特集 ○ 癌ワクチン—最近の進歩 ○

癌胎児性抗原 Glypican-3 を標的とした癌ワクチン療法

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要旨 われわれは，Glypican-3（GPC3）が肝細胞癌（HCC）に高発現する新規癌胎児性抗原であり，腫瘍マーカーとして有用であること，またBALB/cマウスにGPC3由来のペプチドを負荷した樹状細胞を免疫すると，GPC3発現腫瘍の増殖が抑制されることを報告している。今回，ヒトCTLが認識するGPC3由来のHLA-A2あるいはHLA-A24拘束性エピトープペプチドを同定し，これらを用いてHCC患者の末梢血単核球（PBMC）からGPC3ペプチド特異的細胞傷害性Tリンパ球（CTL）が誘導できるか否かを検討した。HLA-A2陽性GPC3陽性HCC患者のPBMCよりGPC3$_{144-152}$ペプチドを用いて，8名中5名から，またHLA-A24陽性GPC3陽性HCC患者のPBMCよりGPC3$_{298-306}$ペプチドを用いて6名中4名から，各CTLエピトープに特異的なCTLを誘導できた。また，これらのCTLはNOD/SCIDマウスに移植した，GPC3を発現するヒトHCC細胞株の増殖を抑制した。これらの結果から，GPC3由来のペプチドは，HCC患者の免疫療法の新たなターゲットとして，その臨床応用が期待できる。

〔Biotherapy 21（1）：62-68, January, 2007〕

Identification of CTL Epitopes of Glypican-3 Useful for Cancer Immunotherapy

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Summary

We previously reported that Glypican-3 (GPC3) was overexpressed specifically in hepatocellular carcinoma (HCC) in humans, and it was useful as a novel tumor marker for HCC. We also reported that the pre-immunization of BALB/c mice with bone-marrow-derived dendritic cells pulsed with the H-2K^d-restricted mouse GPC3$_{298-306}$ (EYILSLEEL) peptide prevented the growth of tumor expressing mouse GPC3.

Because of similarities in the binding peptide motifs between H-2K^d and HLA-A24 (A*2402), the GPC3$_{298-306}$ peptide thus seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC. Therefore, we investigated whether or not the GPC3$_{298-306}$ peptide could induce GPC3-specific CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A24 (A*2402)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice (Tgm) to identify the HLA-A2 (A*0201)-restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients. We found that the GPC3$_{144-152}$ (FVGEFFTDV) peptide could induce peptide-specific CTLs in HLA-A2.1 (HHD) Tgm without inducing autoimmunity. In 5 out of 8 HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3$_{144-152}$ peptide-

specific CTLs were generated from PBMCs by *in vitro* stimulation with the peptide. The GPC3$_{298-306}$ peptide-specific CTLs were also generated from PBMCs in 4 of 6 HLA-A24^{+} GPC3^{+} HCC patients, and the inoculated CTLs could attack the human HCC tumor mass implanted into NOD/SCID mice.

Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Key words: Cancer immunotherapy, CTL, HCC, Glypican-3 (GPC3)
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はじめに

肝細胞癌（HCC）の罹患数は，欧米およびアジア諸国において依然として増大している。HCCは治療後も高頻度に再発を繰り返すため予後不良な癌であり，肝炎，肝硬変から発生したごく初期の癌に対する早期治療法や治療後の再発予防のために有効な補助療法の確立が望まれる。Glypican-3（GPC3）はHCCに高発現し，腫瘍免疫のターゲットとして理想的な癌胎児性抗原である。われわれは，HCCに対する免疫療法の新たなターゲットとしてGPC3に着目し，その有用性に関して前臨床試験を終了し，臨床試験を開始する予定である[1-3]。

Ⅰ．HCCに対する免疫療法

慢性肝炎，肝硬変患者におけるHCCの発症予防やHCC術後における術後化学療法は，未だ開発途上にある。HCCに対する免疫療法についても，1990年代よりlymphokine activated killer（LAK）cells，tumor infiltrating lymphocytes（TIL），peripheral blood mononuclear cell（PBMC）を用いた養子免疫療法，DCワクチン療法，AFP由来ペプチドワクチン療法など試みられているが，未だ標準的な治療法として確立されていない[4]。HCCにおいて高発現する癌特異的抗原も多数報告されており，各施設でその有用性が検討されている[4]（表1，2）。

Ⅱ．新規癌胎児性抗原（GPC3）

われわれは，東京大学医科学研究所ヒトゲノム解析センターの中村祐輔博士らとの共同研究により，癌部と非癌部のcDNAマイクロアレイ解析を用いてHCC特異的に高発現する遺伝子としてGPC3を同定した（図1）。

1．GPC3の構造と機能

膜結合型のGlypicanファミリーは，現在までのところ6種類が報告されている[5]。GPC3は，580アミノ酸からなる60 kDのコア蛋白質にヘパラン硫酸プロテオグリカンの糖鎖修飾が加わった膜蛋白質で，C末端がGPIアンカーにより形質膜に結合している。Piliらは，X染色体（Xq26）連鎖疾患である巨人症の一種Simpson-Golabi-Behmel症候群（SGBs）において，GPC3の遺伝子変異を報告している。また，GPC3ノックアウトマウスでも，SGBsと同様に巨大化などの表現型を示すことがわかっている。GPC3の機能としては，ある種の腫瘍細胞では増殖を抑制したり，あるいはアポトーシスに関連があると報告されている[6]。近年，GPC3コア蛋白質が直接Wntと結合することによりWnt signalを活性化し，HCCの増殖を促進することが報告されている[7]。

2．HCC組織におけるGPC3の発現

われわれは，遺伝子の発現量の差が，その遺伝子産物である蛋白質量の差として反映されているか否かをRT-PCR法ならびに組織切片における免疫染色法を用いて確認した（図2）。肝臓組織は，胎児期においてGPC3を発現するが出生後発現しなくなり，HCCにおいては再び発現するためGPC3は癌胎児性蛋白質としての性格を有しており，恐らく胎児の発生に重要な役割を担っていると推測される。一般に，癌胎児性蛋白質は腫瘍の進行において重要な役割を担っているとは考えられていないが，腫瘍マーカーまたは免疫療法の標的として使用されてきた。

表1 HCCに高発現する癌抗原に関する過去の報告のまとめ*
(Butterfield, L.H.: *Gastroenterology*, 2004. を基に情報を追加)

Study (Year)	GPC-3	MAGE-1	MAGE-2	MAGE-3	MAGE-4	MAGE-10	MAGE-12	SSX-1	NY-ESO1
Yamashita (1996)		80							
Kariyama (1999)		78		42					
Tahara (1999)		68	30	68		30	30		
Chen (2001)								80	
Mou (2002)		70		53					
Luo (2002)		19		24	4			38	0
Chen (2003)		66		70	20	36			40
Nakatsura (2003)	80								
Korangy (2004)									24

*：HCC組織における各腫瘍抗原mRNAの発現頻度（%）

表2 HCC対するワクチン療法の臨床試験に関する過去の報告のまとめ（文献[1]より）

Strategy	Author (Year)	Patients	Setting	Responses
Dendritic cells (DC vaccine)	Ladhams, *et al* (2002)	2 metastatic	GM/IL-4 DC + tumor	1 patient slowed tumor growth
	Iwashita, *et al* (2003)	10 unresectable	GM/IL-4 DC + tumor lysate + TNF + KLH	1/10 MR
	Stift, *et al* (2003)	2 HCC of 20 total	GM/IL-4 DC + tumor lysate + TNF + IL-2	No PR or CR
AFP peptidse	Butterfield, *et al* (2003)	6 stage Ⅳa and Ⅳb	AFP peptidse in Montanide adiuvant	No PR or CR

ワクチンを基盤とした免疫療法に関する臨床試験のみを抜粋した。
GM：GM-CSF, KLH：keyhole limpet hemocyanin, AFP：α-fetoprotain, MR：minor response,
PR：partial response, CR：complete response

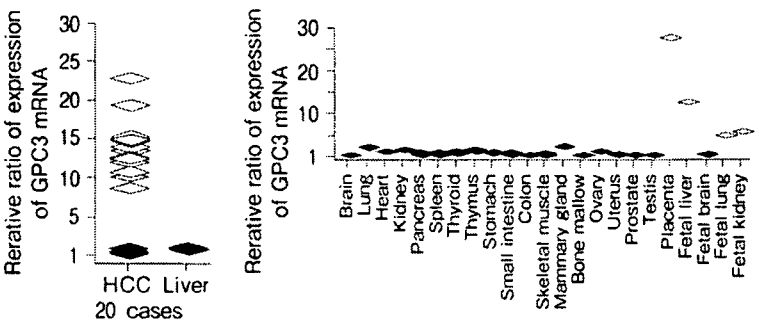

図1 HCC 20例の癌部，非癌部および多様な正常臓器におけるGPC3遺伝子発現のcDNAマイクロアレイ解析データ（東京大学医科学研究所ヒトゲノム解析センター：中村祐輔博士らの研究成果，Okabe, H., *et al.*: *Cancer Res.* 61: 2129, 2001.）
HCC患者20例の癌部と非癌部における23,040種類の遺伝子の発現を比較検討し，発現の比が5以上の遺伝子を16種類選んだ。さらに胎生期の4臓器を含む23臓器の正常組織において，各遺伝子の発現プロファイルを解析して，胎生期の組織あるいは免疫学的に隔離された胎盤や精巣にしか発現しない遺伝子GPC3を探しだした。GPC3は，HCC患者20例中16例で癌部/非癌部の発現の比が5以上（平均396.2）で，胎盤や胎生肝，胎生腎に発現する以外はほとんどの成人正常臓器に発現を認めない癌胎児性抗原をコードする遺伝子であった。

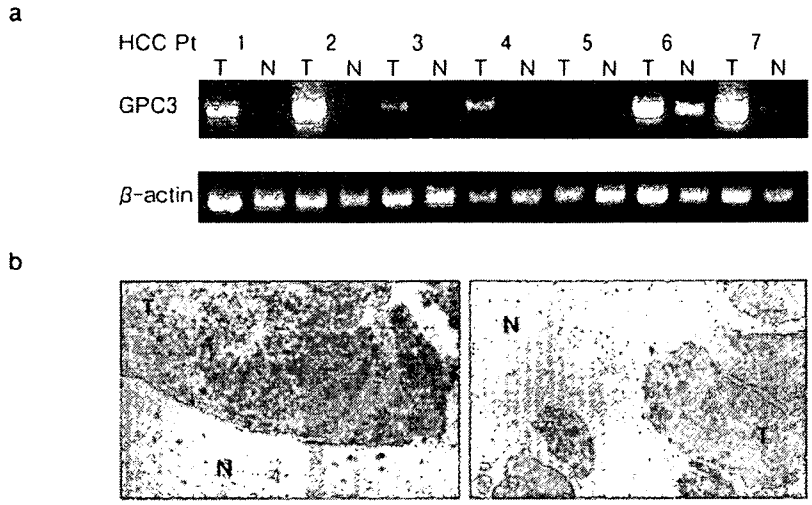

図2　HCC組織におけるGPC3の発現
a：HCC組織の癌部（T），非癌部（N）におけるGPC3mRNAの発現の有無をRT-PCR法にて検討し，癌部においてのみGPC3の発現を認めた。
b：HCC組織切片におけるGPC3蛋白質の発現を，抗GPC3抗体を用いた免疫組織学的解析により確認した。

