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G.知的財産権の出願・登録状況

特になし

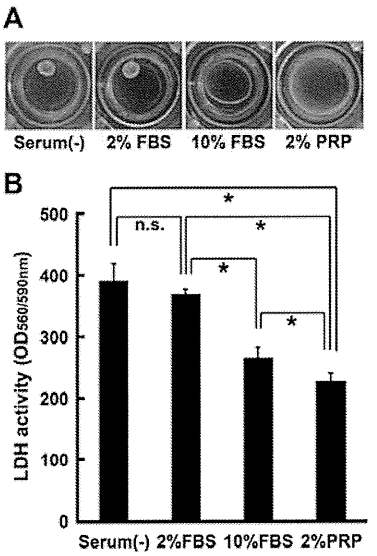

Fig.1. 3 次元培養における PRP の影響

A: 24 時間培養後のゲル写真を示す。2%PRP によりゲルの縮小が抑制された。

B: 培養液中の LDH 活性。2%PRP で有意に減少した。(*P<0.05)

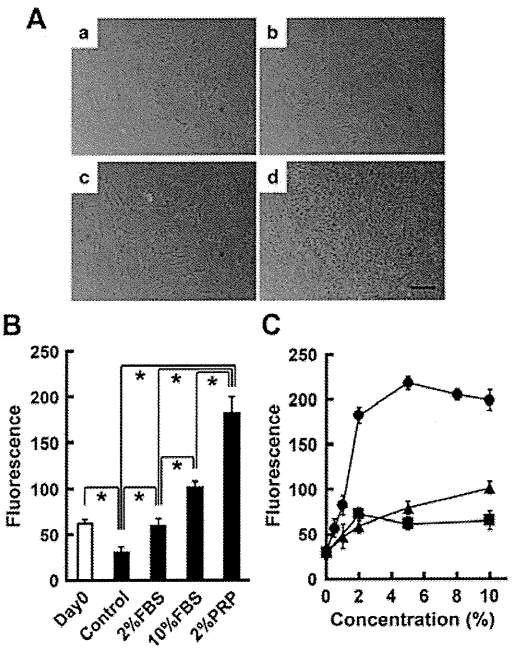

Fig.2　PRP が細胞増殖に及ぼす影響

A:前脂肪細胞を 10cm ディッシュに播種後、3 日間それぞれの培地で培養した後の光学顕微鏡像。a:無血清、b:2%FBS、c:10%FBS、d:2%PRP

B:96 穴プレートで 3 日間培養した後の細胞増殖を、DNA 量により定量化し比較した。2%PRP は高い細胞増殖を認めた。

C: 細胞増殖の PRP 濃度依存性を検討した。PRP は比較的低濃度でも高い細胞増殖を認めた。

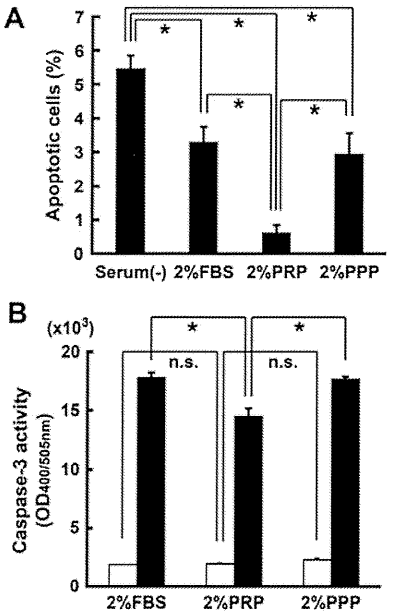

Fig.3　PRP がアポトーシスに及ぼす影響

A: それぞれの培地で 8 時間培養後のアポトーシス細胞数を、フローサイトメーターで測定した。2%PRP では、アポトーシス細胞の割合が減少した。(*P<0.05)

B: TNF-αと cycloheximide を添加し、3 時間培養した後の細胞上清中の caspase-3 活性を測定した。薬剤刺激による caspase-3 の活性化を、2%PRP は低下させた。(*P<0.05)

