抗TNF-α抗体療法

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☞ *Point*

- クローン病では、血清、糞便および粘膜固有層におけるTNF-α活性の上昇や産生細胞の増加が報告され、活動期末梢血においてLPSあるいはOK-432の刺激によりマクロファージのTNF-α産生能が亢進していることが報告されている。
- 抗TNF-α抗体には、抗TNF-αキメラモノクローナル抗体(infliximab)、CDP571抗体(humicade)、recombinantヒトTNF-α receptor(p75)－Fc結合蛋白(etanercept)およびヒト型抗TNF-αモノクローナル抗体(adalimumab)などがある。
- 1997年Targanらが行った、108例の中等症以上のクローン病の患者を対象としたPhase Ⅱb/Ⅲ trialでは、5mg/kgが投与量として適当ではないかとしている。
- クローン病の合併症としての瘻孔治療効果に関しても、infliximabの優れた成績が1999年Presentらにより報告されている。
- infliximab投与の継続に伴ってATIの出現頻度は高まり、反復投与では実に61％に出現を認め、臨床効果の減弱や効果持続期間の短縮などが報告されている。
- しかし、最近infliximab投与前にhydrocortisoneを点滴投与することによってATIのlevelを有意に低下できることが報告され、投与方法の改善などが期待されている。

本稿ではクローン病の病態に関与するサイトカインについて概略するとともに、特に生物工学的治療薬(biologics)の代表格として近年注目されている抗TNF-α抗体を用いたTNF-α阻害によるクローン病の治療成績を中心に述べる。

クローン病（図1）

クローン病は腹痛・下痢・下血、全身倦怠感、発熱、肛門病変などを主訴とし、再燃と緩解を繰り返す原因不明の炎症性腸疾患である。後に述べるさまざまな免疫学的研究により、徐々にその病因・病態が解明されつつあり、これに即した有効な治療法が開発されているが、いまだ根本的な治療法は存在していないのが現状である。

クローン病においては主にマクロファージや樹状細胞の活性化に伴うIL-1、IL-6、IL-12、IL-18、TNF-α、IFN-γなどのTh1優位のサイトカイン

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産生により病態が形成されるものと考えられている。これらのなかでIL-6はT・B細胞、線維芽細胞、血管内皮細胞、マクロファージなど多彩な細胞から産生され、活動性クローン病炎症粘膜におけるmRNAならびに蛋白、さらに血清レベルで上昇していることが報告されている。TNF-αがIL-6産生を誘導する作用を有することを考えると、クローン病での病態にTNF-αとIL-6が協調して深く関わっている可能性がある。IL-12は細菌、lipopolysaccharide (LPS)などの刺激により単球/マクロファージ系細胞より産生され、NK細胞、T細胞に作用してIFN-γの産生を誘導する。一方IL-18についても本症の病態形成と密接な関係があることが知られており[2,3]、Pizarroやわれわれの研究室では、クローン病炎症粘膜上皮細胞や活性化マクロファージにおいてIL-18を強く発現することを報告した。さらにIL-12ならびにIL-18のノックアウトマウスでの検討にて、IFN-γ産生能がきわめて低下していることは、*in vivo*におけるIFN-γ産生はIL-12とIL-18のsynergisticな作用が重要であることが予想されている[6]。

このようにいくつかのサイトカインの研究がクローン病の病因・病態解明のために行われ、それらのサイトカインを標的とした治療が開発されている。例えばIL-6のレセプターを標的とした抗IL-6レセプター抗体はわが国で開発され、クローン病に対する治研が行われた(詳細は他項を参照されたい)。その他の抗サイトカイン療法のなかでも近年クローン病で最も注目され臨床応用が進んでいるサイトカインがTNF-αである。TNF-αは血管内皮に働き、セクレチンの発現を高めて炎症細胞を炎症の場にリクルートさせ、浮腫、血液凝固活性を亢進し、微小循環障害をきたすとともに、血管内皮細胞や上皮細胞のIL-18産生を亢進させ、同時に肉芽腫形成にも関与する。クローン病では、血清、糞便および粘膜固有層におけるTNF-α活性の上昇や産生細胞の増加が報告され、さらに活動期末梢血においてLPSあるいはOK-432の刺激によりマクロファージのTNF-α産生能が亢進していることが報告されている。また、炎症局所におけるホーミング受容体の発現を亢進させ、炎症細胞浸潤を引き起こすことも知られている。クローン病においてはTNF-αが消化管の炎症の惹起ならびに持続にきわめて重要なfactorである。

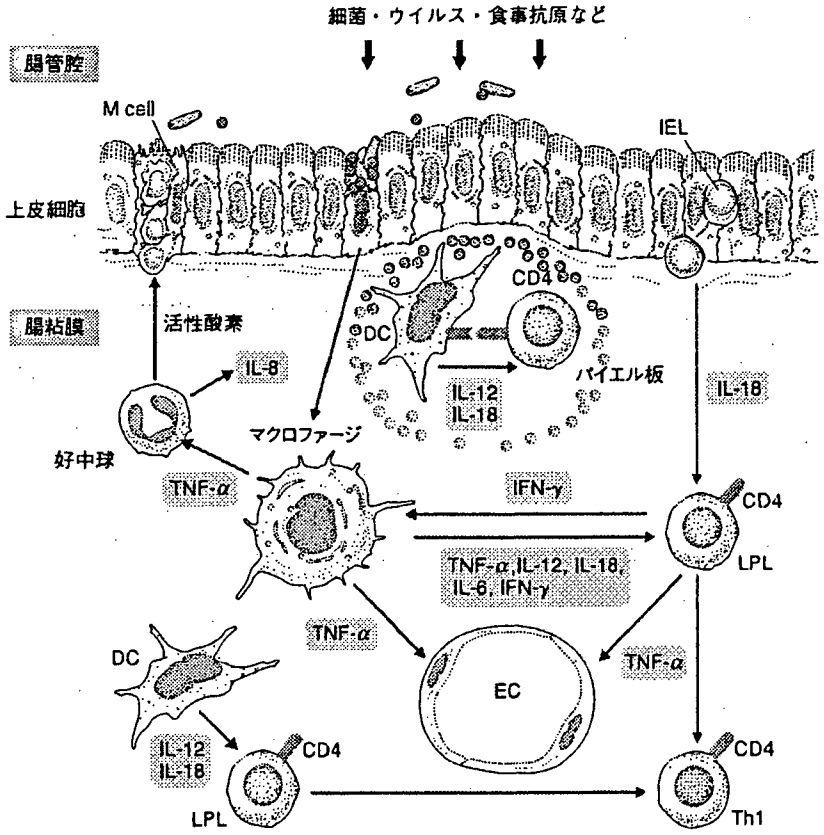

図1　サイトカインよりみたクローン病の病態

IEL：上皮間リンパ球、LPL：粘膜固有層リンパ球、DC：樹状細胞、EC：血管内皮細胞

抗TNF-α抗体療法

抗TNF-α抗体には、抗TNF-αキメラモノクローナル抗体(infliximab)、CDP571抗体(humicade)、recombinantヒトTNF-αreceptor (p75)-Fc結合蛋白(etanercept)およびヒト型抗TNF-αモノクローナル抗体(adalimumab)などがある[4](図2)。この抗体の作用機序としては、TNF-αや可溶性TNF-α自体を中和してTNF-αの作用を抑えることよりも、マクロファー

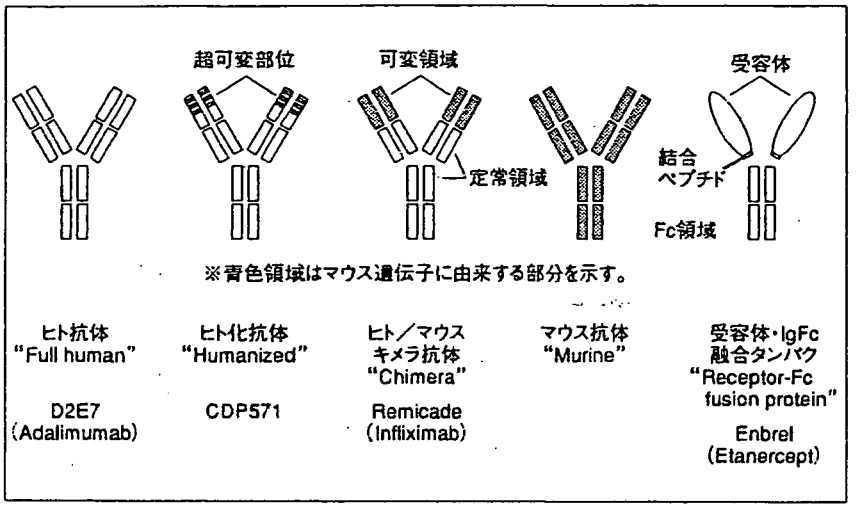

図2　各種TNF-α抗体の分子構造(文献4より改変引用)

	プラセボ群	infliximab投与群		
		5mg/kg	10mg/kg	20mg/kg
組み入れ患者数	25	27	28	28
CDAI				
治療前	288±54	312±56	318±59	307±50
第2週	272±75	182±79*	238±92*	217±90*
第4週	271±82	166±76*	226±115*	211±107*
IBDQ				
治療前	128±29	122±29	116±23	118±28
第4週	133±28	168±36*	146±41*	149±35*
CRP(mg/L)				
治療前	12.6±13.9	22.1±23.6	23.2±34.2	22.4±23.9
第2週	16.4±18.9	4.2±3.0*	6.7±7.3*	8.7±13.8*
第4週	14.8±16.6	5.7±9.3*	12.1±18.6*	6.9±11.6*

CDAI；Crohn's disease activity index, IBDQ；Inflammatry Bowel Disease Questionnaire
*；$p<0.05$

表1　クローン病の緩解導入に対するInfliximabの効果(文献8より改変引用)