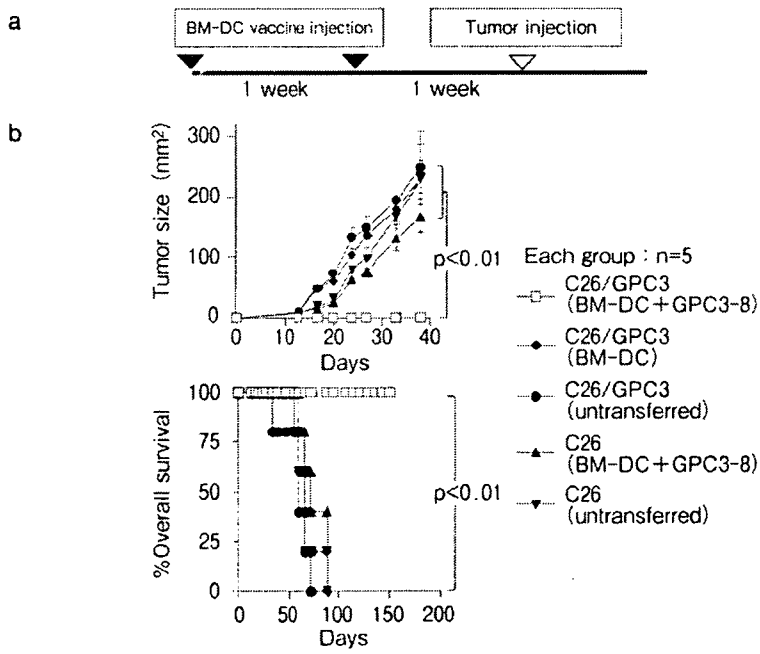

図3　マウスにおけるGPC3ペプチドを負荷したBM-DCワクチンによる腫瘍抑制効果
a：実験のプロトコール。BM-DCは1週間ごとに2回，BALB/cマウスの腹腔内に5×10^5個を投与した。その1週間後に腫瘍を3×10^1個背部の皮下に移植した。
b：抗腫瘍効果の検討。GPC3-8ペプチドを負荷したBM-DCワクチンを投与した後に，同系マウス由来の大腸癌細胞株C26に，マウスGPC3遺伝子を強制発現させた細胞株（C26/GPC3）を皮下移植した群では腫瘍の拒絶が認められた（上段）。さらに同群マウスにおいて，生存期間の著明な延長が観察された（下段）。

表3 HLA-A2あるいはHLA-A24陽性HCC患者（それぞれPt-A2, Pt-A24）の約50%において，GPC3特異的なCTLが誘導された

Patients	Age	Gender	State of tumor†	GPC3 expression#	HKA expression★	CTL induction*
Pt-A2-1	80	F	IIIa	+	+	+
Pt-A2-2	72	M	II	+	+	+
Pt-A2-3	67	F	II	ND	ND	+
Pt-A2-4	54	M	I	+	+	+
Pt-A2-5	57	M	I	ND	ND	−
Pt-A2-6	66	M	I	−	−	−
Pt-A2-7	54	M	IIIa	+	+	−
Pt-A2-8	73	M	II	ND	ND	+
Pt-A2-9	68	F	IIIa	+	+	−
Pt-A2-10	54	M	II	+	+	−
Pt-A24-1	60	M	IVa	+	+	+
Pt-A24-2	57	M	IVa	+	+	−
Pt-A24-3	75	F	IIIa	+	+	+
Pt-A24-4	59	M	IIIa	ND	ND	+
Pt-A24-5	52	M	IVb	−	+	−
Pt-A24-6	65	M	I	ND	ND	+
Pt-A24-7	61	M	I	ND	ND	+
Pt-A24-8	74	M	II	ND	ND	−
Pt-A24-9	59	M	IVb	−	−	−
Pt-A24-10	69	M	IVa	+	+	−
Pt-A24-11	72	M	II	−	+	−
Pt-A24-12	61	M	IIIa	+	+	+

†：TNM分類を用いた，#：免疫染色を用いて腫瘍周囲組織と比較した，★：免疫染色にて膜が染色された場合を陽性とした，*：GPC3発現HCC細胞株HepG2に対する細胞傷害活性が，E/T比20で20%以上観察された場合にCTLを誘導できたと判断した。

3. 腫瘍マーカーとしてのGPC3の有用性

われわれは，ELISA法を用いてHCC患者の約40%の血清中のGPC3蛋白について検討したところ，HCC患者の血清中にのみ検出されるが，健常人，その他の良性肝疾患患者や他の癌種の患者ではまったく検出されなかった。さらにHCC切除手術後に血清GPC3は著明に減少したことから，GPC3がHCCの腫瘍マーカーとして有用であることを報告した[8]。HCCの腫瘍マーカーとしてのGPC3の有用性に関しては，他施設からも同時期に報告されているが[9,10]，早期診断や治療効果の判定などの臨床応用が期待される。

Ⅲ. 抗腫瘍免疫療法のターゲットとしてのGPC3の有用性

1. マウスにおける腫瘍免疫の解析

発現の組織特異性が優れていることから，Nakatsuraらは，この新規癌胎児性抗原GPC3が理想的な腫瘍拒絶抗原になり得るかどうかを検討した。日本人の約60%が所有するHLA-A24とBALB/cマウスのclass I分子のK^dに結合するペプチドの構造モチーフは，非常に一致していることがわかっている。さらに，ヒトとマウスのGPC3では，アミノ酸配列のレベルで95%以上のホモロジーを認めることから，ヒトとマウスのGPC3でアミノ酸配列が完全に一致し，HLA-A24，K^dのいずれにも結合し得るGPC3由来のペプチドを合成した。これらをBALB/cマウスに免疫して解析し，K^d分子に結合して細胞傷害性Tリンパ球（CTL）に提示される（K^d拘束性）

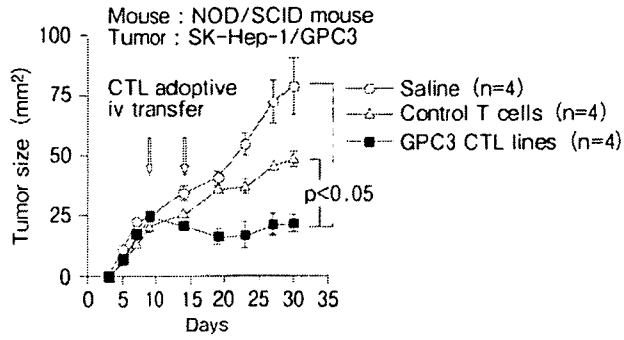

図4　GPC3発現ヒトHCC細胞株に対する養子免疫療法の有効性
免疫不全マウスであるNOD/SCIDマウスに移植したGPC3発現ヒトHCC細胞株に対する，ヒトGPC3特異的CTLの養子免疫による抗腫瘍効果を検討した。GPC3エピトープペプチドで誘導したヒトCTLを投与すると，コントロール群に比べ有意に腫瘍の増大が抑制された。NOD/SCIDマウスの背部の皮下に，ヒトHCC細胞株SK-Hep-1にGPC3遺伝子を強制発現させたSK-Hep-1/GPC3を1×10^7個移植し，5×5 mmの大きさになったところでCTLを8×10^7個iv投与した。HCC患者のPBMCをGPC3エピトープペプチドで刺激して誘導したCTL投与群（■），コントロールとしてHIVエピトープペプチドで誘導したCTL投与群（△），生理食塩水のみを投与した群（○）の間で比較すると，GPC3特異的CTL投与群ではコントロール群に比べ，有意に腫瘍増殖が抑制された。

CTLエピトープペプチドを同定した[1]。このエピトープペプチドを負荷した骨髄由来の樹状細胞（BM-DC）ワクチンをBALB/cマウスの腹腔内に予防的に投与した場合，コントロール群に比べ，GPC3発現マウス大腸癌腫瘍の増殖は著明に抑制された（図3）。このエピトープペプチドはHLA-A24によっても提示され，ヒトでも同様にCTLエピトープとなる可能性があると思われた。またMotomuraらは，マウスGPC3を遺伝子導入したマウスES細胞より分化誘導した樹状細胞（ES-DC-GPC3）を樹立した。ES-DC-GPC3をマウスに免疫することにより，*in vivo*においてGPC3特異的なCTLが誘導され，移植されたGPC3発現腫瘍の増殖転移が抑制されることを報告している[2]。

2. HCC患者における腫瘍免疫の解析

日本人のHLA class I対立遺伝子のうち，HLA-A24（A*2402）は日本人の約60％が所有し，HLA-A2（A*0201）は約30％が所有する，ありふれた対立遺伝子である。そこで，ヒトとマウスのGPC3に保存されたアミノ酸配列をもつペプチドで，HLA-A2（A*0201）に結合すると推定されるGPC3由来の9～10個のアミノ酸からなるペプチドを9種類選択した。このうち，HLA-A2トランスジェニックマウス（HLA-A2 Tgm）に最も強く，GPC3特異的なCTLを誘導できるエピトープペプチドをELISPOTアッセイにて検討した結果，ペプチドA2-3：$GPC3_{144-152}$がCTLエピトープ候補として同定された。このGPC3 A2-3ペプチドを負荷したBM-DCを2回免疫したHLA-A2 Tgmでは，重要臓器（脳，皮膚，心，肺，肝，腎）への自己免疫反応は生じておらず，その安全性が示唆された。

HLA-A2拘束性CTLエピトープペプチド$GPC3_{144-152}$と，HLA-A24拘束性CTLエピトープペプチド$GPC3_{298-306}$を用いて，HLA-A2またはHLA-A24陽性のHCC患者のPBMCから，ペプチド特異的CTLの誘導を試みた。その結果，それぞれのペプチドについてペプチド特異的にCTLが誘導され，HLA-A2陽性GPC3陽性HCC患者のPBMCより$GPC3_{144-152}$ペプチドを用いて8名中5名から，またHLA-A24陽性GPC3陽性HCC患者のPBMCより$GPC3_{298-306}$ペプチドを用いて6名中4名から，各CTLエピトープ特異的なCTLを誘導できた（表3）。

さらに，NOD/SCIDマウスにGPC3遺伝子を

強制発現させたヒトHCC細胞株SK-Hep1/GPC3を皮下注射して生着させた後に，HLA-A2拘束性エピトープペプチドGPC3$_{144-152}$あるいはHLA-A24拘束性エピトープペプチドGPC3$_{298-306}$で刺激することにより，HCC患者のPBMCより誘導されたヒトCTL株を養子免疫した。GPC3エピトープペプチドにて誘導したCTL株を静脈内投与したNOD/SCIDマウスでは，コントロールのT細胞株あるいは生理食塩水のみを投与した群と比較して，有意差をもって腫瘍の増殖抑制が観察された（図4）。現在，国立がんセンター東病院にてHCC患者を対象にして，これらのペプチドを用いた癌免疫療法の臨床試験を計画中である。

おわりに

GPC3由来のCTLエピトープは，HCCの免疫療法の新たなターゲットとして，その臨床応用が期待される。腫瘍の免疫逃避に対抗するためには多様な腫瘍拒絶抗原のレパートリーを確立することが望まれる。GPC3がその一つとして，これを用いた免疫療法がHCCの再発，発症防止に寄与することを期待したい。

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0914-2223/07/¥500/論文/JCLS

STEM CELLS

EMBRYONIC STEM CELLS

Genetically Manipulated Human Embryonic Stem Cell-Derived Dendritic Cells with Immune Regulatory Function

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Key Words. Dendritic cells • Embryonic stem cells • Cell differentiation • Cell therapy