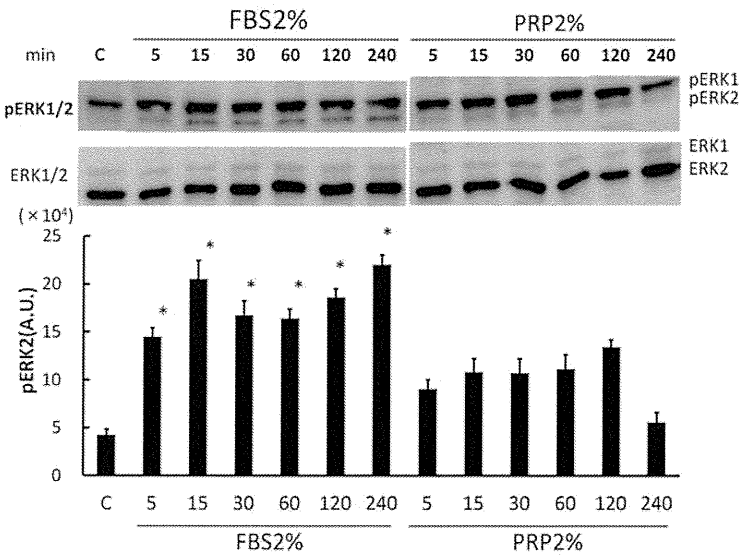

Fig.4. TNF-α/cycloheximide 処理後の ERK1/2 のリン酸化動態

TNF-αと cycloheximide を添加し、経時的に細胞を回収し、ERK1/2 のリン酸化の状態を Western blotting を用いて調べた。FBS2%では早期に見られる ERK1/2 のリン酸化が、PRP2%では抑制された。(*P<0.05)

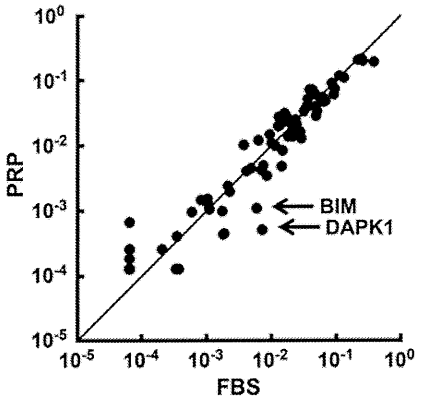

Fig.5　アポトーシス関連遺伝子 84 個の PCR array

2%FBS と 2%PRP で 3 日間培養後の遺伝子発現の変化を PCR array を用いて比較した。DAPK1、BIM の 2 つの遺伝子が、2%PRP で発現が著明に低下していた。

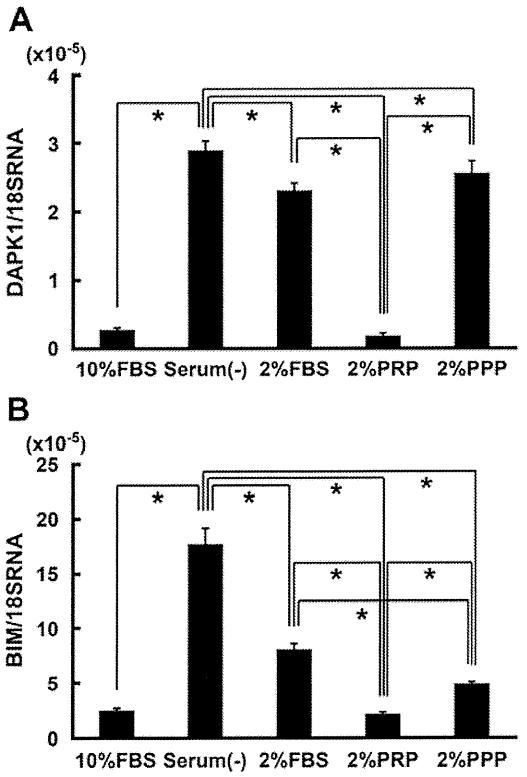

Fig.6　DAPK1, BIM の Real-time PCR による定量解析

それぞれの培地で 3 日間培養後の DAPK1（A）、BIM（B）の mRNA 発現。DAPK1、BIM ともに 2%PRP では有意に発現が低下していた。

(*P<0.05)

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

TGF-β/Smad3 シグナルによる脂肪細胞分化抑制機構

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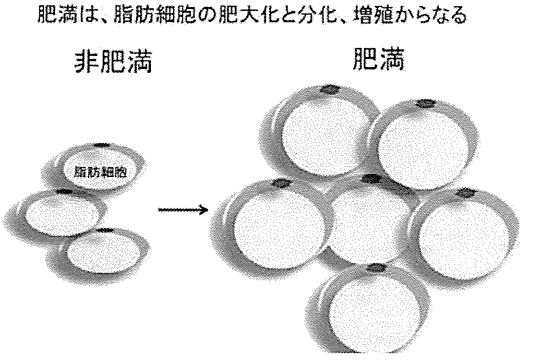
研究協力者　藤本昌紀（千葉大学大学院医学研究院細胞治療内科学）