ジの細胞表面上の膜結合型TNF-αに作用して、活性型マクロファージをアポトーシスに陥らせることにより、Th1系のサイトカイン産生を抑制する、などのメカニズムが考えられている。Infliximabの構造は、75％がヒト、25％はマウス由来で、humicadeが95％がヒト、5％がマウス由来で構成されている。実際にこの両抗体をヒトに投与すると、ヒト抗キメラ抗体(Human Antichimeric Antibody；HACAもしくはAntibodies to Infliximab；ATI)ができることが報告されたため、この解決策として100％がヒト由来であるetanerceptの導入が期待された。しかし米国での大規模なRCTで、その治療効果について否定的な成績が出たことより、現時点ではinfliximabが臨床の場で主として用いられている。しかしATIの問題もあり将来的にはヒト型抗TNF-αモノクローナル抗体(adalimumab)に期待が寄せられている。以下にクローン病に対する抗TNF-α抗体療法の治療成績につき概説する。

1．クローン病への応用

1993年オランダにてステロイドやその他の薬剤に抵抗性の大腸型クローン病の12歳の少女に抗TNF-αマウス・ヒトキメラ型モノクローナル抗体infliximabが投与されたのがクローン病に使用された最初の症例であり、その劇的な効果が世界で初めて報告された[5]。

2．緩解導入効果

この報告に続き、まず緩解導入を目的に、1995年にVan Dullemenら[6]は、ステロイド抵抗性の活動性クローン病の患者10人の内8人に10mg/kg、残りの2人に20mg/kgのinfliximabを1回静脈内投与するpilot studyを行った。その結果なんら副作用を認めることなく投与後4週以内に8例にCDAI(Crohn's disease activity index)の正常化(CDAI150以下)を認め、さらにその8例は4週後の内視鏡検査にてほとんどの潰瘍性病変の治癒が認められ、単回投与での臨床的効果は約4ヵ月間持続したと報告された。

最初のdose-responnse studyは1996年McCabeRPら[7]によって、infliximabの投与量を1、5、10、20mg/kgの群に分けた振り分け試験にて行われた。その結果1mg/kg投与では効果の持続が有意に短く、これをもとに

次のPhase Ⅱb/Ⅲtrialの投与量が決定された。

1997年Targanら[6]は、Phase Ⅱb/Ⅲtrialとして108例の中等症以上のクローン病の患者に現行治療(ステロイドなど)を継続したまま二重盲検法無作為化比較試験を行った。Infliximabを5、10、20mg/kg群に分けて1回静脈内投与しプラセボ群と比較したところ、投与後4週の時点で改善が認められた例は、プラセボ群で17%であったのに対し、5mgの群で81%、10mgの群で50%、20mgの群では64%に認められプラセボ群と比較し有意な改善を認めた。またCDAIが150以下の緩解に至った例は、infliximab投与群の33%に認められ、プラセボ群の4%に比べ有意に優れていた(表1)。

さらに改善のみられなかった群にinfliximab(10mg/kg)の再静脈内投与を施行したが、1回目にinfliximabを投与し効果が得られなかった群においては、改善率34%、緩解率17%と初回プラセボ投与群の成績と有意差を認めず、infliximabに抵抗性の群の存在が示唆された。また投与量に関しては5mg/kgと20mg/kgとの間に有意な差はなく、今後5mg/kgが投与量として適当ではないかとしている。

この結果infliximabの緩解導入効果に関する有用性は、ほぼ確定された。Infliximabの緩解導入効果の有用性は広く認知されるに至り米国においては、1998年に米国食品医薬品局(FDA)の認可を受け市販されている。またこれらの結果よりクローン病においてTNF-αが病因の中心的役割をなすという仮説がより強く裏付けられたかたちとなった。しかし単回投与では、その効果は比較的長期に認められるものの、そのほとんどは再燃が避けられないことも明らかとなった。

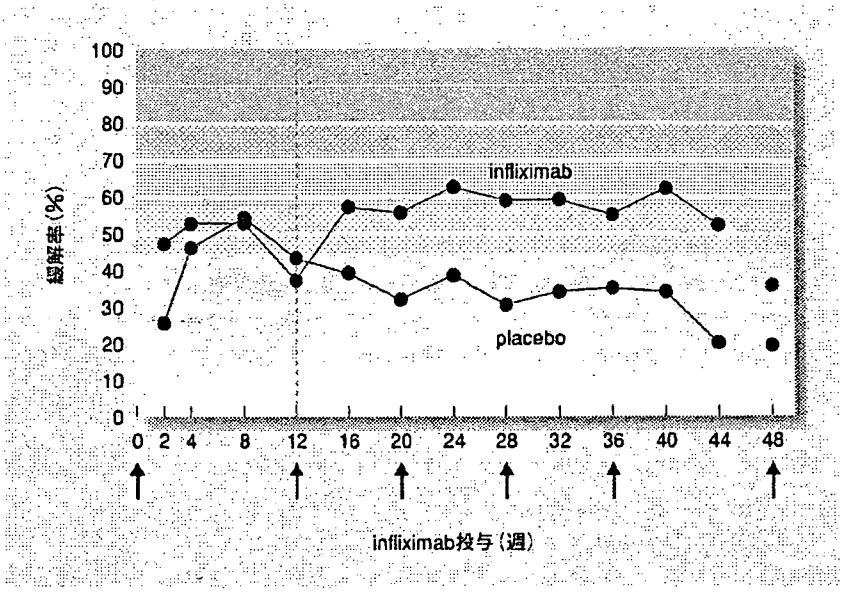

図3 Infliximab連続投与によるクローン病の効果(文献10より改変引用)

3. 反復投与による緩解維持効果

緩解維持を目的とした反復投与の安全性と効果についてRutgeertsら[9]がTarganら[8]のPhase Ⅱb/Ⅲrialの続きとして報告している。彼らはPhase Ⅱb/Ⅲtrialでinfliximabの投与にて有効であったクローン病患者73例を対象として、infliximabを8週ごとに10mg/kgを4回繰り返し静注し、投与終了後8週の段階で最終的な評価を行った。その結果、緩解維持率はinfliximab群で52.9%とプラセボ群の20%に比し有意に優れていた(図3)。また臨床効果維持率でもinfliximab群では62%であったのに対し、プラセボ群では37%であった。そしてinfliximab投与群のうち、6-MPやazathioprineなどの免疫抑制剤を同時投与(治験組み込み以前から)されたグループの臨床効果維持率は75%であり、免疫抑制剤の同時投与のないグループの50%に比べて良好な傾向を認めたとしている。

維持療法の有効性については、さらに大規模な試験(A Crohn's Disease Clinical Trial Evaluating Infliximab in a New Long-Term Treatment Regimen Ⅰ:ACCENT Ⅰ)が実施された。ACCENT Ⅰ[10]では中等度～重度のクローン病患者573例(CDAI220以上)にinfliximab 5mg/kgを単回投与し、2週目に有効性が認められた患者を、プラセボを2および6週目とその後8週毎に46週目まで投与する群(Group Ⅰ)、2および6週目とその後8週毎にinfliximab 5mg/kgを46週目まで投与する群(Group Ⅱ)、2および6週目にinfliximab 5mg/kgを投与し、その後8週毎に46週目までinfliximab 10mg/kgを投与する群(Group Ⅲ)に無作為に振り分けた。有効性評価対象例335例の、54週後の有効率(CDAIが25%以上および70点以上低下した場合を有効と定義)および緩解

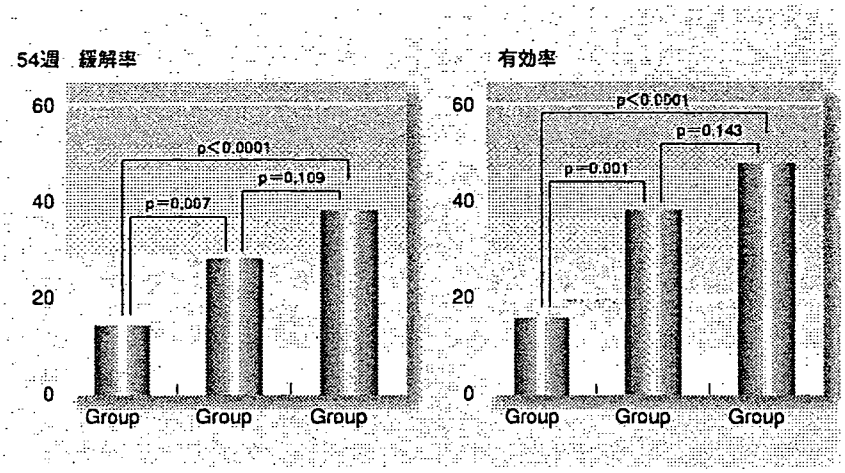

図4 Infliximab維持療法における有効率および緩解率(文献10より改変引用)

	プラセボ群	infliximab 5mg/kg群	infliximab 10mg/kg群
組み入れ患者数	31例	31例	32例
脱落患者数	4例	1例	1例
第一次目標到達率(排膿瘻孔50%以上減少)	26%	68%	56%
完全緩解率(排膿瘻孔の消失)	13%	55%	38%
CDAI(第18週)中央値	160	104	123
PDAI(第18週)中央値	7.0	4.0	5.0
infliximabの副反応(重症)	—	1例	4例

表2 クローン病の瘻孔に対するinfliximabの効果(文献12より改変引用)

率(CDAI150点未満を緩解と定義)は、infliximab 5mg/kgまたは10mg/kg投与群(Group Ⅱ、Ⅲ)の方がプラセボ投与(Group Ⅰ)に比べて有意に高かった(図4)。さらにACCENT Ⅰの別の解析からは、導入療法として単回投与よりも3回投与するほうが効果が高いことや、infliximabでの維持療法がステロイドの減量や中止に有効であることも判明している。また最近の報告では症状の有無によらず、8週おきに計画的に投与するほうが、症状再燃にあわせてepisodicに投与するよりもATIの産生も低く有効性が高いことが判明している[11]。