ABSTRACT

Genetically manipulated dendritic cells (DC) are considered to be a promising means for antigen-specific immune therapy. This study reports the generation, characterization, and genetic modification of DC derived from human embryonic stem (ES) cells. The human ES cell-derived DC (ES-DC) expressed surface molecules typically expressed by DC and had the capacities to stimulate allogeneic T lymphocytes and to process and present protein antigen in the context of histocompatibility leukocyte antigen (HLA) class II molecule. Genetic modification of human ES-DC can be accomplished without the use of viral vectors, by the introduction of expression vector plasmids into undifferentiated ES cells by electroporation and subsequent induction of differentiation of the transfectant ES cell clones to ES-DC. ES-DC introduced with invariant chain-based antigen-presenting vectors by this procedure stimulated HLA-DR-restricted antigen-specific T cells in the absence of exogenous antigen. Forced expression of programmed death-1-ligand-1 in ES-DC resulted in the reduction of the proliferative response of allogeneic T cells cocultured with the ES-DC. Generation and genetic modification of ES-DC from nonhuman primate (cynomolgus monkey) ES cells was also achieved by the currently established method. ES-DC technology is therefore considered to be a novel means for immune therapy. STEM CELLS *2007;25:2720–2729*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem (ES) cells are characterized by pluripotency and infinite propagation capacity, and the methods for genetic modification of ES cells, including targeted gene modification, have been well-established. This laboratory and others have devised methods to generate dendritic cells (DC) in vitro from mouse ES cells [1, 2]. The functions of mouse ES cell-derived DC (ES-DC), including stimulation of allogeneic T cells, processing and presentation of antigenic proteins, and migration upon in vivo transfer, are comparable to those of DC generated in vitro from bone marrow cells [3]. This laboratory has also established a strategy for the genetic modification of mouse ES-DC [1]. Expression vectors were introduced into ES cells by electroporation, and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC. Studies using mice have demonstrated that in vivo transfer of genetically engineered mouse ES-DC is very useful for modulating immune responses both positively and negatively. It is possible to induce anticancer immunity [3–6] and prevent autoimmune disease [7, 8] in mouse models with genetically engineered ES-DC.

In the present study, looking toward future clinical application of ES-DC technology, a method was developed to generate ES-DC from human ES cells. The morphology and the results of functional and flow cytometric analyses indicate that human ES-DC possess the characteristic features of DC. cDNA microarray analysis revealed that the change of gene expression profile during generation and maturation of human ES-DC partially mimics that of monocyte-derived DC (Mo-DC). The currently established method was also applicable to cynomolgus monkey (*Macaca fascicularis*) ES cells.

MATERIALS AND METHODS

Cell Lines, Cytokines, and Reagents

The use of human ES cells was done in accordance with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells (2001) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, after approval by the Institutional Review Board. The human ES cell lines KhES-1 and KhES-3 have recently been established and maintained on mouse

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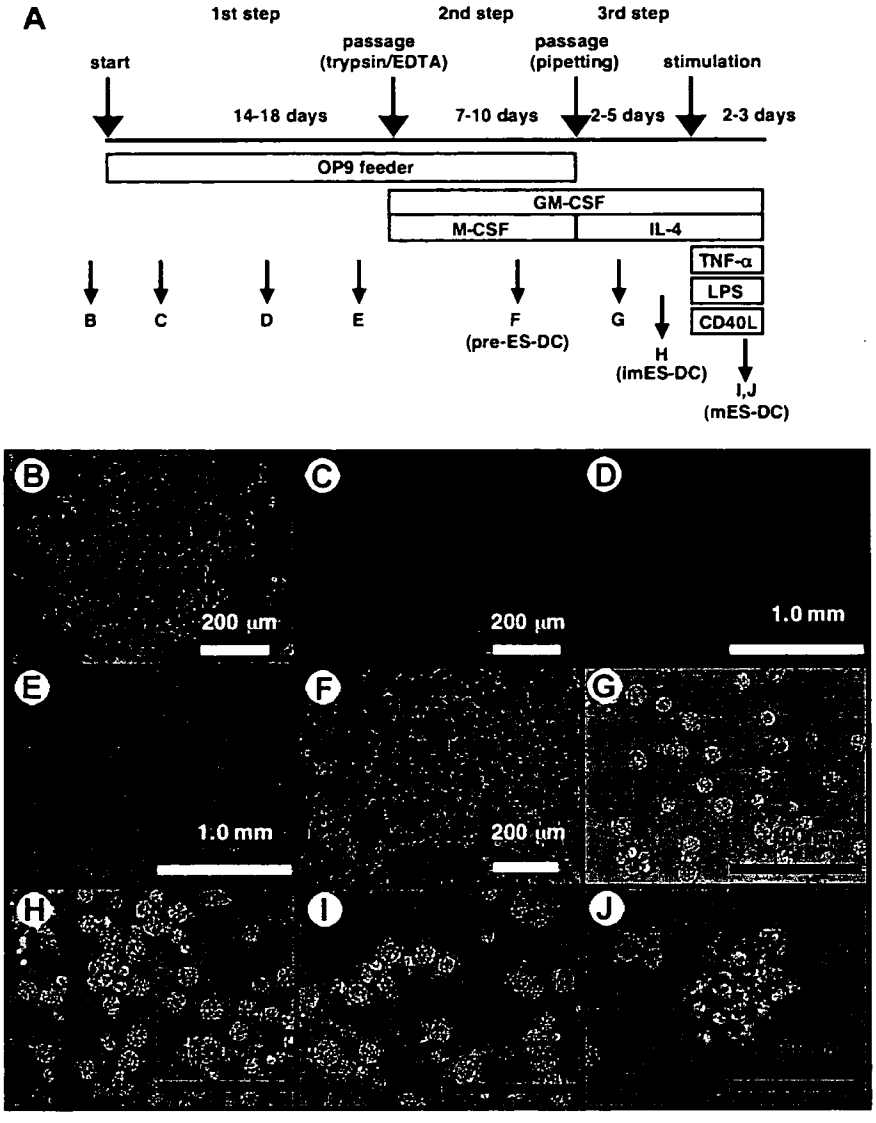

Figure 1. Culture protocol and morphological changes of human embryonic stem (ES) cell-derived cells during differentiation culture. **(A)**: The schedule for the culture to induce differentiation of human ES cells into ES-DC is schematically depicted. **(B)**: Undifferentiated human ES cells on primary embryonic fibroblast feeder layer. **(C–E)**: ES cell-derived cells on day 3 **(C)**, day 11 **(D)**, and day 15 **(E)** in the first step. **(F)**: Cells on day 6 in the second step. **(G–J)**: Cells on day 1 **(G)**, day 3 **(H)**, and day 6 **(I, J)** in the third step. Cells shown in **(I, J)** had been stimulated with TNF-α plus LPS for 2 days. Abbreviations: ES-DC, embryonic stem cell-derived dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; imES-DC, immature embryonic stem cell-derived dendritic cells; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; mES-DC, mature embryonic stem cell-derived dendritic cells; TNF-α, tumor necrosis factor α.

primary embryonic fibroblast (PEF) feeder layers as previously described [9, 10]. Mouse-derived hematopoietic stromal cell line OP9 was treated with mitomycin C (10 μg/ml) for 1 hour before plating onto gelatin-coated tissue culture dishes to make feeder cell layers. The establishment and maintenance of cynomolgus monkey ES cell line CMK6 was also reported [11, 12]. Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin-4 (IL-4), tumor necrosis factor α (TNF-α), and soluble CD40-ligand were purchased from Peprotech (London, http://www.peprotech.com). Lipopolysaccharide (LPS) from *Escherichia coli* and OK-432 were purchased from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) and Chugai Pharmaceutical (Tokyo, http://www.chugai-pharm.co.jp/hc/chugai_top_en.jsp), respectively.

Induction of Differentiation of ES Cells into ES-DC

The procedure for differentiation culture was composed of three steps (Fig. 1A). Step 1 was as follows: undifferentiated ES cells maintained on PEF were rinsed with phosphate-buffered saline (PBS) and treated with dissociation solution containing 1 mg/ml collagenase, 0.25% trypsin, and 20% knockout serum replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) in PBS [10] and cultured on OP9 feeder cell layers in minimum essential medium-α supplemented with 20% fetal calf serum (FCS) and 2-mercaptoethanol (50 μM). Culture of cells was continued for 14–18 days with human ES cells and for 11–13 days with cynomolgus monkey ES cells, and the medium was changed once every 3 days. At the end of this step, the cells were rinsed with PBS, treated with trypsin-EDTA (PBS containing 0.25% trypsin and 1 mM EDTA) for 30–40 minutes, and recovered. After resuspension in culture medium, the cells were plated onto culture dishes and incubated for 2–4 hours. Thereafter, floating or weakly adherent cells were recovered by pipetting, and any firmly adherent cells were discarded. Step 2 was as follows: after being passaged through nylon mesh (Cell Strainer 100 μm; BD Biosciences, Bedford, MA, http://www.bdbiosciences.com), cells recovered from one 90-mm dish were plated in two dishes with freshly prepared OP9 feeder layers. On the following day, the culture medium was exchanged with a medium containing GM-CSF (100 ng/ml) and M-CSF (50 ng/ml). The culture was continued for 7–10 days, depending on the propagation of floating cells on the feeder layers. Step 3 was as follows: ES cell-derived floating cells were recovered by pipetting; resuspended in RPMI 1640 medium containing 10% FCS, GM-CSF (100 ng/ml), and IL-4 (10 ng/ml); and cultured in Petri dishes ($3–5 \times 10^5$ cells per dish) without a feeder layer (Locus, Tokyo). To induce maturation, IL-4 (10 ng/ml), TNF-α (10 ng/ml), LPS (3 μg/ml), and, in some experiments, soluble CD40-ligand (20 ng/ml) or OK-432 (10 μg/ml) were simultaneously added on day 3 or 5 of this step, and the culture was continued for an additional 2–3 days. Differentiating cells were microscopically analyzed on an inverted microscope (IX70; Olympus, Tokyo, http://www.olympus-global.com).

Flow Cytometric Analysis

The following monoclonal antibodies (Ab) conjugated with fluorescein isothiocyanate or phycoerythrin were purchased from BD Pharmingen (San Diego, http://www.bdbiosciences.com/index_us.shtml) or eBioscience Inc. (San Diego, http://www.ebioscience.com): anti-human histocompatibility leukocyte antigen (HLA)-DR (clone L243, mouse IgG2a); anti-HLA-A, B, and C (clone G46–2.6, mouse IgG1); anti-human CD80 (clone L307.4, mouse IgG1); anti-human CD83 (clone HB15e, mouse IgG1); anti-human CD86 (clone FUN-1, mouse IgG1); anti-human CD40 (clone 5C3, mouse IgG1); anti-human B7-H1/programmed death-1-ligand-1 (PD-L1) (clone MIH1, mouse IgG1); and anti-human CD74 (clone M-B741, mouse IgG2a). As isotype-matched controls, mouse IgG2a (clone G155–178) and mouse IgG1 (clone MOPC-21) were used. The cell samples were treated with FcR-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) for 10 minutes, stained with the fluorochrome-conjugated Ab for 30 minutes, and washed three times with PBS/2% FCS. Intracellular staining with anti-CD74 monoclonal Ab was done by using IntraPrep (Beckman Coulter, Marseille, France, http://www.beckmancoulter.com). Stained cell samples were analyzed on a FACScan flow cytometer, and, in some experiments, the DC fraction was gated by forward and side scatters.

