reagents (Amersham Biosciences).

Cytotoxicity assay. We tested the biological activities of the chimeric toxin using UT-7, and its derivative, UT-7/GR [19]. While UT-7/GR expresses G-CSF receptors, UT-7 does not. UT-7 or UT-7/GR cells were washed 2–3 times with phosphate-buffered saline and then cultured at a density of 2.5×10^4 cells/well in 96-well plates in Iscove's modified Dulbecco's medium containing 10% fetal calf serum. Varying concentrations of G-CSF-PE40 were added to the wells and the cells were cultured for 2 days. Aliquots of 10 μl of WST-1 solution (TaKaRa Bio) were added to each well and 4 h later the OD at 440–600 nm of each well was read with a Spectra Max 340 (Molecular Devices, Sunnyvale, CA). In the competition assay, cells were incubated with various concentrations of G-CSF-PE40 and a constant concentration (12.5 μg/ml) wild-type human G-CSF (Fig. 4A) or a constant concentration (1.0 μg/ml) of G-CSF-PE40 and various concentrations of competitive wild-type human G-CSF (Fig. 4B). Tritiated leucine uptake assay was performed to test protein synthesis as described previously [10,20–22].

Receptor binding assay. G-CSF receptor binding of G-CSF-PE40 was performed as described previously [23–25]. Briefly, 2.5×10^5 UT-7/GR and 100 ng/ml of biotin-labeled G-CSF with or without various concentrations of G-CSF-PE40 were incubated for 1 h on ice. After washing with phosphate-buffered saline containing 5% fetal calf serum, cells were incubated with 10% streptavidin–phycoerythrin conjugate (Becton–Dickinson, San Jose, CA). We confirmed that a 100-fold excess of unlabeled G-CSF completely competed for biotin-labeled G-CSF binding. Fluorescence intensity was measured with LSR (Becton–Dickinson). The data were collected for 10,000 events. Then, gated cells were analyzed using CellQuest software (Becton–Dickinson). No apoptotic population was detected after short-term incubation with G-CSF-PE40 (data not shown).

Animal models. C57/BL mice were obtained from the Animal Center, Kyushu University (Fukuoka, Japan). We injected 3.5 μg/day of G-CSF-PE40 into C57/BL mice intraperitoneally for 3 continuous days and then examined neutrophil count, lymphocyte count, hemoglobin level, and thrombocyte counts. SJL-J mice and the L-103 leukemia cell line were kind gifts from Dr. Tamura (Chugai Pharmaceutical). The mice and cells were maintained as described previously [13,26,27]. The SJL-J mice were transplanted with 1×10^5 L-103 cells, and then 114 ng/body/day of G-CSF-PE40 was administered intraperitoneally for the following 3 continuous days. Overall survival of the L-103 leukemia-transplanted mice was monitored.

Results

Construction, expression, and purification of G-CSF-PE40

The expression vector for G-CSF-PE40 was constructed essentially as described previously (Fig. 1A) [10,20–22]. The resulting fusion protein, G-CSF-PE40, was composed of an amino-terminal methionine residue, amino acids 1–174 of mature human G-CSF fused to amino acids 253–613 of PE through a three-amino acid linker, lysine–alanine–phenylalanine (Fig. 1B). The calculated molecular mass of G-CSF-PE40 was 66 kDa. BL21(λDE3) cells transformed with pGL411 were treated with isopropyl β-D-thiogalactoside, collected, and processed as described in Materials and methods. Aliquots were dissolved in SDS–PAGE sample buffer (whole-cell extract) and analyzed by electrophoresis. A protein of approximately 66 kDa was induced (Fig. 2A), and the antigenicity of the protein was confirmed by Western blotting using anti-G-CSF antibody and anti-PE40 antibody (Fig. 2B). G-CSF-PE40 was highly concentrated into an inclusion body (Fig. 2C). We purified the protein from the inclusion body to a purity of more than 95% as determined by visual estimation (Fig. 2D). This highly purified protein was used to determine the bioactivity of G-CSF-PE40.