4. 瘻孔治療効果

クローン病の合併症としての瘻孔治療効果に関しても、優れた成績が1999年Presentら[13]により報告されている。その報告によれば、クローン病の合併症として3ヵ月以上にわたって排膿を有する腹壁瘻もしくは肛門周囲瘻を有する患者94例を対象としてプラセボおよびinfliximabを5mg/kg、10mg/kg投与する群に振り分け、0、2、6週の3回静脈注射とし、排膿を有する瘻孔の50%以上の減少で検討すると5mg/kg群で68%、10mg/kg群で38%であったのに対しプラセボ群では26%であった。さらにすべての瘻孔閉鎖率では5mg/kg群で55%、10mg/kg群で38%であったのに対しプラセボ群では13%であり有意差をもって効果を認めた(表2)。また瘻孔閉鎖期間の平均は約3ヵ月であった。瘻孔性クローン病患者におけるインフリキシマブの維持療法を検討したACCENT Ⅱ試験では、その維持効果が公表されている

5. 副作用

クローン病、関節リウマチの治験におけるinfliximabを投与された患者についてまとめると、infliximab投与に伴う一般的な副反応としては、頭痛、嘔気、上気道感染を76%に認めプラセボ投与群の57%と比べやや頻度が高い傾向があった[13]。Infliximabの投与時および投与後2時間以内の急性期に何らかの副反応が起こる率は、初回投与時は、わずか7%であるのに対し、2回目の投与では10%に認められた。やや頻度が高まるのは感作されることによるものと考えられ、それを裏付けるようにヒト抗キメラ抗体(Human Antichimeric Antibody ; HACAもしくはAntibodies to Infliximab ; ATI)陽性の群では投与早期の副反応を36%に認めるのに対し、HACA陰性の群では11%であった。しかしそのほとんどは非特異的かつ投与を中止するほどのものではなかった。またATI(Antibodies to Infliximab)に関しては、低値ではあるものの患者の13%に出現を認め、投与の継続に伴ってその出現頻度は高まり、反復投与では実に61%に出現を認め、臨床効果の減弱や効果

持続期間の短縮などが報告されている[14]。最近infliximab投与前にhydrocortisoneを点滴投与することによってATIのlevelを有意に低下できることが報告され、投与方法の改善などが期待されている[15]。そのほか、患者の9%に二本鎖DNAに対する抗体の出現を認めたが、その他のSLEに特徴的な抗核抗体の出現や低補体血症は認めず、SLEの発症は認められなかった[14]。これまでの結果においては、infliximabは重大な副作用がほとんどなく大変有用と考えられていたが、最近infliximabと結核発症の関連が報告され注意を促されている[16]。さらに心不全を悪化させる可能性や治癒過程でのイレウスの発症の報告、多発性硬化症の発生や悪化の報告などもあり、長期投与に伴う抗キメラ抗体の出現や悪性疾患の発生、感染症などを含め安全性に関しては、今後さらなるデータの蓄積と検討が不可欠と考えられる。

おわりに

TNF-α阻害によるクローン病の治療は米国ではすでに一般的な治療法となっている。わが国でもinfliximabのクローン病への保健適応が認可され、その効果が期待されている。今後は、医療経済や栄養療法など他の治療法とのかかわりを含めinfliximabの適応とその位置付けをより明確にしていくことが大切と考えられる。Infliximabはクローン病の慢性炎症機序の中心にせまる治療法と考えられるが、いまだ不明な点も多く、病因の解明と根本的な治療法の開発が待たれる。

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ORIGINAL ARTICLE

Impact of cytomegalovirus serostatus on outcome of unrelated cord blood transplantation for adults: a single-institute experience in Japan

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Abstract

Cytomegalovirus (CMV) disease is one of the major infectious complications after allogeneic hematopoietic stem cell transplantation (SCT). Several studies have shown that CMV-seropositive patients have a substantial survival disadvantage after bone marrow transplantation (BMT) or peripheral blood SCT (PBSCT). Between August 1998 and February 2006, 101 adult patients underwent myeloablative cord blood transplantation (CBT) from unrelated donors at our institution. Sixteen and 85 patients were CMV-seronegative and CMV-seropositive, respectively, prior to CBT. Outcomes of CBT were compared between CMV-seronegative and CMV-seropositive patients. The cumulative incidences of neutrophil engraftment at 60 d after CBT did not differ between CMV-seronegative and CMV-seropositive patients (100% and 94%, $P = 0.09$); however, the cumulative incidence of platelet engraftment at 100 d was higher in CMV-seronegative patients than CMV-seropositive patients (100% vs. 86%, $P < 0.005$). The cumulative incidence of CMV antigenemia at 100 d was lower in CMV-seronegative patients than CMV-seropositive patients (0% vs. 77%, $P < 0.001$); however, the cumulative incidences of CMV disease did not differ between CMV-seronegative and CMV-seropositive patients (0% vs. 1%, $P = 0.84$). The probabilities of disease-free survival at 2 yr also did not differ between CMV-seronegative and CMV-seropositive patients (92% vs. 72%, $P = 0.16$). The outcomes of CBT for CMV-seropositive patients as well as CMV-seronegative patients in our series were favorable. This might be due to effective antiviral therapy for CMV infection. Large-scale studies are needed to determine the impact of recipient CMV serostatus on the outcome of CBT for adults.

Key words Cytomegalovirus; antigenemia; cord blood transplantation

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Cytomegalovirus (CMV) disease, particularly interstitial pneumonia, is one of the major complications after allogeneic hematopoietic stem cell transplantation (SCT) (1, 2). Ganciclovir therapy for preventing CMV disease has reduced the incidence of early CMV disease after SCT. In the recent era of effective antiviral therapy, the impact of recipient CMV serostatus on the outcome of SCT has been studied (3–5). Several studies have shown that CMV-seropositive patients have a substantial survival disadvantage after bone marrow transplantation (BMT) or peripheral blood SCT (PBSCT). The survival disadvantage has been suggested to be largely restricted to profoundly immunosuppressed SCT patients, such as those receiving T-cell-depleted grafts and/or grafts from human leukocyte antigen (HLA)-mismatched or unrelated donors. Increased mortality in CMV-seropositive recipients is probably due to both direct and indirect effects of CMV infection as well as drug toxicities.

Umbilical cord blood transplantation (CBT) from an unrelated donor has recently been utilized as an alternative therapy for patients who do not have suitable donors for BMT or PBSCT (6, 7). Cord blood (CB) lymphocytes are functionally and phenotypically immature when compared with adult blood lymphocytes (8); therefore, infectious complications including viral infections are suggested to occur frequently in patients after CBT. Our previous study showed that the probabilities of developing positive CMV antigenemia and requiring preemptive ganciclovir therapy after CBT were 79% and 67%, respectively, in 24 CMV-seropositive patients (9); however, no patients developed CMV disease. In four CMV-seronegative patients, none developed positive CMV antigenemia. In the present study, we compared the outcomes of CBT between 16 CMV-seronegative patients and 85 CMV-seropositive patients.

Patients and methods

Patients

Between August 1998 and February 2006, 101 adult patients underwent unrelated CBT following a myeloablative conditioning regimen including 12 Gy total body irradiation (TBI) at The Institute of Medical Science, The University of Tokyo. Sixteen and 85 patients were CMV-seronegative and CMV-seropositive, respectively, prior to CBT. Patient characteristics are shown in Table 1. CMV-seronegative patients were younger than CMV-seropositive patients (median age, 32 yr vs. 40 yr; $P = 0.014$). The other characteristics did not differ significantly between the two groups (data not shown).

Table 1 Patient characteristics

	Negative	Positive
No. patients	16	85
Age, yr		
Median	32	40
Range	16–49	16–55
Gender, *n*		
Male	9	46
Female	7	39
Disease, *n*		
AML	12	42
ALL	2	15
CML	1	3
MDS	1	7
NHL	0	8
Disease status, *n*		
Low risk	10	37
High risk	6	48
TNC, $\times 10^7$/kg		
Median	2.60	2.38
Range	2.04–3.98	1.16–5.29
CD34, $\times 10^5$/kg		
Median	0.88	0.94
Range	0.27–1.53	0.15–8.97
HLA matching, *n*		
5/6 or 4/6	8	65
3/6 or 2/6	8	20
ABO incompatibility, *n*		
Identical	4	23
Minor	5	28
Major	4	18
Bidirectional	3	16
Preparative regimen, *n*		
TBI + CY + AraC	14	63
TBI + CY	2	9
TBI + FLU + AraC	0	8
TBI + FLU + L-PAM	0	3
GVHD prophylaxis, *n*		
CSP + MTX	16	82
CSP	0	3

Negative indicates cytomegalovirus-seronegative; Positive, cytomegalovirus-seropositive; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; TNC, total nucleated cell; Identical, ABO-identical; Minor, minor ABO-incompatible; Major, major ABO-incompatible; Bidirect, bidirectional ABO-incompatible; TBI, total body irradiation; CY, cyclophosphamide; AraC, cytarabine; FLU, fludarabine; L-PAM, melphalan; CSP, cyclosporin; MTX, methotrexate.

Characteristics of the grafts

The median numbers of total nucleated cells (TNCs) before freezing were 2.60×10^7/kg (range, 2.04–3.98) in CMV-seronegative patients and 2.38×10^7/kg (range, 1.16–5.29) in CMV-seropositive patients ($P = 0.37$) (Table 1). The median numbers of CD34-positive cells were 0.86×10^5/kg (range, 0.27–1.53) in CMV-seronegative patients and 0.94×10^5/kg (range, 0.15–8.97) in CMV-seropositive patients ($P = 0.37$). The matching of HLA-A and -B was confirmed by low-resolution typing methods, and the matching of HLA-DRB1 was confirmed by high-resolution typing methods. All CB grafts were from HLA-mismatched unrelated donors.