Enzyme-Linked Immunosorbent Assay to Detect Cytokine Production by ES-DC

Cells were cultured in 96-well flat-bottomed culture plates (1.2×10^5 cells in 150 μl of medium per well) in the presence or absence of soluble CD40-ligand, LPS, or OK432. After 60 hours of culture, supernatant was collected, and the concentration of TNF-α and IL-12 p70 was measured by using enzyme-linked immunosorbent assay (ELISA) kits (Pierce, Rockford, IL, http://www.piercenet.com).

Allogeneic T-Cell-Stimulation Assay

Mononuclear cells were isolated from heparinized peripheral blood of a human or a cynomolgus monkey housed in the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden, http://www.amersham.com). T cells were purified using the Pan T cell isolation kit for humans or the kit for nonhuman primates (Miltenyi Biotec). The T cells (4×10^4/well) were cocultured with graded numbers of x-ray-irradiated (40 Gy) stimulator cells in RPMI 1640 medium supplemented with 10% human plasma in 96-well round-bottomed culture plates for 5 days. [^{3}H]-Methyl-thymidine (247.9 GBq/mmol) was added to the culture (0.037 MBq/well) for the last 16 hours. At the end this time, the cells were harvested onto glass fiber filters (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com), and the incorporation of [^{3}H]-thymidine was measured by scintillation counting. In the experiment using PD-L1-transfectant ES-DC, anti-PD-L1 blocking Ab (clone MIH1; eBioscience) or control mouse IgG1 Ab (eBioscience) was added to the culture (10 μg/ml).

Recombinant Antigenic Protein

A DNA fragment encoding human glutamic acid decarboxylase (GAD65) p96–174 protein fragment was cloned into the prokaryotic expression vector pGEX-4T-3 (Amersham Biosciences), to generate a vector for glutathione *S*-transferase-fused GAD65 protein fragment (GST-GAD). The induction of the production of recombinant protein in *E. coli* (DH5α) and the extraction of the recombinant protein from bacterial inclusion bodies was done according to Frangioni and Neel [13]. The purification of the recombinant protein with glutathione-agarose (Sigma-Aldrich) was done as described in our previous report [14, 15]. The purity and integrity of the recombinant protein was confirmed by SDS-polyacrylamide gel electrophoresis. The protein was concentrated and separated from small peptide fragments, if any, with Centricon-10 (Millipore, Bedford, MA, http://www.millipore.com), and the solvent was changed from the elution buffer to the culture medium by dialysis.

Antigen Presentation Assay

A human CD4$^+$ T-cell clone, SA32.5, recognizing GAD65p111–131 in the context of HLA-DR53 molecule (DRA*0101+DRB4*0103) was established and maintained as previously described [16]. In the assay with the synthetic peptide, ES-DC stimulated with TNF-α (10 ng/ml) plus LPS (3 μg/ml) were harvested, incubated in the presence of peptide (6 μM) for 3 hours, washed four times with culture medium, and x-ray-irradiated (35 Gy). A T-cell proliferation assay was set up in a 96-well flat-bottomed culture plate with SA32.5 T cells (3×10^4 cells per well) and graded numbers of the peptide-loaded ES-DC in RPMI 1640 medium supplemented with 10% human plasma. In the assay with recombinant protein, the indicated amount of GST or GST-GAD protein was added to the coculture of SA32.5 T cells (3×10^4 cells per well) and irradiated ES-DC (1×10^4 cells per well). After 48 hours of culture, [^{3}H]-thymidine was added, and then after an additional 16 hours of culture, the cells were harvested and the incorporated radioactivity was counted.

Plasmid Construction

cDNA for human PD-L1 was isolated by polymerase chain reaction (PCR) with Pyrobest DNA polymerase (Takara, Osaka, Japan, http://www.takara.co.jp) using cDNA clone CS0DI011, purchased from Invitrogen (Carlsbad, CA, http://www.invitrogen.com), as a template. Double-stranded oligo DNA (5′-atgaacattttacttcagtatgtg-gtgaaaagtttcgat-3′) coding for GAD65p115–127 (the core epitope for SA32.5 T-cell clone) was ligated to human invariant chain (Ii)-based epitope presentation vector pCI [17] to generate GAD65-epitope-fused Ii. A cDNA fragment for HLA-DRB4*0103 was generated by reverse transcriptase (RT)-PCR from RNA isolated from peripheral blood mononuclear cells positive for *HLA-DRB4*0103*. The coding DNA fragments were cloned into a mammalian expression vector, pCAG-IRES-Neo, which is driven by the CAG promoter and includes an internal ribosomal entry site (IRES)-neomycin-resistance gene cassette [3].

Transfection of ES Cells

Human ES cells were harvested using CTK solution, dissociated into clusters of 50–100 cells by pipetting, and washed twice with Dulbecco's modified Eagle's medium (DMEM). The cells harvested from two 90-mm culture dishes with subconfluently growing ES cells were suspended in 0.1 ml of DMEM and mixed with 50 μg of linearized plasmid DNA dissolved in 0.1 ml of PBS in a 4-mm-gap cuvette. The electroporation of human ES cells was performed at 150 V and 200 μF on a Gene Pulser (Bio-Rad, Hercules, CA, http://www.bio-rad.com). The transfection of cynomolgus monkey ES cells was done as previously described [18], with some modifications. Cynomolgus monkey ES cells were harvested after treatment with trypsin-EDTA. ES cells ($1–1.5 \times 10^7$) suspended in 0.7 ml of DMEM were mixed with 50 μg of plasmid DNA in 0.1 ml of PBS in a 4-mm-gap cuvette. Electroporation was done at 250 V and 500 μF. After electroporation, the ES cells were cultured on G418-resistant PEF feeder layers in 90-mm culture dishes or six-well plates. Selection with G418 (150 μg/ml) was done from 2 to 4 days after the transfection, and G418-resistant ES cell colonies were picked up using a micropipette under microscopic observation on days 15–18 for human ES cells and on day 11 for monkey ES cells. The transfectant clones were transferred to 24-well culture plates with PEF and expanded in the presence of G418. ES cell transfectant clones with relatively high levels of expression of the transgene were selected on the basis of the resistance to a high dose (1–3 mg/ml) of G418 and the results of the RT-PCR analysis. Thereafter, the clones were subjected to the differentiation procedures. At the proper stages of differentiation, the cells were screened to select ES cell clones that highly expressed the transgene after differentiation, based on a flow cytometric analysis for PD-L1 and Ii transfectant human ES cells and on the antigen-presenting capacity for HLA-DRB4 transfectant cynomolgus monkey ES cells.

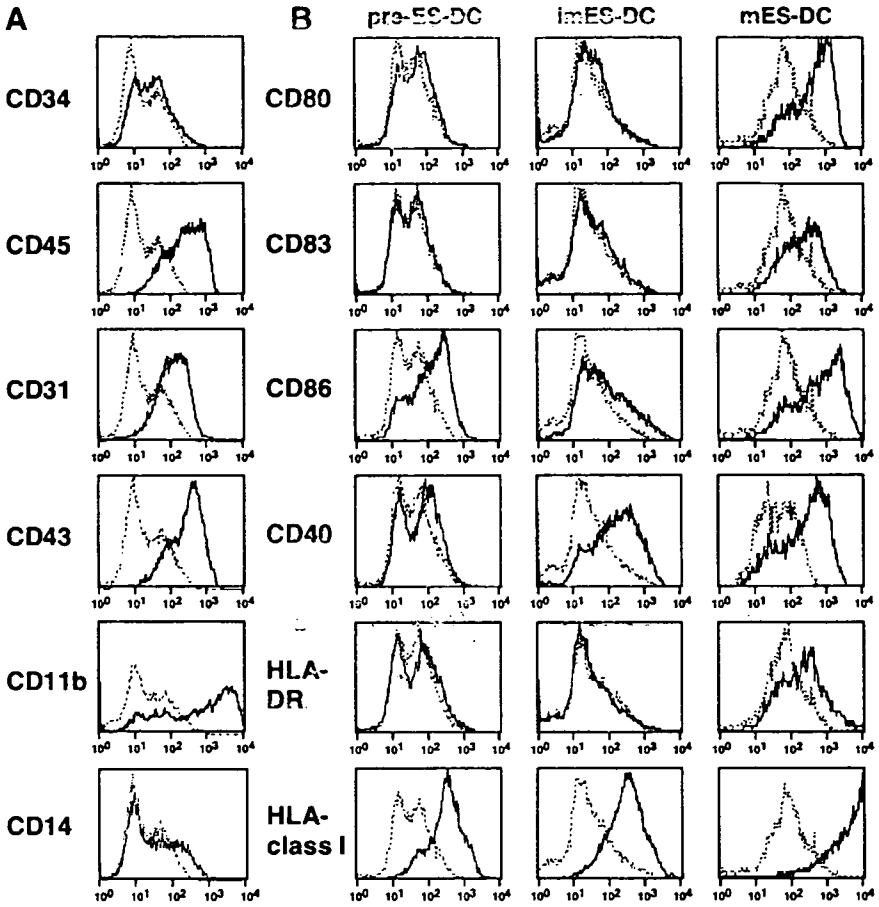

Figure 2. Cell surface phenotypes of human ES-DC. **(A):** ES cell-derived floating cells harvested on day 6 in the second step were analyzed for the cell surface expression of CD34, CD45, CD31, CD43, CD11b, and CD14. **(B):** ES cell-derived cells harvested on day 8 in the second step (pre-ES-DC) and from the third step before (imES-DC) and after (mES-DC) addition of maturation stimuli were analyzed for the cell surface expression of CD80, CD83, CD86, CD40, HLA-DR, and HLA class I. Staining profiles with specific antibody (Ab) (thick lines) and isotype-matched control Ab (thin, broken lines) are shown. Abbreviations: ES-DC, embryonic stem cell-derived dendritic cells; HLA, histocompatibility leukocyte antigen; imES-DC, immature embryonic stem cell-derived dendritic cells; mES-DC, mature embryonic stem cell-derived dendritic cells.

RT-PCR for Detection of the Transgene-Derived Transcripts

cDNA was synthesized from total cellular RNA with random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). The following PCR primer sets were used: 5′-gctggattacatcaaagcactgaa-3′ and 5′-caacaaagtctggcttatatccaa-3′ for hypoxanthine-guanine phosphoribosyl transferase and 5′-ctgactgaccgcgttactcccaca-3′ and 5′-ttggttatagatgtatctgatcaggt-3′ for transgene-derived DRB4 transcript.

RESULTS

Differentiation of Human ES Cells to ES-DC

Based on previous experience in the generation of dendritic cells from mouse ES cells [1] and also based on the findings in a preliminary study using cynomolgus monkey ES cells, the feeder cell-coculture method was adopted for the generation of dendritic cells from human ES cells, instead of the embryoid body (EB)-based method. The human ES cell line selected was KhES-1; this line exhibited the highest growth rate among the three lines of human ES cell lines established in a recent study [9, 19]. For feeder cells, three lines of mouse stromal cell lines (ST2, OP9, and PA6) were evaluated for their capacity to induce hematopoietic differentiation of KhES-1 ES cells, and OP9 had the best yield among them (data not shown).

The protocol for the differentiation culture to generate ES-DC from human ES cells developed in the current study is composed of three steps, as shown in Figure 1A. At the beginning of the differentiation culture, undifferentiated ES cells maintained on mouse PEF feeders (Fig. 1B) were harvested using dissociation solution CTK [9] and plated on OP9 feeder cell layers (step 1). Next, the ES cells grew and formed clusters composed mostly of epithelial cell-like large flat cells (Fig. 1C, 1D). Clusters of round, cobblestone-like cells also appeared at approximately day 8, and those resembled the mesodermally differentiated cell clusters observed in hematopoietic differentiation culture of mouse ES cells [1, 20]. The size and number of round cell clusters gradually increased, and by around day 15, they covered 20%–30% of the surface area (Fig. 1E).