G-CSF-PE40 is cytotoxic to cells expressing G-CSF receptors

G-CSF-PE40 showed cytotoxicity against UT-7/GR cells, which express a large number of G-CSF receptors (Fig. 3A) [19]. After incubation with 1.0 μg/ml G-CSF-PE40 for 48 h, the cytoplasm of most cells contained

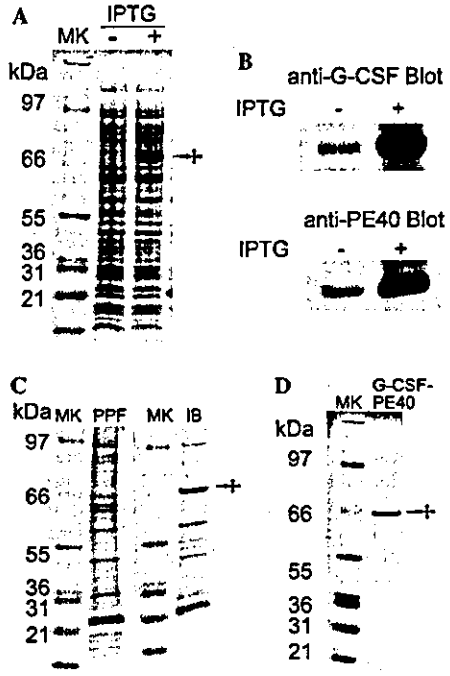

Fig. 2. SDS–PAGE of G-CSF-PE40 expressed in *E. coli* BL21(λDE3). A Coomassie blue-stained gel (A) and the results of immunoblotting with an antibody to G-CSF (B, upper) or PE (B, lower) are shown. Molecular masses of the standards are indicated in kDa. Arrows indicate G-CSF-PE40, which migrated to a position corresponding to a mass of approximately 66 kDa. (C) The expressed G-CSF-PE40 fractionated into the periplasmic fraction (PPF) and the inclusion body (IB) were visualized by Coomassie blue staining, which indicated that expressed G-CSF-PE40 was concentrated in the inclusion body rather than the periplasmic fraction. (D) Highly purified G-CSF-PE40 indicated more than 95% purity on visual inspection. We utilized this purified G-CSF-PE40 in subsequent analyses. *Abbreviations:* MK, multimark molecular size marker (Invitrogen); IPTG, isopropyl β-D-thiogalactoside; PPF, periplasmic fraction; IB, inclusion body.

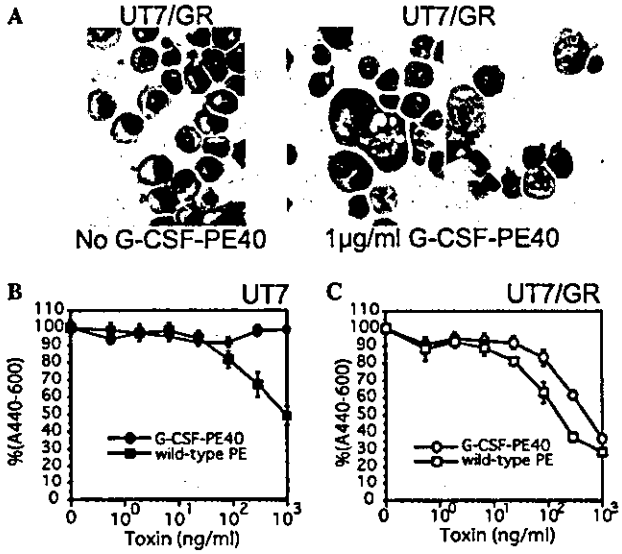

Fig. 3. Cytotoxicity of G-CSF-PE40. (A) UT-7/GR cells cultured for 48 h with (right panels) or without (left panel) 1.0 μg/ml of G-CSF-PE40 were visualized with Wright–Giemsa stain. Both pictures were obtained under equal power (400×). Most cells incubated with G-CSF-PE40 carried clear vesicles in their cytoplasm, and the nuclei of some cells were scattered. (B) The effects of G-CSF-PE40 and wild-type PE on UT-7 without G-CSF receptor expression. While wild-type PE was toxic to this cell line, G-CSF-PE40 was not. (C) The effects of G-CSF-PE40 and wild-type PE on UT-7/GR with G-CSF receptor expression. Both wild-type PE and G-CSF-PE40 were toxic to this cell line.

transparent granules, and in some cells, the nuclei seemed scattered (Fig. 3A).