Transplantation procedures and supportive care

Transplantation procedures and supportive care were described previously (10). Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine (3 mg/kg/d) and a short course of methotrexate (15 mg/m^2 on day +1 and 10 mg/m^2 on days +3 and +6). To facilitate neutrophil recovery, recombinant human granulocyte colony-stimulating factor was administered intravenously at a dose of 5 μg/kg/d from day +1 after CBT. All patients received 1000 mg/d acyclovir

orally from day −3 to day +35 to prevent herpes simplex virus infection.

All platelet concentrates were obtained from single-donor apheresis. All packed red blood cells (RBCs) and platelets transfused were leukoreduced by filtration. In addition, four of 16 CMV-seronegative patients were transfused with only CMV-seronegative blood components.

Preemptive ganciclovir therapy

Cytomegalovirus infection was monitored using an antigenemia assay twice a week after engraftment during the hospital stay, and at each hospital visit after discharge. The antigenemia assay consisted of direct immunostaining of polymorphonuclear leukocytes with monoclonal antibodies C10/C11 (Clonab CMV; Biotest, Dreieich, Germany) directed against the lower matrix protein pp65. We used two preemptive strategies for preventing CMV disease. Prior to May 2002, we used risk-adapted preemptive ganciclovir therapy (9). In brief, ganciclovir was initiated when two or more positive cells per 3×10^5 cells on two slides were detected in patients at high-risk for CMV disease, defined as those developing grade II–IV acute GVHD and those receiving more than 0.5 mg/kg prednisolone. In patients other than the above, ganciclovir was initiated when four or more positive cells per 3×10^5 cells on two slides were detected. Ganciclovir was initially administered at a dose of 5 mg/kg twice daily for 14 d, and then continued at a dose of 5 mg/kg once daily. Ganciclovir therapy was discontinued when neutropenia with an absolute neutrophil count (ANC) of $< 1 \times 10^9/l$ developed, or when negative results on two consecutive tests were obtained. From May 2002, we used another preemptive strategy with ganciclovir (11). Irrespective of the risk for CMV disease, preemptive ganciclovir therapy was initiated when any positive cells were detected. In addition, the initial dose of ganciclovir was reduced to 5 mg/kg once daily, which was half the dose in the previous method. Ganciclovir therapy was discontinued according to the same criteria as above.

Definition

Neutrophil engraftment was defined as an ANC exceeding $0.5 \times 10^9/l$ for three consecutive days. Platelet engraftment was defined as a platelet count exceeding $20 \times 10^9/l$ for three consecutive days without platelet transfusion. Acute GVHD was graded according to previously published criteria (12). Patients who survived more than 100 d after CBT with sustained donor hematopoiesis were considered at risk for the development of chronic GVHD (13).

Cytomegalovirus disease was diagnosed as described previously (14). Briefly, CMV pneumonia was defined by the presence of signs and symptoms of pulmonary disease together with the detection of CMV in bronchoalveolar lavage fluid or lung tissue samples. CMV disease of the central nervous system (CNS) is defined by the identification of CNS symptoms together with the detection of CMV in cerebrospinal fluid or brain biopsy specimens. CMV disease of other organs was diagnosed by biopsy with clinical signs and symptoms.

Low-risk diseases were defined as acute leukemia and lymphoma in the first and second complete remission, myelodysplastic syndrome in the early phase, and chronic myelogenous leukemia in the first chronic phase. High-risk diseases were defined as those other than the above.

Statistical methods

Patient characteristics in two groups were compared using the Mann–Whitney *U*-test or Fisher's exact test. Cumulative incidences were estimated in a competing risks setting, death being treated as a competing event (15). In multivariate analysis, a Cox proportional hazards model was used to assess the independent effect of risk factors on the end-points. We used the stepwise variable selection procedures at a significant level of 5%. The following factors were studied: age, gender, disease status, CMV serostatus, TNC dose, CD34-positive cell dose, HLA matching, and ABO incompatibility. Transplantation-related mortality (TRM) was evaluated using the Kaplan–Meier method as well as the cumulative incidence. Disease-free survival (DFS) was evaluated using the Kaplan–Meier method, and differences between curves were compared using the log-rank test. A two-sided P of < 0.05 was considered statistically significant.

Results

Engraftment

All 16 CMV-seronegative patients and 78 of 85 CMV-seropositive patients achieved donor-derived neutrophil engraftment within 60 d after CBT. The median days of neutrophil engraftment were day +21 (range, +19 to +26) and +22 (range, +16 to +46), respectively, after CBT. Of the remaining seven CMV-seropositive patients, three patients developed autologous hematopoietic recovery and four patients died before neutrophil engraftment. The cumulative incidences of neutrophil engraftment at 60 d after CBT were 100% and 94%, respectively [hazard ratio (HR), 1.62; 95% confidence interval (CI), 0.93–2.83; $P = 0.09$] (Fig. 1A). All CMV-seronegative

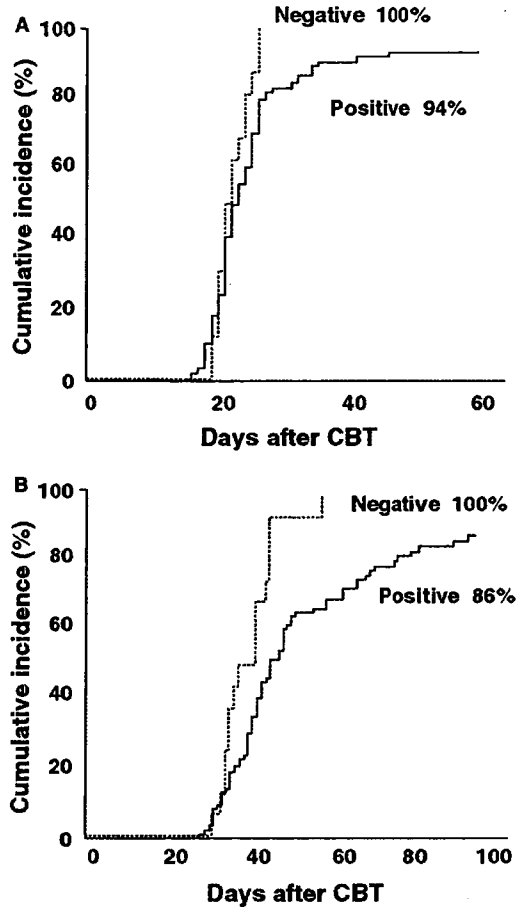

Figure 1 (A) Cumulative incidences of neutrophil engraftment at 60 d after CBT. (B) Cumulative incidences of platelet engraftment at 100 d after CBT. Positive, CMV-seropositive; Negative, CMV-seronegative.

patients and 73 CMV-seropositive patients achieved platelet engraftment within 100 d after CBT. The median days of platelet engraftment were day +37 (range, +29 to +54) and day +40 (range, +26 to +88), respectively. Of the remaining 12 CMV-seropositive patients, three patients developed autologous hematopoietic recovery, and four patients died before neutrophil and platelet engraftment, and five patients who achieved neutrophil engraftment died before platelet engraftment. The cumulative incidence of platelet engraftment at 100 d was significantly higher in CMV-seronegative patients than CMV-seropositive patients (100% vs. 86%; HR, 2.29; 95% CI, 1.29–4.08; $P < 0.005$) (Fig. 1B). In addition, multivariate analysis showed that the larger CD34-positive cell dose was also significantly associated with rapid neutrophil and platelet engraftment (HR, 1.80; 95% CI, 1.18–2.76; $P < 0.01$; and HR, 1.61; 95% CI, 1.03–2.52; $P < 0.05$, respectively).

GVHD

Seven CMV-seronegative patients and 46 CMV-seropositive patients developed grade II–IV acute GVHD after CBT. The cumulative incidences of grade II–IV acute GVHD at 100 d were 50% and 53%, respectively (HR, 0.98; 95% CI, 0.46–2.09; $P = 0.96$) (Fig. 2A). Twelve CMV-seronegative patients and 63 CMV-seropositive patients at risk developed chronic GVHD after CBT. The cumulative incidences of chronic GVHD at 2 yr were 79% and 75%, respectively (HR, 0.63; 95% CI, 0.33–1.19; $P = 0.15$) (Fig. 2B).

CMV infection

No CMV-seronegative patients developed positive CMV antigenemia after CBT. In contrast, 66 CMV-seropositive patients developed positive CMV antigenemia within 100 d after CBT. The median onset of antigenemia in

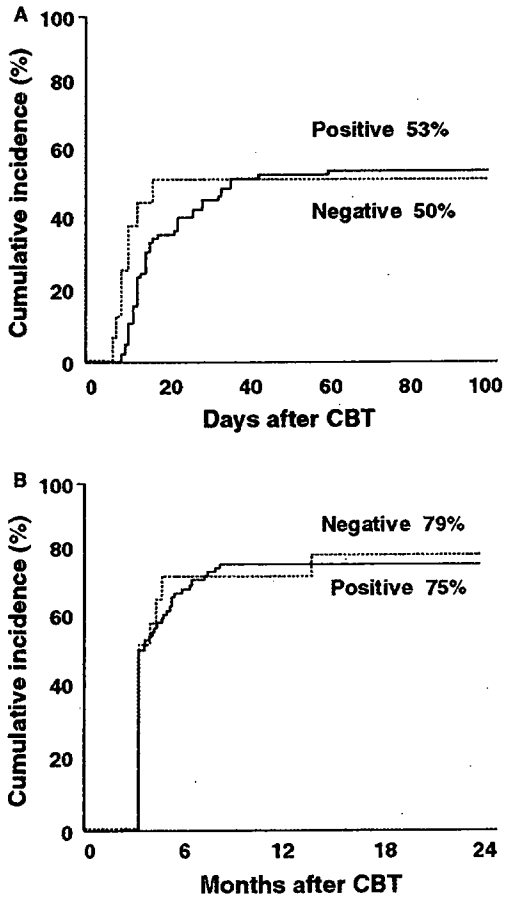

Figure 2 (A) Cumulative incidences of grade II–IV acute GVHD at 100 d after CBT. (B) Cumulative incidences of chronic GVHD at 2 yr after CBT.