On days 15–18 of the first step, cells were recovered from the dishes using trypsin/EDTA and isolated nonadherent cells, and then they were seeded onto freshly prepared OP9 cell layers, to begin the second step. On the next day, the culture medium was exchanged for medium containing GM-CSF and M-CSF. Thereafter, small round cells, floating or loosely adhering to the feeder layer, appeared and gradually increased in number (Fig. 1F). The growth of the round cells depended primarily upon GM-CSF, thus suggesting that they grew in response to that factor. The cells were recovered and analyzed for their expression of hematopoietic cell lineage markers by flow cytometry (Fig. 2A). The cells expressed CD34 and CD45, thus indicating that they followed a hematopoietic cell lineage. They also expressed CD31, CD43, and CD11b, thus collectively indicating a commitment to a myeloid cell lineage. The double peaks seen in the histograms in Figure 2 reflect the heterogeneity of the analyzed cells in size and intensity of autofluorescence.

On days 7–10 of the second step, the floating or loosely adherent cells were harvested by pipetting and transferred to Petri dishes without feeder cells. We then cultured the cells in the presence of GM-CSF and IL-4 to start the third step. Following this passage, the cells changed their morphology from round to irregular shapes, and some had protrusions (Fig. 1G).

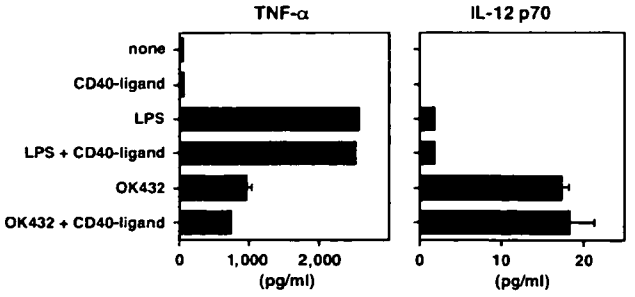

Figure 3. Production of TNF-α and IL-12 by embryonic stem cell-derived dendritic cells (ES-DC). ES-DC were recovered from the culture (third step, day 4) and replated (1.2×10^5 cells per 150 μl in 96-well culture plates) in the presence or absence of soluble CD40-ligand (20 ng/ml), LPS (3 μg/ml), or OK432 (10 μg/ml) as indicated. After 60 hours, supernatant was collected, and concentration of TNF-α and IL-12 p70 was measured by enzyme-linked immunosorbent assay. Data are indicated by mean value + SD of duplicate cultures. Abbreviations: IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α.

Cells with protrusions gradually increased, and more than 50% of the cells exhibited DC-like irregular shapes after 2–3 days (Fig. 1H). The floating cells expressed CD86 and CD40 but scarcely expressed CD80 or CD83 (Fig. 2B). Expression of HLA-DR at this stage differed between experiments.

Figure 1I and 1J shows the cells after the simultaneous addition of TNF-α, LPS, soluble CD40-ligand, and IL-4. Generally, they exhibited longer protrusions than before the stimulation, and some of the protrusions were veil-like. Many of the cells formed aggregates. Flow cytometric analysis showed the increased expression of CD86 and the expression of CD80, CD83, and HLA-DR (Fig. 2B). Collectively, the cells exhibited the characteristics of DC in their morphology and expression of surface molecules, and thus they were designated human ES-DC.

Production of IL-12 and TNF-α by ES-DC was measured by ELISA (Fig. 3). Production of TNF-α was profoundly induced by either LPS or OK432. OK432, but not LPS, induced the production of IL-12, consistent with the reports that OK432 is an efficient inducer of IL-12 [21, 22]. Addition of CD40-ligand showed little effect on the production of these cytokines by human ES-DC.

ES cell-derived floating cells first appeared during the second step of the culture for differentiation (pre-ES-DC) and could readily be isolated by the pipetting procedure. Their morphology, pattern of expression of surface molecules, and T-cell-stimulation capacity (described below) continuously changed until the final maturation. To determine the change in gene expression associated with such changes in the phenotypes, the gene expression profiles of pre-ES-DC, immature ES-DC, and mature ES-DC were analyzed using cDNA microarrays. For reference purposes, human peripheral blood monocytes and immature and mature Mo-DC were also analyzed. The data for genes with relevance to immune functions were selected from the total microarray data and are shown in supplemental online Table 1. Consistent with the results of flow cytometric analysis (Fig. 2B), upregulation of the expression of genes encoding cell surface molecules such as HLA class I, HLA class II, CD86, and CD40, along with differentiation of ES-DC, was observed. In addition, expression of the genes related to DC function, including CD74/invariant chain, CCR7, and CCL17/TARC, was increased during the differentiation. Clustering analysis indicates similarity between change of the gene expression pattern from monocytes to immature Mo-DC and that from pre-ES-DC to immature ES-DC, as well as that from immature Mo-DC to mature Mo-DC and that from immature ES-DC to mature ES-DC (supplemental online Fig. 1).

The protocol of differentiation culture described thus far was originally developed using the KhES-1 line of human ES cells. This differentiation procedure was also applied to KhES-3, another human ES cell line. KhES-3 differentiation was similar to KhES-1 except that KhES-3 differentiated slightly more quickly than KhES-1, and a first-step culture of 14–15 days was sufficient for the differentiation of KhES-3.

Function of Human ES-DC

The capacity of the human ES-DC to stimulate T cells was examined based on the proliferative response of allogeneic T cells cocultured with ES-DC (Fig. 4A). ES cell-derived floating cells recovered from the second step (pre-ES-DC) showed little capacity to induce a response of T cells. In contrast, ES-DC following the third step before the addition of maturation stimuli (immature ES-DC) showed a weak but definite stimulation, and following exposure to the maturation stimuli (mature ES-DC) they showed a strong capacity to stimulate allogeneic T cells to proliferate.

Next, the antigen-presenting capacity of ES-DC was examined. KhES-1 is positive for the *HLA-DRB4*0103* gene encoding the β chain of HLA-DR53 molecule. Presumably, ES-DC derived from KhES-1 should express the DR53 molecule, and their ability to present antigen to DR53-restricted $CD4^+$ T cells was determined. As shown in Figure 4B, KhES-1-derived ES-DC preloaded with GAD65-derived synthetic peptide stimulated GAD65-specific DR53-restricted human T-cell clone SA32.5 to proliferate. To examine the capacity to process antigenic protein and present epitope, recombinant protein was used as the antigen (Fig. 4C). The SA32.5 T-cell clone cocultured with the ES-DC in the presence of recombinant GAD65 protein also showed a proliferative response, thus indicating that ES-DC processed the antigenic protein and presented the epitope derived from the protein in the context of HLA class II molecules.

Genetic Modification of Human ES-DC

Previous research established a strategy for the genetic modification of mouse ES-DC [1]. Briefly, the expression vectors were introduced into ES cells by electroporation, and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC. The following experiments were performed to determine whether or not this strategy could be applicable to human ES cells.

PD-L1/B7-H1 is known to downmodulate responses of T cells upon interaction with the ligand, PD-1 on T cells [23]. An expression vector for human PD-L1 was introduced to KhES-1 by electroporation. The expression vector used was pCAG-INeo, driven by the CAG promoter and containing an IRES-neomycin-resistance gene cassette (Fig. 5A). Among the transfectant clones, 23 ES cell clones showing resistance to high does of G418 (2 mg/ml) were selected and subjected to the ES-DC-differentiation culture.

The expression of PD-L1 of the transfectant clones was examined by a flow cytometric analysis at the stage of immature ES-DC, harvested on day 2 of the third step of the differentiation culture. Although even nontransfectant ES-DC evidently expressed PD-L1 after maturation (data not shown), only a small population of them expressed PD-L1 at this stage (Fig. 5B, K1ES-DC). On the basis of the results of the analysis, one transfectant clone, KhES1-PD28, expressing the highest level of PD-L1 after the differentiation into immature ES-DC, was selected (Fig. 5B). Allogeneic T cells cocultured with immature ES-DC-PD28 showed a significantly lower response than those cocultured with nontransfectant immature ES-DC ($p < .05$; Fig.

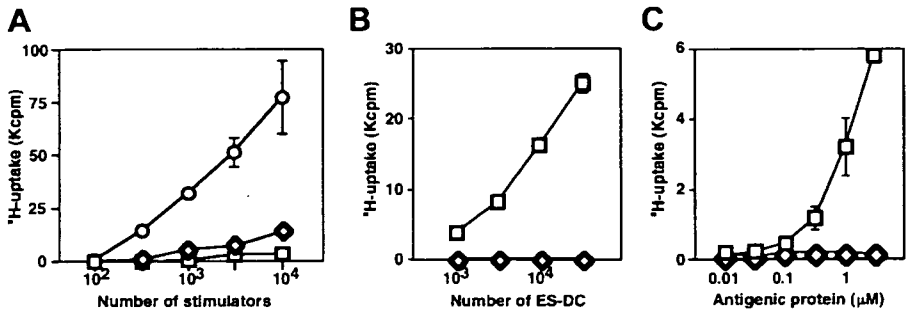

Figure 4. Stimulation of allogeneic T cells and antigen presentation by human ES-DC. **(A):** The indicated numbers of mature ES-DC (circles), immature ES-DC (diamonds), and pre-ES-DC (squares) were x-ray-irradiated (40 Gy) and cocultured with allogeneic human peripheral blood T cells (4×10^4 cells per well) in a 96-well round-bottomed culture plate for 5 days. Proliferation of T cells in the last 16 hours of the culture was measured based on [^{3}H]-thymidine uptake. The data are indicated as the mean value ± SD of duplicate cultures. **(B):** The indicated numbers of KhES-1-derived mature ES-DC prepulsed with glutamic acid decarboxylase (GAD) $65_{111-131}$ peptide (squares) and those left unpulsed (diamonds) were cocultured with a GAD65-specific, HLA-DR53-restricted human CD4$^+$ T-cell clone, SA32.5 (3×10^4 T cells per well) for 3 days. Proliferation of the T cells in the last 16 hours of the culture was measured by [^{3}H]-thymidine uptake. **(C):** Mature KhES-1-derived ES-DC (1×10^4 cells per well) were cocultured with SA32.5 T cells (3×10^4 cells per well) in the presence of the indicated concentrations of glutathione *S*-transferase (GST)-GAD65 recombinant protein (squares) or GST protein (diamonds) for 3 days. Proliferation of the T cells in the last 16 hours of the culture was measured by [^{3}H]-thymidine uptake. Abbreviation: ES-DC, embryonic stem cell-derived dendritic cells.

5C). The proliferation-reducing effect of the transgene-derived PD-L1 was abrogated by the addition of anti-PD-L1 blocking Ab ($p < .01$), ruling out the possibility that the introduction of the PD-L1 expression vector impaired the differentiation of ES-DC. Collectively, these results suggest that forced expression of PD-L1 on ES-DC downmodulated the proliferative response of cocultured allogeneic T cells via the interaction of PD-L1 with PD-1 on the T cells.