To examine the receptor specificity, we administered G-CSF-PE40 and wild-type PE to UT-7 cells, which do not express G-CSF receptors, and UT-7/GR cells, which show high-level expression of G-CSF receptors [19]. Wild-type PE was almost equally cytotoxic to both cell lines independent of G-CSF receptor expression. On the other hand, G-CSF-PE40 was cytotoxic only to UT-7/GR and showed no effect on UT-7 (Figs. 3B and C). These results simply suggest the receptor specificity of the cytotoxicity of G-CSF-PE40.

Cytotoxicity of G-CSF-PE40 is G-CSF receptor-specific

To confirm the G-CSF receptor specificity of G-CSF-PE40, we conducted three further assays. First, we neutralized the cytotoxicity of G-CSF-PE40 by the addition of 12.5 μg/ml of wild-type G-CSF (Fig. 4A). The presence of a constant concentration of G-CSF neutralized the cytotoxicity of G-CSF-PE40. The cytotoxicity of a constant concentration (1.0 μg/ml) of G-CSF-PE40 was also suppressed by various concentrations of wild-type G-CSF (Fig. 4B). Wild-type human G-CSF at a concentration of 1.0 μg/ml neutralized 1.0 μg/ml G-CSF-PE40 almost completely. This suggested reduced receptor affinity of G-CSF-PE40 compared with that of wild-type human G-CSF. Receptor binding was tested using biotin-labeled human G-CSF. Biotin-labeled G-CSF bound to cells expressing G-CSF receptor can be detected by flow-cytometry [23–25]. The binding was suppressed by G-CSF-PE40 in a concentration-dependent manner. Figs. 4C and D show mean fluorescence intensity and percent of gated population, representing the mean amount of biotin-labeled G-CSF bound to UT-7/GR cells and the proportion of cells with biotin-labeled G-CSF, respectively. As biotin-labeled G-CSF and wild-type G-CSF bind to the same binding sites in a competitive manner, suppression of biotin-labeled G-CSF by G-CSF-PE40 indicates binding between G-CSF-PE40 and G-CSF receptor. The data described in this section suggest that G-CSF-PE40 binds to G-CSF receptors, and its cytotoxicity is mediated through G-CSF receptor binding. It is possible that G-CSF stimulates proliferation of myeloid leukemia cells. However, enhanced cell-cycle progression of leukemia cells by G-CSF signal can result in chemosensitivity to S-phase specific agents such as cytosine arabinoside

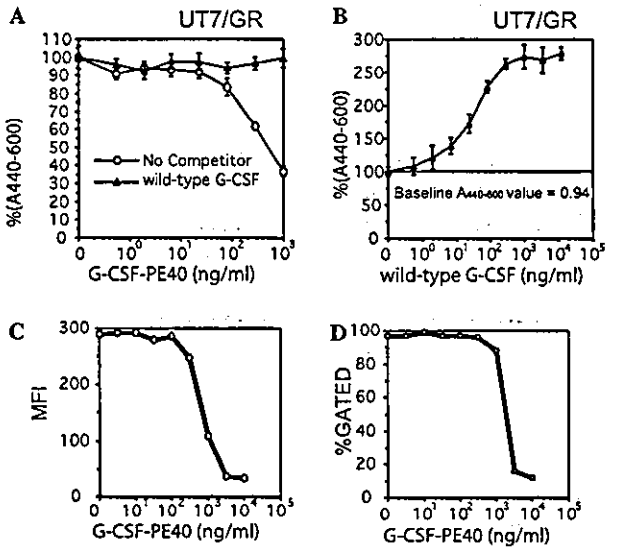

Fig. 4. G-CSF receptor specificity of G-CSF-PE40 on UT-7/GR determined by competitive assay using wild-type G-CSF or biotin-labeled G-CSF. (A) A constant concentration (12.5 μg/ml) of wild-type G-CSF competitively inhibited the cytotoxicity of G-CSF-PE40 up to 1 μg/ml. (B) The cytotoxicity of a constant concentration (1.0 μg/ml) of G-CSF-PE40 was neutralized by a high concentration of wild-type G-CSF. (C) The mean fluorescence intensity (MFI) that represents biotin-labeled G-CSF bound to G-CSF receptor expressed on the UT-7/GR cell surface was competitively suppressed by G-CSF-PE40 in a dose-dependent manner. (D) Similarly, %GATED represents the population of cells binding biotin-labeled G-CSF through their G-CSF receptor. The number of these cells was reduced by G-CSF-PE40 in a dose-dependent manner.