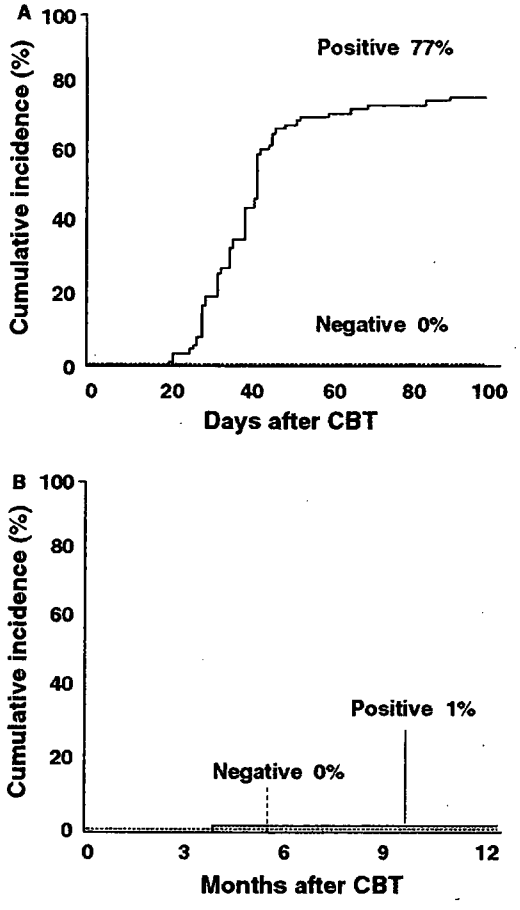

Figure 3 (A) Cumulative incidences of CMV antigenemia at 100 d after CBT. (B) Cumulative incidences of CMV disease at 1 yr after CBT.

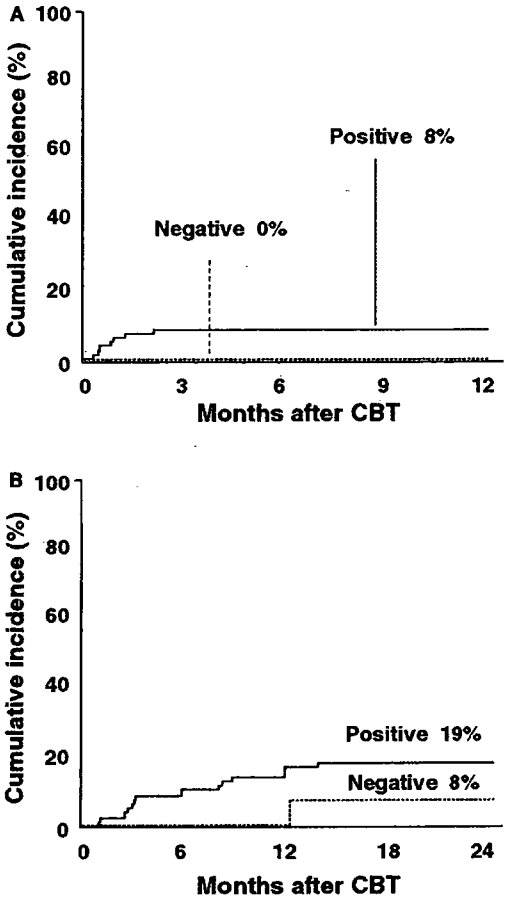

Figure 4 (A) Cumulative incidences of transplantation-related mortality at 1 yr after CBT. (B) Cumulative incidences of relapse at 2 yr after CBT.

CMV-seropositive patients was day +39 (range, +20 to +91) after CBT. The cumulative incidence of CMV antigenemia at 100 d was significantly lower in CMV-seronegative patients than CMV-seropositive patients (0% vs. 77%, $P < 0.001$) (Fig. 3A).

No CMV-seronegative patients developed CMV disease. One CMV-seropositive patient (1%) developed CMV disease of the CNS on day +111, which directly caused her death. The cumulative incidences of CMV disease at 1 yr did not differ significantly between CMV-seronegative and CMV-seropositive patients (0% vs. 1%, $P = 0.84$) (Fig. 3B).

TRM

Within 1 yr after CBT, no CMV-seronegative patients and seven CMV-seropositive patients died without leukemia relapse. CMV disease was considered to be the cause of death in one CMV-seropositive patient, as described above. The main causes of death in the remaining six patients were organ toxicity in three patients and infection in the remaining three patients. No patients died of GVHD within 1 yr after CBT. The cumulative incidences of TRM at 1 yr did not differ significantly between CMV-seronegative and CMV-seropositive patients (0% vs. 8%, $P = 0.23$) (Fig. 4A). The probabilities of TRM at 1 yr which were estimated using the Kaplan–Meier method also did not differ significantly between CMV-seronegative and CMV-seropositive patients (0% vs. 8%, $P = 0.24$).

Relapse

Leukemia relapse occurred in two CMV-seronegative patients at 12 and 54 months after CBT, and 16 CMV-seropositive patients at a median of 4 months (range, 1–36) after CBT. The cumulative incidences of relapse at 2 yr did not differ significantly between CMV-sero-

negative and CMV-seropositive patients (8% vs. 19%; HR, 0.28; 95% CI, 0.04–2.15; $P = 0.22$) (Fig. 4B).

Survival

The probabilities of DFS at 2 yr after CBT were 92% in CMV-seronegative patients and 72% in CMV-seropositive patients (Fig. 5A). The probabilities of DFS at 2 yr did not differ significantly between CMV-seronegative and CMV-seropositive patients (HR for death or relapse, 0.24; 95% CI, 0.03–1.79; $P = 0.16$). Multivariate analysis showed that high-risk disease status was significantly associated with a lower probability of DFS at 2 yr (HR, 4.92; 95% CI, 1.66–14.6; $P < 0.005$).

Discussion

In the present study, we compared the outcomes of CBT between CMV-seronegative and CMV-seropositive patients. The outcomes of CBT for CMV-seropositive patients as well as CMV-seronegative patients in our series were favorable; therefore, statistical analyses did not show significant differences for the cumulative incidences of neutrophil engraftment, CMV disease, and TRM at 1 yr, and the probability of DFS at 2 yr between CMV-seronegative and CMV-seropositive patients after CBT. In addition, the results showing no significant differences in TRM and DFS between two groups may be due to the small patient numbers, particularly of CMV-seronegative patients.

The most striking finding was a marked higher incidence of antigenemia after CBT in CMV-seropositive patients than CMV-seronegative patients (77% vs. 0%, $P < 0.001$). In BMT recipients, the infusion of bone marrow cells from a CMV-seropositive donor was associated with the early recovery of CMV-specific T-cell responses after BMT, which indicates the important role of primed CMV-specific T cells from the donor marrow inoculum for the early recovery of CMV-specific immunity of recipients (16). Therefore, the lack of CMV-specific T cells in the infused CB units as well as the immunological immaturity of CB lymphocytes might be associated with the delayed recovery of CMV-specific immunity after CBT. However, CMV disease occurred only in one CMV-seropositive patient after CBT. Preemptive ganciclovir therapy might effectively reduce the incidence of CMV disease in CMV-seropositive patients after CBT.

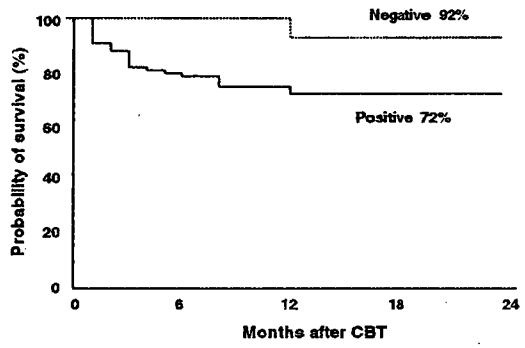

Figure 5 Probability of DFS at 2 yr after CBT.

Previous studies have suggested that CMV-seropositive BMT or PBSCT recipients, particularly those receiving T-cell-depleted grafts and/or grafts from HLA-mismatched or unrelated donors, have a substantial survival disadvantage after SCT (3–5). A higher incidence of infectious complications including CMV disease or severe acute GVHD is considered to be likely responsible for the poor outcome in CMV-seropositive recipients. In CBT recipients, the impact of CMV serostatus on various outcomes has not been studied in detail. In a recent study on CBT for adult patients (median age, 29 yr), the probabilities of DFS at 2 yr after CBT did not differ significantly between 55 CMV-seronegative and 106 CMV-seropositive patients (35% vs. 23%, $P = 0.17$) (17); however, the impact of CMV serostatus on outcomes other than survival was not shown. Other previous studies also did not clearly show the association between CMV serostatus and the outcomes of CBT for adult patients (6, 7).

The outcomes of CBT in CMV-seronegative patients in our series were considered to be excellent; however, the outcomes in CMV-seropositive patients, including the cumulative incidences of CMV disease and TRM at 1 yr, and the probability of DFS at 2 yr, were also favorable. This might be partly due to effective antiviral therapy for CMV infection. In addition, 99 of 101 recipient and donor pairs in the present study were Japanese. The lesser genetic diversity in a single ethnic population might be associated with the favorable outcomes of CBT for CMV-seropositive patients, such as a lower rate of fatal acute GVHD. As the CMV-seronegative population is small in Japanese adults, our study included only 16 CMV-seronegative patients. Large-scale studies are needed to determine the impact of CMV serostatus on the outcome of CBT for Japanese adults.