ES-DC carrying an epitope-presenting vector and expressing recombinant human invariant chain (Ii/CD74), which included GAD65p115–127 in the class II-associated invariant chain peptide region, were also generated (Fig. 5D). It was expected that the epitope could be efficiently targeted to the major histocompatibility complex (MHC) class II pathway [17]. Using a protocol similar to that used for the generation of PD-L1 transfectants, the vector was introduced into KhES-1 ES cells, and a transfectant clone, KhES-1-Ii23, highly expressing transgene-derived recombinant CD74, was selected by a flow cytometric analysis at the pre-ES-DC stage. The expression of CD74 was detected even in the nontransfectant pre-ES-DC, reflecting intrinsic expression of CD74 (Fig. 5E). The transfectant exhibited an increased expression of CD74 in comparison to the nontransfectants, thus indicating additional expression of the molecule derived from the transgene. The ability of the transfectant ES-DC, ES-DC-Ii23, to stimulate the GAD epitope-specific T-cell clone SA32.5 in the absence of antigenic peptide or protein was next examined. As a result, ES-DC-Ii23 stimulated SA32.5 T cells and induced their proliferation, thus demonstrating functional expression of the epitope-presentation vector in the transfectant ES-DC (Fig. 5F). The in vivo transfer of ES-DC transfected with this antigen-presenting vector is therefore expected to be useful for controlling the immune response in an antigen-specific manner [7].

Generation and Genetic Modification of Cynomolgus Monkey ES-DC

The differentiation protocol established using human ES cells was then applied to nonhuman primate ES cells. An ES cell line derived from cynomolgus monkey, CMK6 [11], was subjected to the ES-DC differentiation culture. Following the transfer to OP9 feeder layers, CMK6 cells grew and differentiated more rapidly than did human ES cells KhES-1 and KhES-3. The optimal duration of the first step of the differentiation culture for CMK6 was 11–13 days, whereas the duration ranged from 14 to 18 days for human ES cells. Figure 6A–6C illustrates the morphological changes of CMK6-derived cells following the second step of the differentiation culture. The surface phenotypes of the CMK6-derived pre-ES-DC, immature ES-DC, and mature ES-DC were then analyzed by flow cytometry (Fig. 6D). The double peaks seen in the histograms in Figure 6D reflect the heterogeneity of the analyzed cells in size and intensity of autofluorescence. Cynomolgus monkey ES-DC had the capacity to stimulate allogeneic cynomolgus monkey T cells (Fig. 6E), as human ES-DC did.

The expression vector for *HLA-DRB4*0103* (Fig. 7A) was introduced to CMK6. An analysis of the partial nucleotide sequence of *DRA* (*CyLA-DRA*) gene of CMK6 showed that the predicted amino acid sequence of the CyLA-DRα chain is very similar to that of HLA-DRα, with only one amino acid difference in α1 domain (GenBank accession no. AY591919). This suggested that the transgene-derived HLA-DRβ chain could associate with the intrinsic CyLA-DRα chain expressed in cynomolgus ES-DC and present an antigen to human T cells. The expression of the transgene before and after the ES-DC differentiation was confirmed by an RT-PCR analysis (Fig. 7B). ES-DC derived from a transfectant ES cell clone, cES-53-23, were prepulsed with synthetic GAD65 peptide and cocultured with the HLA-DR53-restricted, GAD65-specific T-cell clone SA32.5. Figure 7C shows that the GAD65 peptide-pulsed transfectant ES-DC stimulated the T cells to proliferate. In contrast, ES-DC originating from parental ES cells prepulsed with the peptide could not stimulate the T-cell clone. In addition, DR53-transfectant cynomolgus ES-DC had the capacity to process and present a protein antigen to the T cells (Fig. 7D). These results demonstrate the antigen-processing and presenting capacity of cynomolgus ES-DC and also the functional expression of the transgene that had been introduced into the ES cells before the differentiation. Thus, the effect and safety of the immune therapy by the in vivo transfer of ES-DC can be examined by preclinical studies using cynomolgus monkeys.

DISCUSSION

To establish the current culture protocol, various culture conditions were tested. As feeder cell lines, three lines of mouse stromal cells, OP9, PA6, and ST2, were comparatively evaluated. As a result, the use of OP9 was thus observed to produce

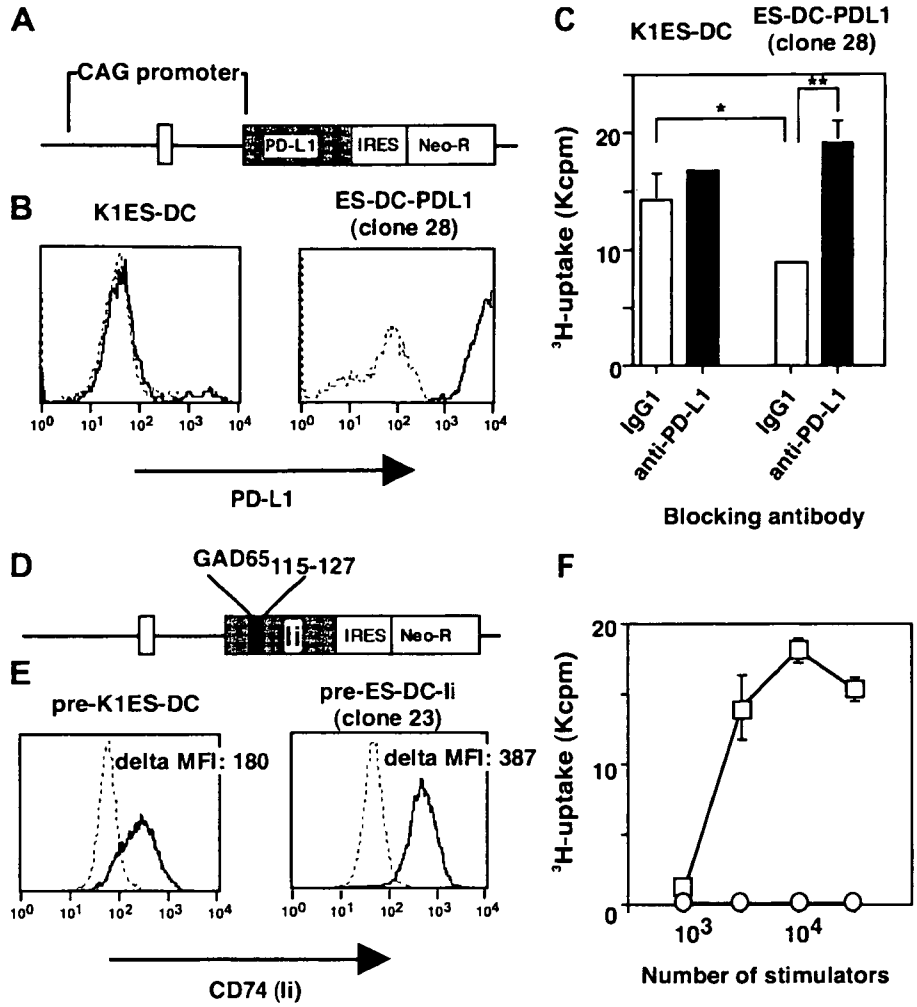

Figure 5. Genetic modification of human ES-DC. **(A):** Structure of the expression vector for human PD-L1. The expression of PD-L1 was driven by the CAG promoter, and the PD-L1-coding sequence was followed by IRES-neomycin-resistance gene (Neo-R), a selection marker. The open box in the CAG promoter indicates exon 1 of the rabbit β-actin gene contained in CAG promoter. **(B):** Transgene-derived PD-L1 expressed in immature ES-DC originated from transfectant embryonic stem (ES) cells was detected by flow cytometric analysis (ES-DC-PD-L1, clone 28). As a control, the staining profile of ES-DC derived from parental ES cell line (K1ES-DC) is shown. Specific stainings with anti-human-PD-L1 monoclonal antibody (Ab) (thick line) and isotype-matched control staining (thin, broken line) are shown. **(C):** The alloreactive response of T cells (4×10^4 cells per well) cocultured with immature ES-DC (1×10^4 cells per well) derived from the PD-L1-transfectant ES cells (ES-DC-PD-L1, clone 28) or those derived from parental ES cell line (K1ES-DC) is shown. The culture was done under the same conditions as those shown in Figure 3A except that anti-PD-L1 blocking Ab or isotype-matched mouse IgG1 was added to the culture. The statistical significance of the differences between the T-cell responses is indicated by asterisks (*, $p < .05$; **, $p < .01$). **(D):** Structure of expression vector for human Ii (Ii/CD74) including GAD65-derived epitope. The class II-associated invariant chain peptide region of the Ii-coding sequence was replaced with an oligo DNA-encoding $GAD65_{115-127}$. **(E):** Intracellular CD74 expressed in pre-ES-DC originated from transfectant ES cell clone (pre-ES-DC-human Ii [hIi], clone 23) and parental ES cell line (pre-K1ES-DC) was detected by a flow cytometric analysis. Specific staining with anti-human-CD74 monoclonal Ab (thick lines) and isotype-matched control staining (thin, broken lines) are shown. The values in the figure indicate the delta MFI between staining with the anti-CD74 and the isotype-matched control Ab. **(F):** SA32.5 T cells (3×10^4 cells per well) were cocultured with the indicated numbers of mature ES-DC-hIi clone 23 (squares) or nontransfectant ES-DC (circles) in the absence of exogenous antigen for 3 days. The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. Abbreviations: delta MFI, difference of mean fluorescence intensity; ES-DC, embryonic stem cell-derived dendritic cells; GAD, glutamic acid decarboxylase; Ii, invariant chain; IRES, internal ribosomal entry site.

the highest yield of ES-DC. Although ST2 also worked as feeder cells in the second step, the yield of ES-DC was approximately half of that obtained using OP9. It was also essential to remove any firmly adherent cells, when transferring the cells from the first to second step, by the procedure described in the Materials and Methods. At the end of the first step, many flat, adherent ES cell-derived cells were observed to form monolayers in the dishes. They probably differentiated into cell lineages other than mesoderm, and unless removed, they grew rapidly in the second step and inhibited the growth of hematopoietic cells.

Previously, two other groups reported the generation of functional antigen-presenting cells or DC from human ES cells. Zhan et al. adapted embryoid body-based induction of hematopoietic differentiation [24]. Slukvin et al. recently reported a method using OP9 [25]. Although there are some similarities between the method of Slukvin et al. [25] and the one reported here, the two methods differ in the following points.