研究要旨　本研究は、患者組織由来の前脂肪細胞に LCAT 遺伝子を導入し、これを自家移植する遺伝子細胞治療法の開発を目的としている。本研究を効率よく完遂するためには、前駆脂肪細胞から成熟脂肪細胞に至る細胞分化の分子メカニズムの解明が必要とされる。本分担研究では、代表的な細胞増殖抑制因子である TGF-βの主流下位シグナルである smad3 に着目し、肥満、脂肪細胞分化における TGF-βシグナルの役割について、smad3 ノックアウトマウスを用いて、検討した。その結果、１）代表的な肥満モデルマウスである ob/ob マウスでは、白色脂肪組織において TGF-β/Smad3 シグナルが亢進している。２）TGF-βは、smad3 を介して、脂肪細胞分化を抑制し、３）高脂肪食負荷を行うと、smad3 ノックアウトマウスは、野生型に比して、インスリン感受性が保たれており、脂肪細胞は小さく、数が多い結果となった。

A．研究目的

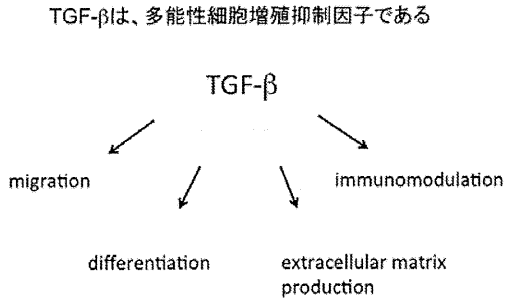
家族性レシチンコレステロールアシルトランスフェラーゼ（LCAT）欠損症は、幼少期から角膜混濁、貧血が発症し、腎不全に至る予後不良かつ病態不明の遺伝性難治性疾患である。根本的治療がなく、欠損タンパク質の持続的補充を可能とする新規の治療法が求められている。これに対し、本研究では、患者脂肪組織由来の初代培養細胞（前脂肪細胞）に LCAT 遺伝子を導入し、自家移植する全く新しい遺伝子細胞治療法の開発を目指している。脂肪組織は、人体の臓器の中で最も巨大かつ再生力にあふれる組織であり、肥満では、脂肪細胞が肥大、分化、増殖していることが知られる（図 1）。脂肪細胞の分化、増殖には、様々な転写因子がカスケードを形成し、制御している（１）。

図1



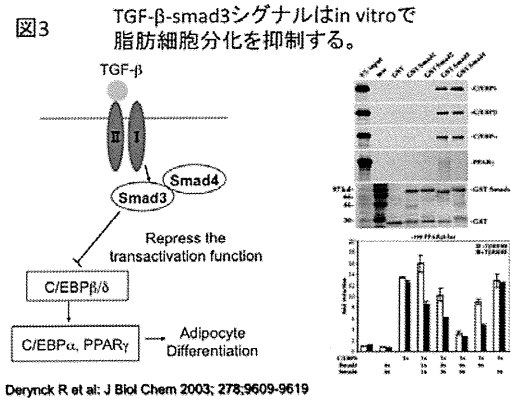
TGF-β (transforming growth factor-β)は、その名のとおり特定の培養条件下で細胞をトランスフォーム,すなわち形質変換/癌化させる因子として 1983 年に血小板などから精製された。しかし、その後の研究から形質変換作用は、他の増殖因子などを介した間接作用であり、むしろ強力な細胞増殖抑制と細胞外マトリックス産生作用などの多彩な作用を有することが認識されるようになった(2)（図 2）。

図2



TGF-βにはⅠ型およびⅡ型、2種類のレセプターがあり,いずれも細胞内にセリン・スレオニンキナーゼ活性を有する。TGF-βとの結合により活性化されたレセプターはSmadとよばれる細胞内シグナル分子のセリン・スレオニン残基をリン酸化する。自身が転写因子であるSmad分子は核内へと移行,各種遺伝子の発現を調節し,細胞生物学的作用をもたらすと考えられている。TGF-βは、脂肪細胞分化を抑制することが知られているが（3）、