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Rapid Communication

Cytogenetic Remissions Induced by Interferon α and Imatinib Mesylate are Immunologically Distinct in Chronic Myeloid Leukemia

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Abstract

We compared immunologic parameters of chronic myeloid leukemia (CML) patients in cytogenetic remission receiving imatinib mesylate (STI) treatment, CML patients receiving interferon α (IFN-α), and healthy volunteers. Each group comprised 14 subjects. Median treatment dosages and durations were 6×10^6 IU/week and 174 months, respectively, for IFN-α and 400 mg/day and 54 months for STI. The numbers of T-cells were significantly lower in the 2 patient groups ($P = .0006$), whereas the 3 groups were comparable with respect to the numbers of natural killer cells. Not only the absolute numbers of monocytes and B-cells but also serum immunoglobulin G (IgG) and IgA titers were significantly lower in the STI group than in the IFN-α group ($P < .0001$). For T-cell subsets, the ratio of CD4 T-cells to CD8 T-cells was significantly lower in the IFN-α group than in the STI group, but the proportion of $CD26^{high}CD4^+$ T-cells among $CD4^+$ cells was significantly higher. Collectively, the 2 therapeutic agents induce a distinct immunologic status in CML patients whose hematopoiesis has returned to normal levels.
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Key words: CML; Cytogenetic remission; Interferon α; Imatinib mesylate

1. Introduction

Imatinib mesylate (STI), now the first-line therapy for chronic myeloid leukemia (CML) in the chronic phase, induces a complete cytogenetic response (CCyR) as well as a major molecular response in the vast majority of patients with newly diagnosed CML [1-3]. The long-term efficacy and feasibility of STI has also been confirmed in the IRIS trial [4], and its sustained administration is recommended for preventing disease progression and recurrence from minimal residual disease, which cannot readily be eradicated with STI alone [5-7]. On the other hand, recent articles have shown that STI inhibits not only the T-cell receptor–mediated proliferation and activation of effector T-cells [8,9] but also the development and function of dendritic cells [10,11], suggesting an immunosuppressive state in CML patients who have been treated continuously with STI. Moreover, a recent observation that interferon (IFN)-resistant or IFN-intolerant CML patients frequently showed hypogammaglobulinemia during the subsequent STI therapy suggests a therapy-related functional deficit in B-cells.

Prior to the STI era, IFN-α was the standard choice for the conventional treatment of CML in the chronic phase and was capable of inducing a CCyR in a sensitive but limited population (10%-20%) of patients with a new CML diagnosis [12,13]. Although the mechanisms involved in the selective inhibition of a Philadelphia chromosome–positive (Ph^+) clone by IFN-α have not yet been clarified, various clinical and laboratory observations strongly suggest that the activation of immunologic effector functions, including T-cell and dendritic cell responses, brought about by IFN-α may contribute to its life-prolonging effect [14].

These data taken together indicate that treatment with STI or IFN-α is likely to cause distinct immunologic alterations in CML patients, especially in therapy-sensitive long-term survivors. In the present study, we evaluated various immunologic

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parameters for CML patients in an STI-induced CCyR and for CML patients with an IFN-α–induced CCyR and detected significant differences between the 2 patient groups in the values for some of these parameters.

2. Patients, Materials, and Methods

2.1. Patients

Fourteen individuals were enrolled in each of 3 groups, the 2 patient groups plus a control group of healthy volunteers. Of the 14 patients in the STI group, 6 had a new CML diagnosis and had started STI treatment, and 8 patients had been treated with IFN-α but switched to STI at the beginning of the domestic release of STI in 2002. The latter 8 patients had already taken STI for approximately 5 years at the commencement of this study. All patients in the STI group have achieved both a CCyR [12] and a major molecular response. All patients in the IFN-α group had new diagnoses and had started IFN-α treatment. Ten of the patients in the IFN-α group are also in a CCyR, and the remaining 4 are very close to a CCyR (the incidence of the residual Ph^+ clone is between 3.0% and 6.7% by repeated fluorescence in situ hybridization analysis of 1000 interphase nuclei and a cutoff rate of 1.5%). The patients' profiles are summarized in Table 1. Peripheral blood cell counts and differential counts of leukocytes were measured with a Sysmex XE 2100 cell counter (Sysmex, Kobe, Japan).

2.2. Flow Cytometry

Peripheral blood was obtained after informed consent had been obtained and was directly stained with antibodies prior to the lysis of red blood cells with Lysing Solution (Beckman Coulter, Miami, FL, USA). Samples were analyzed by multicolor flow cytometry with an Epics XL instrument (Beckman Coulter) according to standard procedures, and results were analyzed with Expo32 ADC software provided with the Epics XL (Beckman Coulter). TruCount tubes (BD Biosciences, San Jose, CA, USA) were used in calculating the absolute number of blood cells staining for CD45, CD3, CD19, and CD56 markers. Fluorescein isothiocyanate (FITC)-conjugated anti-CD8α, anti-CD4–FITC, phycoerythrin (PE)-conjugated anti-CD4, ECD (PE–Texas red)-conjugated anti-CD3, RD1-conjugated anti-CD56, and PC5 (PE–cyanine 5)-conjugated anti-CD45 monoclonal antibodies were purchased from Beckman Coulter, and anti-CD4–PE, anti-CD25–FITC, and anti-CD19–FITC were from BD Biosciences. Anti-CD26–PE and anti-Foxp3–PE were obtained from eBioscience (San Diego, CA, USA).

2.3. Statistical Analysis

Flow cytometry results were analyzed with JMP 6.0.2 software (SAS Institute, Cary, NC, USA). Statistical analyses were performed with Wilcoxon and Kruskal-Wallis tests, with a P value of .05 regarded as statistically significant.

3. Results and Discussion

Prior to the approval of STI, all of the patients in the IFN-α group had already achieved major cytogenetic responses, and IFN-α has been gradually tapered to a minimal dose to maintain their responses. The standard dose has principally been continued in the STI group. Accordingly, the treatment durations for the 2 groups are markedly different, but the age distributions for the 2 groups are comparable. In both patient groups, white blood cell counts were well controlled at levels that were moderately lower than those in the control group of healthy volunteers (5.50×10^9/L in the control group, 4.18×10^9/L in the IFN-α group, and 3.95×10^9/L in the STI group). Red blood cell counts and hemoglobin levels were significantly reduced in the STI group compared with the other 2 groups ($P < .0001$), and platelet counts were lower in the IFN-α group than in the control group ($P < .01$) (Table 1).

TruCount tubes were used in the calculation of the absolute number of T-cell subsets and other immune cells. The total num-

Table 1.
Characteristics of the Patients and Healthy Control Individuals*

	Control (n = 14)	IFN-α (n = 14)	STI (n = 14)	P
Male/female sex, n	10/4	12/2	9/5	—
Age, y†	41 (30-63)	50 (39-73)	54 (29-65)	—
Treatment†				
Duration, mo	—	174 (61-235)	54 (7-61)	—
Dosage	—	6×10^6 IU/wk ($3\text{-}18 \times 10^6$ IU/wk)	400 mg/d (300-400 mg/d)	—
Blood data‡				
WBC, $\times 10^9$/L	5.50 ± 1.21	4.81 ± 1.36	3.95 ± 0.89	.0056
RBC, $\times 10^{10}$/L	479.7 ± 33.4	435.7 ± 55.1	357.9 ± 45.0	<. 0001
Hemoglobin, g/dL	14.4 ± 0.9	13.7 ± 1.5	11.8 ± 1.5	<. 0001
Platelets, $\times 10^9$/L	223 ± 59	153 ± 50	185 ± 23	.0057

*IFN-α indicates interferon α; STI, imatinib mesylate; WBC, white blood cells; RBC, red blood cells.
†Age and treatment data are presented as the median (range).
‡Blood data are presented as the mean ± SD. Asterisks indicate statistically significant difference.