In both methods, human ES cells were cocultured with OP9 feeder cells at the initial differentiation step (the first step). However, the duration of this culture step in our method (14–18 days) is significantly longer than the 10 days in the method of Slukvin et al. [25]. In our system, cells with morphology indicating mesodermal differentiation first appeared on day 8 or 9, and the extension of the first step of culture to days 14–18 significantly improved the yield of hematopoietically differentiated cells (Fig. 1D, 1E). In addition, we pretreated OP9 cells

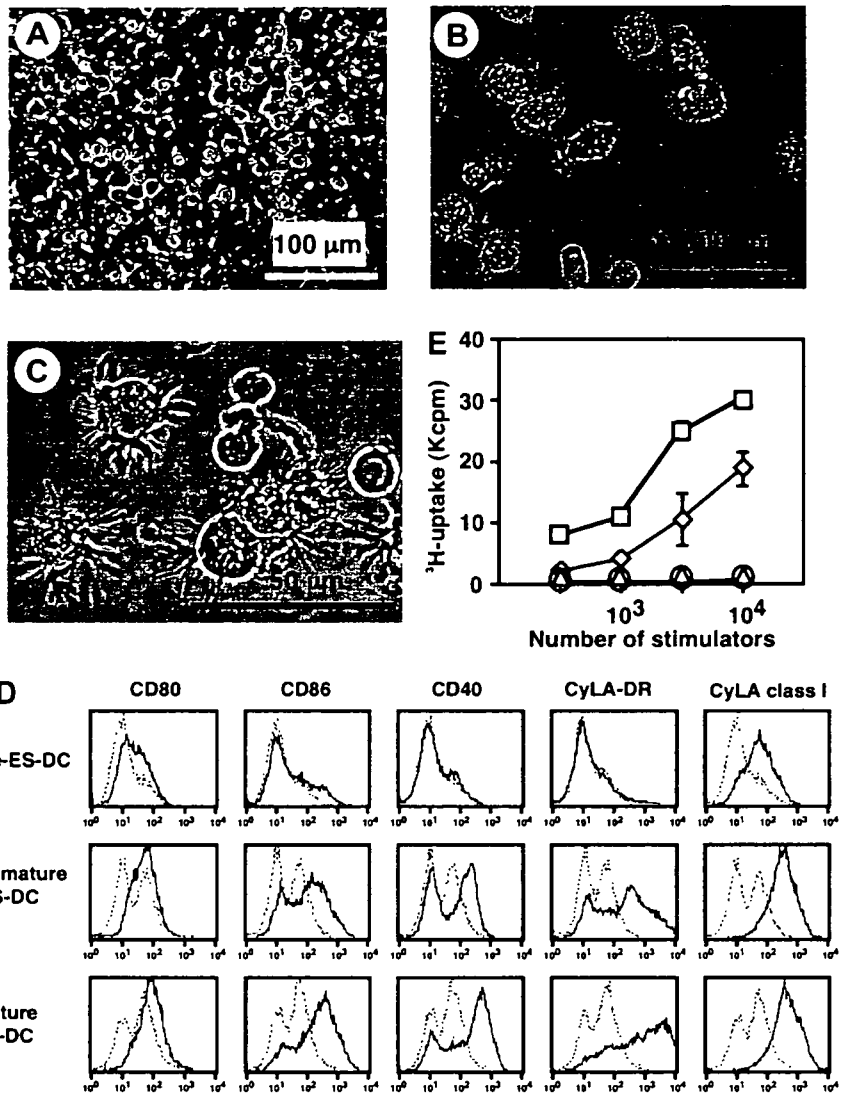

Figure 6. Generation of ES-DC from cynomolgus monkey embryonic stem (ES) cells. **(A–C):** The morphologies of cynomolgus monkey ES cell-derived differentiating cells (pre-ES-DC) at day 7 in the second step **(A)** and those in the third step before **(B)** and after **(C)** the addition of maturation stimuli are shown. **(D):** Cynomolgus monkey ES cell-derived cells harvested on day 8 in the second step (pre ES-DC) and from the third step before (immature ES-DC) and after (mature ES-DC) addition of maturation stimuli were analyzed for the cell surface expression of CD80, CD86, CD40, CyLA-DR, and CyLA class I. Staining patterns with specific monoclonal antibody (thick lines) and isotype-matched controls (thin, broken lines) are shown. **(E):** The indicated numbers of mature ES-DC (squares), immature ES-DC (diamonds), pre-ES-DC (circles), and undifferentiated cynomolgus ES cells (triangles) were x-ray-irradiated (40 Gy) and cocultured with allogeneic cynomolgus monkey peripheral blood T cells (4×10^4 cells per well) in a 96-well round-bottomed culture plate for 5 days. The proliferative responses of T cells in the last 16 hours of the culture were measured based on the [^{3}H]-thymidine uptake. Abbreviation: ES-DC, embryonic stem cell-derived dendritic cells.

with mitomycin C before use as feeder cells, and this was essential for efficient generation of hematopoietic cells. Treatment with mitomycin C may not only inactivate the mitosis of OP9 but also enhance the capacity of OP9 to support hematopoietic differentiation [26].

In the method of Slukvin et al., cells harvested from the first step of culture were directly transferred to 2-hydroxyethyl methacrylate-coated culture containers for the second step of culture [25]. In our method, cells harvested from the first step of culture were incubated in tissue culture-coated dishes for 2–5 hours to remove adherent cells. Removal of cells committed to nonmesodermal lineages by this procedure is essential. In addition, the second step of culture was also done with OP9 feeder in our method.

After the second step of culture, removal of dead cells and aggregated cells may be necessary in the method of Slukvin et al., as described in their report [25]. Indeed, we observed many dead cells, as well as DC-like cells, when we tried that method. In our method, most of recovered cells after the second step were viable, and removal of dead cells was not necessary.

The issues of safety and efficacy are critical for the establishment of ES-DC therapy. It is presumed that preclinical in vivo studies with the nonhuman primates will be required. Therefore, the ability to generate ES-DC from cynomolgus monkey ES cells is also considered to be important. It is probable that ES-DC can be generated from the ES cells of other nonhuman primates used in medical research, such as the rhesus monkey (*Macaca mulatta*) [27] and the common marmoset (*Callithrix jacchus*) [28]. For clinical application of the ES-DC technology, development of a feeder-free differentiation method may be required. Embryoid body-mediated differentiation methods may be one way to resolve this issue. In the mouse system, induction of mesodermal differentiation of ES cells using type IV collagen-coated culture plates has been reported [29, 30]. Several molecules have been reported to be involved in support of hematopoietic cell growth or differentiation by stromal cells [31–33]. Information on the molecular basis of the interaction between differentiating ES cells and feeder cells is valuable for the development of a feeder-free differentiation system.

Considering clinical applications, manipulation of function of ES-DC by genetic modification without use of viral vectors, demonstrated in the present study, has a significant advantage. However, random integration of multiple copies of transgenes into various genomic loci of ES cells is accompanied by risks such as activation of cellular oncogenes. Thus, a method to integrate transgenes into intended loci of the genome of human ES cells needs to be established.

Previously, we demonstrated a method for efficient targeted integration of expression vectors into specific genomic sites of mouse ES cells, using exchangeable gene-trap vector with Cre-

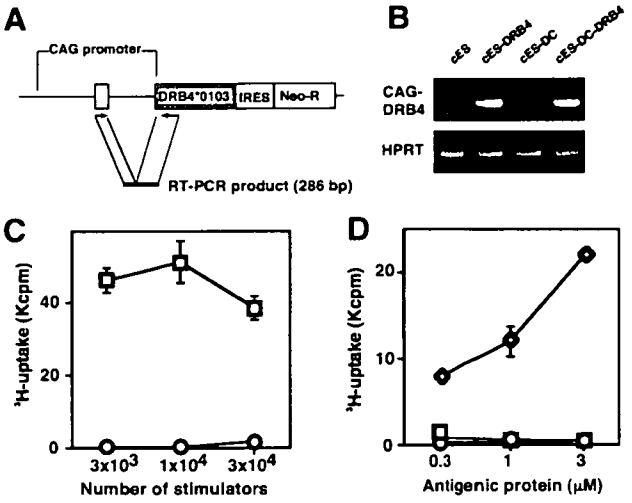

Figure 7. Antigen presentation to human T cells by genetically modified cES-DC. **(A):** The structure of HLA-DRB4*0103 expression vector is shown. The open box indicates the noncoding first exon of rabbit β-actin gene included in the CAG promoter. RT-PCR with PCR primers indicated by arrowheads generated PCR products of 286 base pairs from the transgene-derived mRNA. **(B):** Results of an RT-PCR analysis of parental cES and a transfectant embryonic stem cell clone (cES-DRB4) and derivative embryonic stem cell-derived dendritic cells (ES-DC) on the expression of transgene-derived mRNA (CAG-DRB4). The PCR products for HPRT transcript amplified from the same cDNA samples are also shown as control. **(C):** The indicated numbers of DRB4-transfectant ES-DC (squares) or nontransfectant ES-DC (circles) were preloaded with $GAD65_{111-131}$ peptide, x-ray-irradiated (40 Gy), and cocultured with SA32.5 T cells (3×10^4 cells per well) for 3 days. The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. **(D):** DRB4-transfectant ES-DC (diamonds) (1×10^4 cells per well) or nontransfectant ES-DC (squares) were cocultured with SA32.5 T cells (3×10^4 cells per well) in the presence of the indicated concentration of glutathione *S*-transferase (GST)-GAD recombinant protein for 3 days. DRB4-transfectant ES-DC and SA32.5 T cells were cocultured also in the presence of GST protein (circles). The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. Abbreviations: cES, cynomolgus embryonic stem cells; cES-DC, cynomolgus embryonic stem cell-derived dendritic cells; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRES, internal ribosomal entry site; RT-PCR, reverse transcriptase polymerase chain reaction.

Lox-mediated recombination system [1]. We are now trying to develop a system for targeted integration of transgenes into human ES cell genome. In this system, at first, gene-targeting vector conveying a drug resistance marker gene flanked by lox sequences is introduced, and then ES cell clones carrying the vector properly integrated by homologous recombination are selected. Subsequently, expression vectors with lox sequences are introduced with the aid of the Cre-Lox recombination system. Integration of a single copy of the transgene into the intended locus can be verified by Southern blot analysis. By this strategy, we can obtain ES cell clones with defined genetic modification, thus avoiding the risks accompanying the random integration of exogenous genes.

Allogenicity caused by differences in the genetic background between human ES cell lines and the recipients is considered to be a critical problem in medical application of ES-DC. We previously reported that mouse ES-DC administered into semiallogeneic recipients, sharing one MHC haplotype with the ES-DC, effectively primed antigen-specific cytotoxic T lymphocytes (CTL), suggesting that ES-DC can survive for a period long enough to stimulate antigen-specific CTL restricted by the shared MHC class I [4]. However, in the same semiallogeneic setting, we also observed five times that injection of no antigen-loaded ES-DC significantly reduced the efficiency of priming of antigen-specific CTL induced by the subsequent injection of antigen-loaded ES-DC (unpublished observations). Thus, repetitive stimulation with ES-DC expressing allogeneic MHC may result in activation and expansion of allogeneic MHC class I-reactive CTL, and in such recipients, subsequently transferred ES-DC may be rapidly eliminated. Repeated immunization may be required in clinical applications, for example, to induce antitumor immunity. Thus, we should resolve the problem of the histoincompatibilty between ES cell lines and recipients.

Methods for targeted gene modification of human ES cells and for targeted chromosome elimination of mouse ES cells have been developed [34–36]. To overcome the problem of histoincompatibility, genetic modification to inhibit expression of endogenous HLA class I in ES-DC may be effective. Deletion of more than 1,000 kilobases of entire HLA class I region of human ES cell genome by gene targeting is infeasible by currently available technology. However, disruption of the genes of molecules necessary for the cell surface expression of HLA class I molecules, such as transporter associated with antigen processing (TAP) or β2-microglobulin (β2M), is presumably feasible. In our plan, we will introduce expression vector encoding the β2M-linked form of recipient-matched HLA class I heavy chain into TAP1- or β2M-deficient human ES cells. We are now testing this strategy by using a mouse system.