図3



生体内での脂肪組織への作用は明らかではなかった。本研究では、TGF-βの主要下流シグナルであるsmad3ノックアウトマウスを用いて、in vivoでのTGF-βの脂肪細胞への作用を検討することとした。

B．研究方法

本研究の目的のために、①代表的な肥満モデルであるob/obマウスでのTGF-βのmRNA及び蛋白発現レベル、りん酸化smad3の発現レベルを同週齢の野生型マウスを対照として、比較検討した。②smad3ノックアウト及び野生型マウスより胎児線維芽細胞を採取し、TGF-β 1ng/ml添加もしくは非添加で インスリン、デキサメサゾン、IBMXにより、脂肪細胞分化を誘導する。8日後まで培養し、oil red O染色、RT-PCR法で遺伝子発現の差異を比較した。③10週齢の♂smad3ノックアウトマウス及び野生型マウスに高脂肪食負荷を行い、体重変化を検討した。そして、負荷後8週でインスリン負荷試験（1U/Kg）を行い、インスリン感受性を比較検討した。脂肪組織を採取し、組織学的比較及び脂肪細胞分化マーカーであるC/EBP, PPARをmRNA発現で比較検討した。

C．研究結果

1.肥満モデルである10週齢♂ob/obマウスでは、TGF-βのmRNA発現が亢進し（図4）、りん酸化smad3が上昇していた（図5）。一方、TGF-β受容体の発現には差を認めなかった。

図4

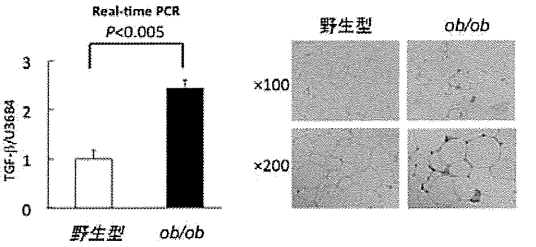
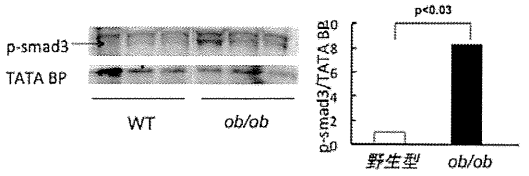


図5

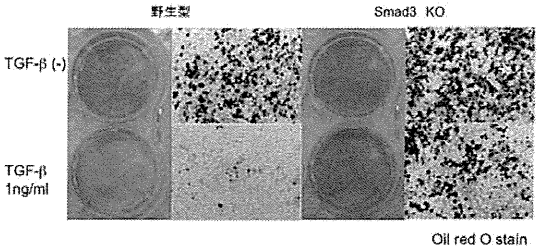
肥満状態では、白色脂肪組織において
TGF-β-smad3シグナルは亢進している



２．マウス胎児線維芽細胞において、TGF-βは脂肪細胞分化を抑制し、oil red O 染色では脂肪蓄積が完全に抑制された。一方、smad3 ノックアウト胎児線維芽細胞では、TGF-βの分化抑制作用は部分的ではあるが、キャンセルされていた（図6）。

図6

TGF-βは、Smad3依存性に脂肪細胞分化を抑制する



３．高脂肪食負荷条件下で、smad3 ノックアウトマウスは、野生型マウスに比して、体重増加が有意に多い。しかしながら、Smad3 ノックアウトマウスと野生型マウスでは、食餌量に差は認めなかった。インスリン感受性は、smad3 ノックアウトマウスで有意に高かった（図 7）。高脂肪食負荷時に、smad3 ノックアウト脂肪組織は、細胞径は小さく、細胞数が増えていた（図 8）。

図7

Smad3 KOマウスは、高脂肪食負荷で太りやすいが、インスリン感受性は保たれている

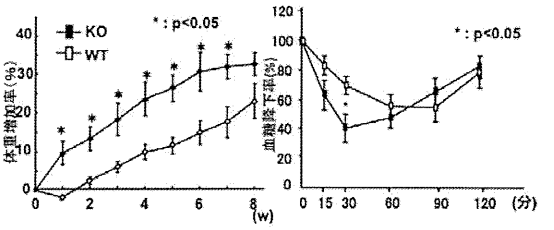
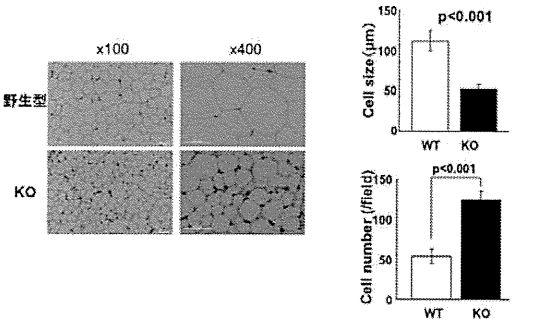