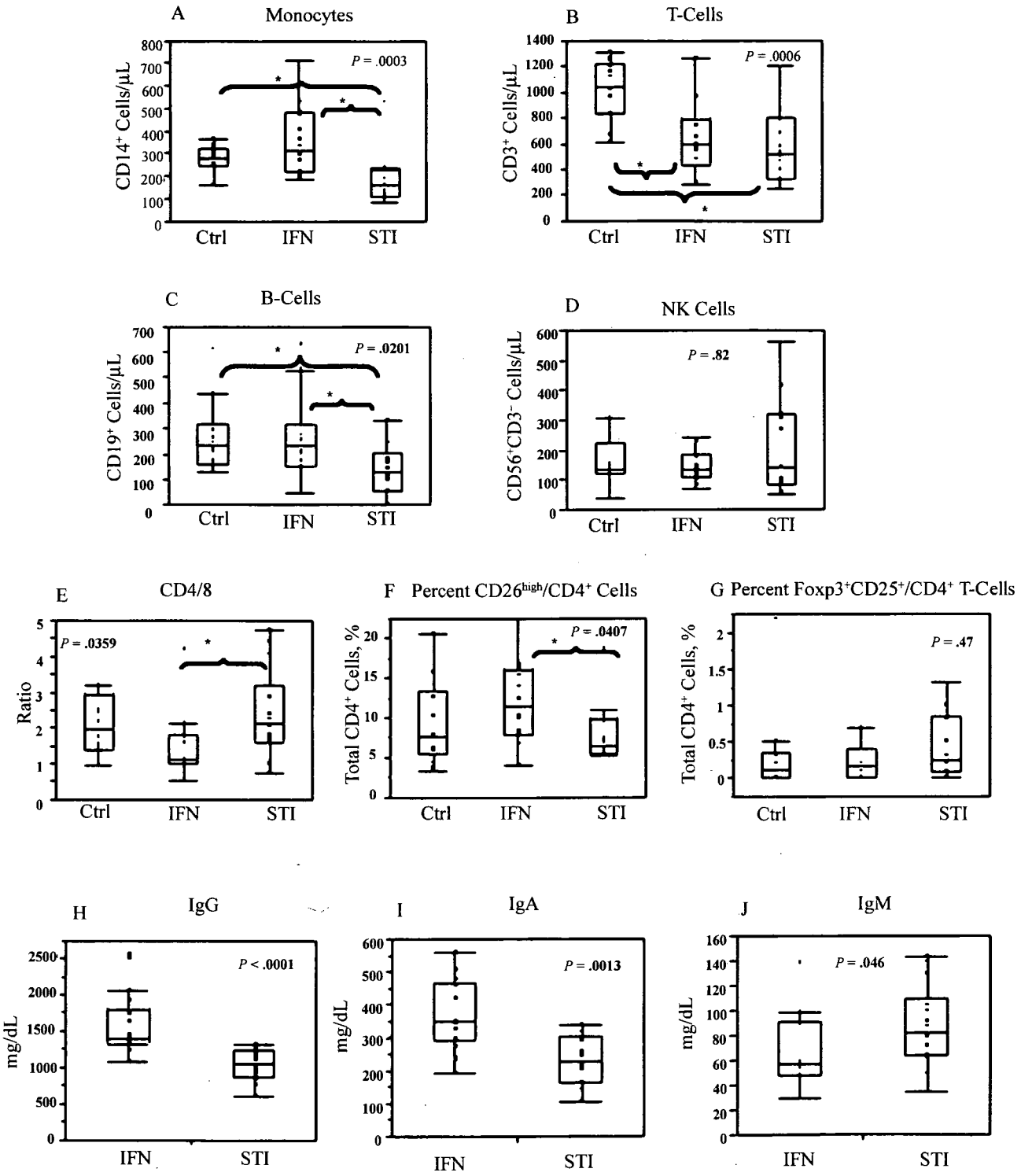

Figure 1. Immunologic parameters in chronic myeloid leukemia patients under interferon-α (IFN-α) or imatinib mesylate (STI) therapy. The absolute numbers of $CD14^+$ monocytes (A), $CD3^+$ T-cells (B), $CD19^+$ B-cells (C), and $CD56^+CD3^-$ natural killer (NK) cells (D) were measured by flow cytometry with TruCount tubes and were evaluated in these 2 groups and the control group of healthy volunteers (Ctrl). The CD4/CD8 T-cell ratio (CD4/8) (E), the proportion of $CD26^{high}$ cells among $CD4^+$ T-cells (F), and the proportion of $Foxp3^+CD25^+CD4^+$ T-cells among $CD4^+$ T-cells (G) were also obtained via flow cytometry. Serum titers for immunoglobulin G (IgG), IgA, and IgM (H-J) were measured simultaneously. Box-and-whisker plots show the median, interquartile range, and 95% confidence intervals. The sample numbers in the 3 groups are the same (n = 14). We used the Wilcoxon or Kruskal-Wallis test with JMP 6.0.2 software (SAS Institute) to calculate the statistical significance of differences between groups.

ber of T-cells was significantly suppressed in both patient groups compared with the control group ($P = .0006$), whereas the numbers of natural killer cells in the 3 groups were not different (Figures 1B and 1D). Only the STI group showed significantly reduced numbers of monocytes and B-cells. In particular, the absolute mean (±SD) B-cell count in the STI group was 1.38 ± 0.94 × 10^8/L, a count approximately one half that of the mean values for the control group (2.67 ± 1.34 × 10^8/L) and the IFN-α group (2.61 ± 1.67 × 10^8/L) ($P = .0201$).

Although the absolute T-cell counts were significantly-lower in the 2 patient groups than in the control group but were comparable with each other, the CD4/CD8 ratios of the 3 groups were significantly different (2.08 ± 0.8 in the control group, 1.5 ± 0.90 in the IFN-α group, and 2.4 ± 1.2 in the STI group; IFN-α versus STI, $P = .0359$) (Figure 1E), clearly indicating a relatively CD8-dominant pattern in the IFN-α group. We also assessed the proportion of $CD26^{high}$ cells among $CD4^+$ cells. CD26 is known to be a dipeptidylpeptidase IV, which is mainly expressed on T-cells among blood cells and is up-regulated upon activation. $CD26^{high}CD4^+$ T-cells are considered to represent effector memory T-cells of a typical type 1 helper T-cell phenotype [15]. Recently, the allergen dose–dependent recruitment of $CD4^+CD26^+CD25^+$ T-cells was noted in a study of a rat model of asthma [16]. Intriguingly, the IFN-α group showed a higher proportion of $CD26^{high}$ cells among $CD4^+$ T-cells than the STI group (12.1% ± 5.0% in the IFN-α group and 7.9% ± 3.6% in the STI group) (Figure 1F), whereas the absolute counts were reduced in the 2 patient groups. Regulatory T-cells (Treg cells), which are characterized by their $Foxp3^+CD25^{high}CD4^+$ phenotype, are implicated in the maintenance of immunotolerance. Treg cells have an attenuated cytokine response to T-cell receptor stimulation and can suppress the proliferation and effector function of neighboring T-cells [17]. The expression of Foxp3, an essential transcription factor required for Treg cell development, is detectable by flow cytometry with an intracellular staining method. We studied whether absolute Treg cell counts or their relative proportions are influenced by the treatment option used. The absolute numbers and relative proportions of Treg cells were not different among the 3 groups (Figure 1G).

To assess the consequences of reduced peripheral blood B-cell counts in the STI group, we compared immunoglobulin A (IgA), IgG, and IgM levels in the 2 patient groups (Figures 1H-1J). Although all values were within the normal ranges, serum IgG and IgA titers were significantly decreased in the STI group compared with the IFN-α group. On the contrary, IgM values were not significantly different. Mean IgG and IgA values were 1563 ± 395 mg/dL and 370 ± 106 mg/dL, respectively, in the IFN-α group and 1032 ± 210 mg/dL and 232 ± 71 mg/dL in the STI group ($P < .0001$, and $P < .0013$, respectively). Therefore, these data suggest that STI therapy relatively impaired B-cell effector function, as has previously been reported [18].

Our data indicate that the 2 CML therapeutic agents induce a distinct immunologic status in CML patients and raise the possibility that immunologic surveillance of residual Ph^+ clones may be defective in STI-treated patients. Finally, periodic monitoring of immunologic parameters is recommended for CML patients undergoing prolonged STI therapy.

Acknowledgments

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Identification and comparative analysis of Pax5 C-terminal isoforms expressed in human cord blood-derived B cell progenitors

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Abstract

We identified three Pax5 isoforms due to alternative splicing of the C-terminal exons of its gene in cord blood (CB)-derived B cell progenitors cultivated on the murine bone marrow stromal (HESS-5) cells. Apart from wild type (wt), one isoform skips exon 9 without subsequent frameshift (del9), while the other has a frameshift insert between exons 8 and 9, resulting in novel C-terminal sequences (ins8′). Quantitative reverse transcription-polymerase chain reaction analysis revealed that wt mRNA could be detected in CB CD34$^+$ cells, but that del9 and ins8′ isoforms only appeared after 1 or 3 weeks of co-culture, respectively. Expression of each isoform mRNA was markedly upregulated during B cell differentiation *in vitro*, and wild type continued to be the most abundant isoform. In a luciferase reporter assay using a synthetic CD19 enhancer, del9 isoform revealed slightly lower activity and ins8′ isoform showed much lower activity, compared with Pax5-wt. Furthermore, retroviral expression of each Pax5 isoform in CB CD34$^+$ cells induced aberrant CD19 expression in a fraction of immature myeloid cells after 1 week of culture, although del9 and ins8′ isoforms showed much less potent activity than Pax5-wt. These results suggest that Pax5-wt is quantitatively and qualitatively dominant over other C-terminal isoforms during human B cell differentiation.

Keywords: Pax5; Alternative splicing; Isoform; Cord blood; CD34; CD19

1. Introduction

B cell differentiation is tightly regulated by the precise usage of stage- and lineage-specific transcription factors including EBF, E2A, and Pax5 [1]. Among them Pax5 specifies B cell lineage commitment over other hematopoietic cell fates [2]. In Pax5-null mice, B cell development is blocked at the pro-B cell stage [2–4]. Ectopic expression of Pax5 in multipotent progenitors does not inhibit their differentiation but extensive proliferation to the myeloid lineage [5,6], and completely blocks T-lineage development in the thymus [7]. Human and murine Pax5 are also known to have a number of splicing variants [8–11] in not only their N-terminal but also C-terminal domain, the latter of which constitutes a transactivation domain as well as an inhibitory domain [12]. However, the functional role of Pax5 in B lymphopoiesis was studied exclusively in mice because of the lack of proper experimental system for humans. Recently, it has been shown that human B lymphopoiesis can be simulated by cultivating cord blood (CB) progenitors on the murine bone marrow stromal cell line such as MS-5 [13,14], OP9 [15], and HESS-5 [16]. Using this system, we could detect expression of three Pax5 C-terminal isoforms including wild type (wt) during early B cell differentiation. We further performed comparative analysis of their transcriptional activities using synthetic and endogenous target genes.

2. Materials and methods

2.1. Preparation of CB CD34$^+$ cells

Umbilical CB units were collected from single-birth, full-term, and normal deliveries following the standards for CB collection developed by the Tokyo Cord Blood Bank and the Institute of Medical Science, the University of Tokyo. CD34$^+$

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cells were purified from mononuclear cells using a $CD34^+$ progenitor cell isolation kit and a MiniMACS separation unit (Miltenyi Biotec GmbH, Glandbach, Germany) and stored in a deep freezer until use. Samples with $CD34^+$ cell purity and viability of at least 95% were used for experiments.

2.2. Cell lines

Hematopoiesis-supporting ability of a murine bone marrow stromal cell line HESS-5 was precisely described elsewhere [16]. HESS-5 cells were maintained at 37 °C in 5% CO_2 in DNA, RNA-free α-modified Eagle's medium (α-MEM) containing 10% horse serum. Hela and 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS).

2.3. Culture of CB $CD34^+$ cells over HESS-5 cells

HESS-5 cells were prepared at 2×10^4 cells/well in 6-well plates 1 week prior to seeding $CD34^+$ cells and were allowed to reach confluence without irradiation. $CD34^+$ cells were plated at a density of 3×10^3 cells/well onto the HESS-5 cell layer in 3 ml of α-MEM containing 10% heat inactivated FBS, 50 ng/ml recombinant human stem cell factor (SCF) and 10 ng/ml recombinant human granulocyte colony-stimulating factor (G-CSF) [13]. The culture medium was totally replaced by fresh medium every week until 9th week. This culture system exclusively produces non-adherent myeloid cells and adherent B lymphoid cells, which migrate beneath the stromal layer. Non-adherent and adherent cells were separately harvested for further analysis.