Acknowledgments

This work was supported in part by Grants-in-Aid 12213111, 14657082, 14570421, 14370115, 16590988, 17390292, 17015035, and 18014023 from MEXT, Japan; the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases launched as a project commissioned by MEXT, Japan; a Research Grant for Intractable Diseases from Ministry of Health and Welfare, Japan; Oncotherapy Science Inc. (to Y.Ni.); and grants from Japan Science and Technology Agency; the Tokyo Biochemical Research Foundation; Uehara Memorial Foundation; and Takeda Science Foundation. We thank Risa Goswammi for the cDNA microarray experiments, the Chemo-Sero-Therapeutic Research Institute for the cynomolgus monkey peripheral blood samples, and Tanabe Seiyaku Co., Ltd. for CMK6.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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はじめに

MHC（主要組織適合遺伝子複合体：major histocompatibility complex）は，マウスを用いた皮膚移植の実験において，移植片生着の成否，すなわち組織適合性を決定する主要な遺伝的因子として同定された。その後の研究により，*MHC*領域に存在する遺伝子によってコードされるMHC分子が，T細胞に対して抗原ペプチドを提示する分子であり，その遺伝子多型が免疫応答の個体差を規定していることが明らかにされた。

免疫系は，感染性微生物の侵入から生体を防御するために不可欠なシステムである。体内に侵入した微生物に由来するタンパク質は，獲得免疫系により，抗原としてアミノ酸配列のレベルで識別される。抗原認識は，個々のリンパ球が発現する抗原特異的なレセプターにより担われている。Bリンパ球（B細胞）の抗原レセプターは，免疫グロブリン（抗体）であり，Tリンパ球（T細胞）の抗原レセプターは，T細胞レセプター（T cell receptor：TCR）である。免疫グロブリンが抗原分子に直接結合するのに対し，T細胞レセプターは，抗原タンパクの限定分解の結果生じたペプチドがMHC分子に結合し，MHC-抗原ペプチドの複合体を形成したものを認識するという特徴がある。

MHC分子は，クラスⅠとクラスⅡの2種類に大別される。MHCクラスⅠとクラスⅡでは，その構造，組織分布，結合する抗原ペプチドの由来，さらに，それを認識するT細胞の種類が異なる。T細胞が，T細胞レセプターを介してペプチドとMHC分子の複合体を認識すると，活性化状態となり，細胞増殖，サイトカインの産生，細胞傷害活性の発現などの反応を示す。そして，MHCクラスⅠとクラスⅡいずれもが，免疫系による抗原の認識と，その後の感染性微生物の排除による生体防御機構において必須の役割を担っている。本稿では，ヒトのMHC（*HLA*：human histocompatibility leukocyte antigen）とマウスの*MHC*（*H-2*）についての知見から，哺乳動物のMHC分子の構造と機能について概説する。なお，本稿ではMHCクラスⅠ分子として，著明な遺伝的多型性を有し，ペプチド抗原の提示を行なう，古典的MHCクラスⅠについてのみ言及する。

MHCクラスⅠの組織分布と構造

MHCクラスⅠは，マウスではH2-K，D，Lの3種類の分子があり，ヒトではHLA-A，B，Cの3種類の分子がある。MHCクラスⅠ分子は，約340個のアミノ酸より成る45kDaのα（重）鎖と，99個のアミノ酸からなる12kDaのβ2ミクログロブリン（β2M）とが非共有結合により結合して細胞膜表面に発現する（図1）。MHCクラスⅠ分子は，すべての有核細胞に発現しており，核の無い細胞のうち，血小板には発現しているが，赤血球には発現していない。MHCクラスⅠ分子のα鎖は，

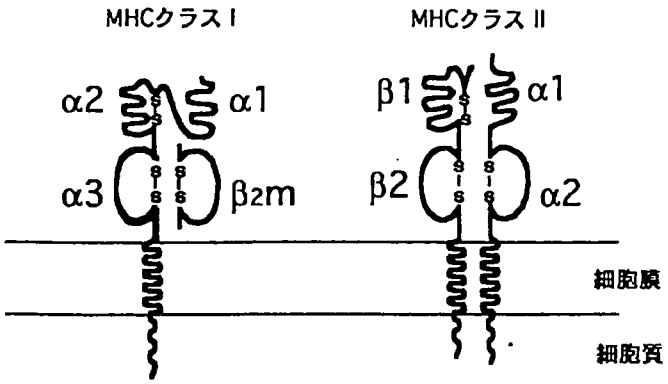

図1　MHC分子の構造

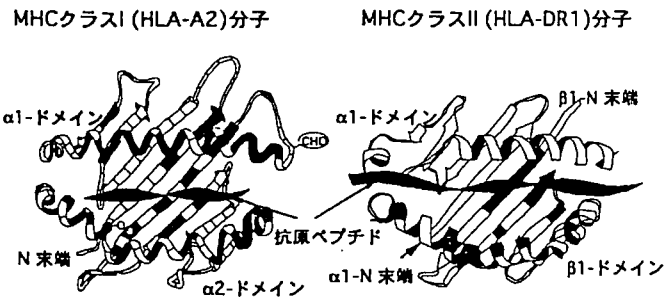

図2　MHCクラスⅠ(HLA-A2)およびクラスⅡ(HLA-DR1)分子の立体構造。この図は，上面（T細胞レセプター側）より見たものであり，黒く塗りつぶしたアミノ酸残基は多型を示す。これらはHLA分子のペプチド収容溝の底面や側壁に多く認められる。

それぞれ約90個のアミノ酸からなるα1，α2およびα3の3つの細胞外ドメインと，膜通過部分(39あるいは40アミノ酸)および短い細胞質内部分(25あるいは28アミノ酸)を有する。α2およびα3ドメインには各々2個ずつのシステイン残基があり，その間に各ドメイン内でジスルフィド(S-S)結合が形成されている。X線結晶解析により数種類のMHCクラスⅠ分子の立体構造が解明され，MHCクラスⅠ分子の先端の部分を構成するα1とα2ドメインには，αヘリックス構造が側壁を，またβシート構造が底面を構成する溝状の構造（ペプチド収容溝）が存在し，この溝にペプチドが結合していることが明らかになった（図2)。

MHCクラスⅠ分子の機能

MHCクラスⅠ分子のペプチド収容溝には，細胞内で合成されたタンパク質に由来するペプチドが結合する。この中には，細胞に感染したウイルスなどのタンパク質に由来するペプチドも含まれる。まず，これらのタンパク質にユビキチンが結合したものが，プロテアソームと呼ばれるタンパク質分解装置に取り込まれ分解される。その結果，タンパク質は9個前後のアミノ酸からなるペプチド断片となり，ペプチドトランスポーター（TAP：transporter associated with antigen processing）により小胞体内腔へと輸送される（図3)。なお，プロテアソームを構成するサブユニットの一部，ならびにTAPの遺伝子は共に，*MHC*遺伝子領域内に存在する。小胞体の内腔で，MHCクラスⅠの重鎖-β2M-ペプチドの複合体が形成され細胞表面へ輸送され，CD8陽性キラーT細胞へ提示される。

MHCクラスⅠに結合するペプチドは8～12個の

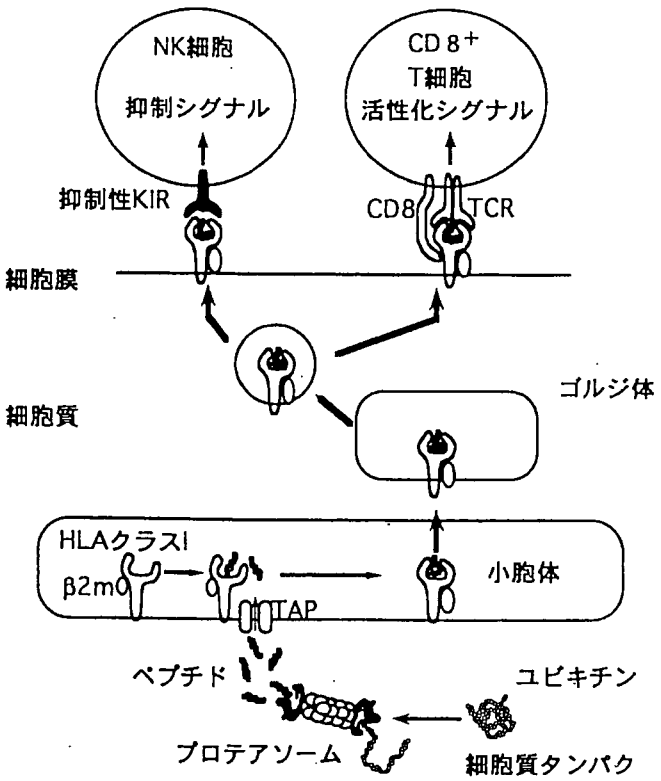

図3　MHCクラスⅠ分子による抗原提示の経路

アミノ酸からなるペプチドで，両端（NおよびC末端側）のアミノ酸残基（アンカー残基）がクラスⅠ分子の先端のペプチド収容溝に存在する窪み（ポケット）にうまく収容されると，ペプチドはMHCクラスⅠ分子に結合する。MHCの型によってポケットの構造が異なっているので，そこに結合できるアンカー残基の種類は，MHCの型によって異なる。

感染等のない正常な細胞では，細胞表面に存在するMHCクラスⅠ分子はその細胞が本来産生するタンパク質に由来するペプチドを結合している。このような自己のMHCクラスⅠ分子と自己由来のペプチドの複合体を認識するT細胞は，胸腺における分化の過程で除かれているか，あるいは機能的に不活性化され反応しない状態になっている（自己に対する免疫寛容）。

ウイルスや細胞内寄生性の細菌，原虫などが感染した細胞では,細胞表面のMHCクラスⅠ分子のごく一部にこれらの感染性微生物に由来する抗原ペプチドが結合している。CD8陽性細胞傷害性（キラー）T細胞は，MHCクラスⅠ分子にこのような非自己抗原ペプチドが結合した複合体を認識して活性化する。活性化キラーT細胞は，パーフォリンを分泌して標的細胞の細胞膜に穴をあけ，さらにグランザイムなどのタンパク質分解酵素を細胞質内に送り込み，アポトーシスを誘導することにより標的細胞を破壊する。この際にキラーT細胞の表面に発現する糖タンパク質であるCD8分子は，標的細胞上に発現するMHCクラスⅠ分子の$\alpha 3$ドメインに結合して，CD8陽性T細胞と標的細胞との間の接着を高めると同時に，T細胞内に活性化シグナルを伝達する。キラーT細胞がウイルスが感染した細胞を破壊することにより，細胞内に存在しているウイルスの増殖が阻止される。このメカニズムは，ウイルス感染に対する重要な防御機構である。また，がん細胞上のMHCクラスⅠに提示されているペプチドの中には，正常細胞では発現していないか，あるいは，極めてわずかしか発現していないタンパク質に由来するものがある。このようながん関連抗原に由来するペプチドを認識するキラーT細胞が体内に存在すれば，そのがん細胞を傷害することが可能である。すなわち，免疫系は，がん細胞特異的ペプチドを目印にして，正常細胞とがん細胞を識別し，がん細胞を攻撃することが可能である。

NK（ナチュラルキラー）細胞は血球系の細胞であるが，代表的な免疫担当細胞であるマクロファージ，樹状細胞，T細胞あるいはB細胞のいずれにも属さない細胞である。NK細胞は特定のウイルスあるいは細菌に感染した細胞，あるいは腫瘍細胞を破壊する。NK細胞の表面にはKIR（killer-cell immunoglobulin-like receptors）と総称される一群のレセプターが発現している。KIRの中にはリガンドを認識した結果としてNK細胞の細胞傷害活性を活性化するものと抑制するものがあるが，抑制性KIRの中には標的細胞上のMHCクラスⅠ分子に結合するものがある。NK細胞が，体内の正常な細胞に発現しているMHCクラスⅠ分子を認識すると，このような抑制性KIRを介してNK細胞内に細胞傷害活性の発現を抑制するシグナルが伝達されるため，このような正常な自己細胞は傷害しない（図3）。一方，ウイルス感染やがん化などにより，MHCクラスⅠ分子を発現しなくなった細胞に対しては，MHCクラスⅠ分子を認識する抑制性KIRが働かないので，NK細胞による攻撃の対象となる。

MHCクラスⅡ分子の組織分布と構造

MHCクラスII分子は，マウスではI-AとI-Eの2種類があり，ヒトではHLA-DR，DQ，DPの3種類の分子がある。MHCクラスII分子は，樹状細胞，マクロファージやB細胞などの，いわゆるプ