(AraC) in vitro and in vivo. We previously discussed the application of this chemosensitization effect in the publications listed below. We also expect that the combination of AraC with G-CSF-PE40 instead of G-CSF may be more attractive regimen against myeloid leukemias [28–30].

G-CSF-PE40 is cytotoxic to neutrophil and leukemia cells in vivo

One of the major obstacles in targeted therapy, such as antibody-based regimens or those using immunotoxins, is the suitability of murine models. As human tumor-associated antigens and other target molecules are generally not present in normal mouse tissues, murine models are usually not applicable for assessment of adverse reactions in such cases [31,32]. On the other hand, as mentioned above, murine G-CSF receptor has been shown to interact with human G-CSF, and therefore murine models will be applicable in many respects to test G-CSF-PE40. To clarify this point, we administered G-CSF-PE40 to healthy and leukemic mice. As normal murine neutrophils express a large number of G-CSF receptors, they may be specific targets of G-CSF-PE40 in vivo. As expected, healthy C57/BL mice administered G-CSF-PE40 showed severe neutropenia, recovery from which took over one month (Fig. 5A). As the period of neutrophil turnover is only a few days, and their recovery took as long as one month, G-CSF-PE40 must have damaged not only neutrophils but also their committed progenitors. The mice also showed mild lymphocytosis and anemia (Figs. 5B and C). As lymphocytes and erythrocytes do not express G-CSF receptors [11], these mild transient reactions may have been nonspecific; e.g., volume overload or nonspecific immune reactions against contaminating molecules. We are currently planning to perform further experiments in a murine model using much more highly purified G-CSF-PE40 to clarify these points. Thrombocyte count was not significantly affected by administration of G-CSF-PE40 (Fig. 5D). Finally, we administered G-CSF-PE40 to SJL-J leukemic mice. These mice inoculated with 1×10^5 L-103 cells usually died of leukemia within 2–3 weeks. The survival period without treatment in this assay was consistent with that reported previously [26]. SJL-J

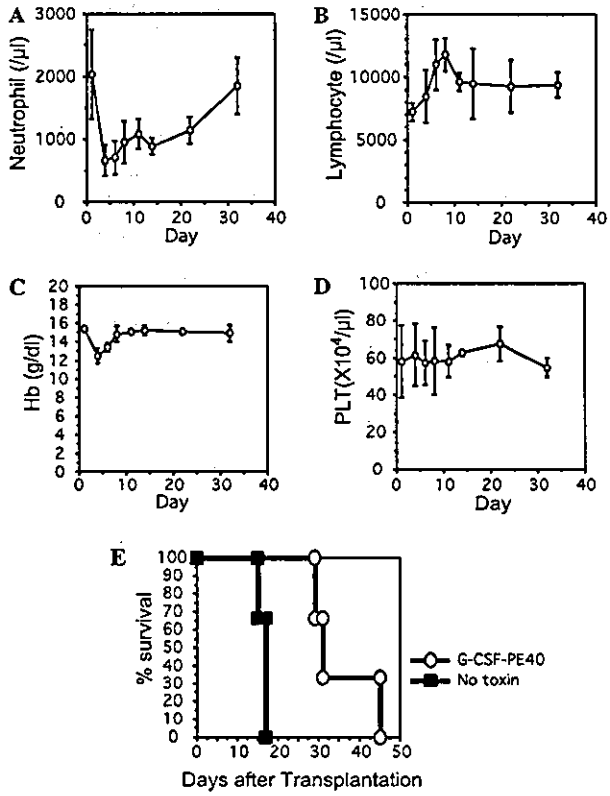

Fig. 5. Effects of G-CSF-PE40 on normal mice (A–D) and myeloid leukemic mice (E). (A–D) Four C57/BL mice were injected with 3.5 μg G-CSF-PE40 on days 1, 2, and 3. The neutrophil counts (A), lymphocyte counts (B), hemoglobin (Hb) concentration (C), and thrombocyte (PLT) counts (D) were monitored. (E) Three SJL-J myeloid leukemic mice were treated with G-CSF-PE40 (-O-) or with vehicle alone (-■-) as described in Materials and methods. The survival after transplantation of L-103 cells is shown in Kaplan–Meyer plots.