2.4. Cloning of Pax5 C-terminal isoforms

Total RNA was extracted from 8 weeks cultured B lymphoid cells after separation from the HESS-5 cell layer. First-strand cDNA was synthesized from 0.5 μg total RNA using Bca PLUS reverse transcriptase (TaKaRa Bio, Ootsu, Japan) and random hexamers. Pax5 isoforms were cloned by polymerase chain reaction (PCR) with the high fidelity PCR kit (TaKaRa Bio) using a pair of primers, 5′-atggatttagagaaaaattatccg-3′ (forward) and 5′-tcagtgacggtcataggcagtg-3′ (reverse). PCR reaction was

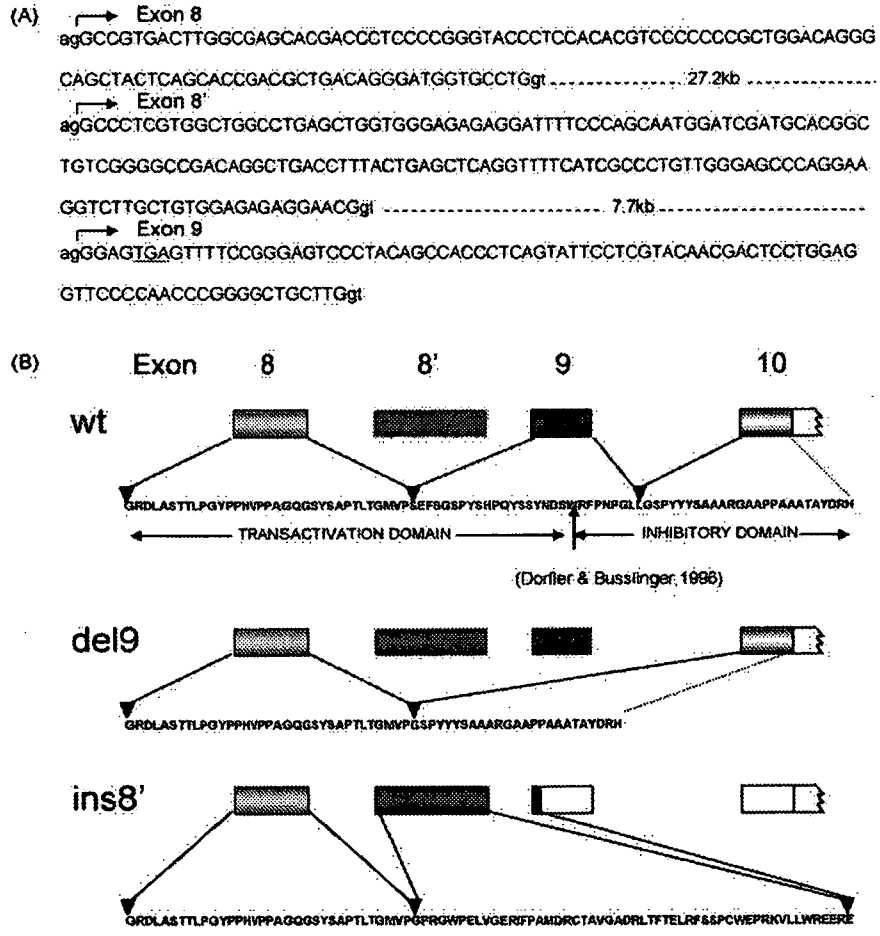

Fig. 1. Novel exon and splicing variants of human Pax5. (A) Nucleic acid sequences of exons 8–9 of the human Pax5 gene: splice donor and acceptor sites are indicated in small letters. Exon 8′ is newly identified in this study. Underlined TGA in exon 9 indicates a novel termination codon by frameshift in ins8′ isoform. (B) Alternative usage of C-terminal exons by Pax5 isoforms and the resulting amino acid sequences written in a single letter. Each exon boundary is indicated (▼).

performed as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 96 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min. The resulting products were cloned into pCR-BluntII (Invitrogen, Carlsbad, CA) and subjected to sequencing analysis using a BigDye terminator cycle sequencing kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.5. Quantitative RT-PCR

Quantitative analysis of mRNA expression of Pax5 isoforms was performed by the Taqman real-time RT-PCR method (Applied Biosystems). Total RNA was extracted from $CD34^+$ cells and B lymphoid cells in culture using an RNeasy micro-kit (Qiagen, Valencia, CA), and was reverse transcribed with SuperScriptII First-strand Synthesis System (Invitrogen). The primers and probes designed for this assay were: exon 8 forward, 5′-tactcagcaccgacgc-3′; exon 8′ forward, 5′-aaggtcttgctgtggagag-3′; exon 10 reverse, 5′-gcggcagcgctataat-3′; exon 9 probe, 5′-attcctcgtacaacgactcctggaggttcc-3′; exon 8/10 probe, 5′-tggtgcctggctcccccta-3′, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as internal RNA standard [17].

2.6. Plasmid construction and protein analysis

Each Pax5 isoform cDNA was subcloned into pcDNA3.1+ (Invitrogen, Carlsbad, CA) and transfected into 293T cells, followed by immunoblot analysis using anti-Pax5 mouse monoclonal antibody (clone N-19; Santa Cruz Biotechnology, Santa Cruz, CA). The reporter plasmid pCD19-Luc [12] was generated by inserting three copies of high-affinity Pax5 binding site from the human CD19 gene into pTAL-Luc plasmid (TaKaRa Bio).

2.7. Luciferase reporter assay

Hela cells were seeded in 12-well plates and transfected using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) with 1000 ng of pCD19-Luc, 50 ng of pcDNA-Pax5 or mock vector and 200 ng of pSV-βGal. Luciferase assay and β-galactosidase assay were carried out 24 h after transfection using the Steady-Glo luciferase assay system and β-galactosidase assay system (Promega, Madison, WI) according to the manufacturer's instruction.

2.8. Transduction of $CD34^+$ cells with Pax5 retrovirus

For retroviral transduction, each isoform of Pax5 cDNA was inserted into pMCs-Ig vector [18], upstream of the IRES-EGFP element. Plat-F packaging cells producing RD114-pseudotyped viral particles [18] were transiently transfected with pMCs-Ig or pMCs-Pax5-Ig, and the culture supernatant was harvested for 72 h after transfection. Viral titer determined by flow cytometry was higher than 1×10^6 infectious units/ml. $CD34^+$ cells were primed for 24 h in Iscove's modified Dulbecco's medium containing 20%FBS, 50 ng/ml SCF, 50 ng/ml Flt-3 ligand, 10 ng/ml thrombopoietin, and 10 ng/ml interleukin-3, and then an aliquot of 2×10^5 cells were transduced in Retronectin (TaKaRa Bio)-coated 24-well plates pretreated with 1 ml/well of viral supernatant for 30 min at room temperature. Half medium was replaced four times during 48 h of infection by fresh supernatant containing a cocktail of cytokines and 5 μg/ml protamine. $EGFP^+CD34^+$ cells were sorted on a FACS Aria (BD Biosciences, Franklin Lakes, NJ), and 9×10^3 cells were cultured on the HESS-5 monolayer in triplicate. Flow cytometric analysis was performed on a FACS Caliber (BD Biosciences).

3. Results and discussion

In our culture system, the number of non-adherent myeloid cells initially increases, reaching a maximum during the 3rd week, and decreases rapidly thereafter [13]. On the other hand, foci of small B lymphoid cells beneath the stromal layer appear during the 2nd week and increase in size until the 6–9th week. The surface marker profile of B lymphoid cells after 6–9 weeks of culture was $CD34^-/CD19^+/CD20^+$ (data not shown), which was compatible with that of small pre-B or immature B cells [13]. By RT-PCR analysis of 8 weeks cultured B cells, we detected

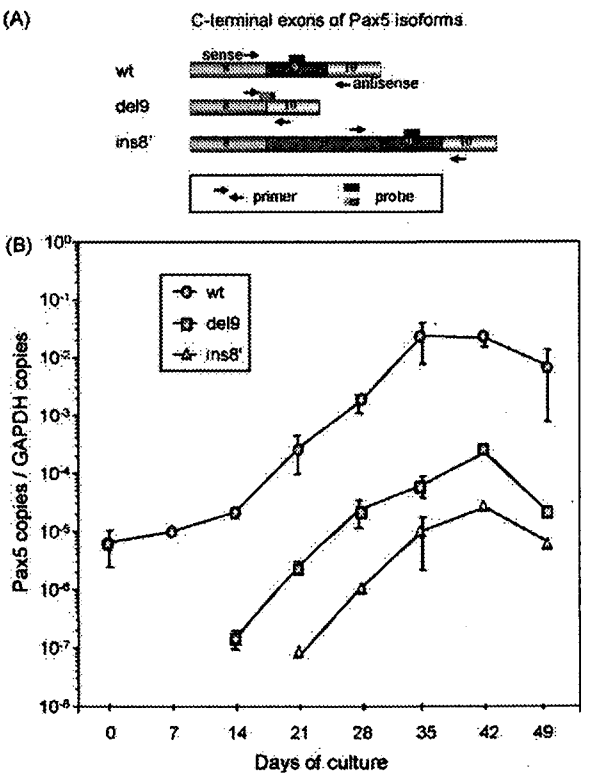

Fig. 2. Quantitative analysis of Pax5 isoforms. (A) Primers and probes used for real-time RT-PCR analysis of Pax5 isoforms. Arrows and boxes indicate the location of primers and probes, respectively. The sequences of primers and probes are described in the text. (B) Changes in mRNA expression of each Pax5 isoform during B cell differentiation from CB $CD34^+$ cells. *X*-axis indicates the culture periods of CB $CD34^+$ cells, and *Y*-axis means the ratio of mRNA copy numbers between each Pax5 isoform and GAPDH as an internal standard. Reactions were performed in triplicate and the results are shown as mean ± S.D.