図8

高脂肪食負荷Smad3 KOマウスの白色脂肪細胞は小さく、数が多い



以上をまとめると、肥満状態では、脂肪組織においてTGF-β/smad3 シグナルは亢進している。そして、TGF-βは、smad3 を介して脂肪細胞分化を抑制し、生体内ではインスリン抵抗性に寄与していた（４）。

D．考察

今年度実施した検討結果によって、脂肪細胞において、TGF-βは smad3 を介して分化を抑制し、インスリン抵抗性の形成に寄与していることが示された。本研究班では、前脂肪細胞を採取、遺伝子導入し、戻し移植をすることでタンパク質欠損症を治療することを目的としている。そのためには、効率のよい前脂肪細胞の採取、培養法が望まれる。最近、smad3 抑制薬が発見され、報告されている（５）。採取できた脂肪組織が少ない場合でも、smad3 抑制薬を用いることで、前脂肪細胞回収を効率化できる可能性が示唆された。

E.結論

肥満状態では、白色脂肪組織に於いてTGF-βの発現は亢進している。

TGF-βは、smad3依存性に脂肪細胞分化を抑制し、肥満状態におけるインスリン抵抗性に寄与する。smad3を何らかの方法で抑制することで、効率よく脂肪細胞を採取できる可能性が示された。

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厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

科学的倫理的配慮に基づく遺伝子治療臨床研究の円滑な実施に関する研究

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研究要旨：遺伝子治療を実施するにあたって適切な臨床研究基盤を整備する必要性がある。そこで、特にデータマネージメントに関する整備を実施し、適切な研究の実施に関する検討を行った。

A. 研究目的

臨床研究実施体制の整備

B. 研究方法

本研究においては、以下の検討を行った。

1．データマネジメントに関する計画立案
2．システムの導入に関する検討
3．システムバリデーションに関する検討

（倫理面への配慮）

本研究は試験実施の準備のため、直接被験者への影響はない。実施される試験については臨床研究に関する倫理指針に基づいて実施する必要がある。

C. 研究結果

1．本学におけるデータマネジメントに係る手順書を参考とした。

＜EDCの場合＞

010データマネジメント業務に関する手順書
010-1 データマネジメント業務に関する手順
010-2 アカウント管理およびEDCトレーニングに関する手順
010-3 DM計画書及びDM報告書の作成に関する手順

＜紙の場合＞

010データマネジメント業務に関する手順書
010-1 データマネジメント業務に関する手順
010-2 症例報告書の見本等の作成に関する手順
010-3 DM計画書及びDM報告書の作成に関する手順

＜登録＞

011被験者の登録に関する手順書

＜割付＞

014治験薬の割付に関する手順書
015緊急コードの開封に関する手順書019
コード開鍵（キーオープン）に関する標準業務手順書

2．本学で導入している2システムについて、遺伝子治療臨床研究における問題点について比較検討を行った。
3．使用するシステムについてのシステムバ

リデーションを実施した。

D. 考察

データマネジメントにおいて対象を遺伝子治療臨床研究とそれ以外の臨床研究において、基本的には大きな違いは存在しない。しかし、研究の早期の相における探索的項目においては、安全性データを含めた網羅的なデータの収集が不可欠であり、いわゆる化合物の安全性を検討する従来の研究手法とは根本的に異なる事より、その結果の意味する事が場合によっては解決不可能な事も存在する。非臨床試験において十分な予測が不可能な事項も多くあり人への遺伝子治療の適応は、予測不可能なデータに関して収集を適切に実施する必要がある。また、その分類においても非臨床試験のデータのみならず類似の試験のデータより推測を行う事が必要と考えられる。

２システムは紙の CRF を用いるか、EDC とするかという点において大きな違いが存在するが後者においては、あらかじめ収集するデータについてより予測性を高めて行う必要性はあるものの、海外との共同の国際試験を実施するにあたり共通の基盤によるデータの収集が可能となる。

システムバリデーションについては試験の信頼性を確保するためにも GAMP5 に基づく手法をとる必要があるが、必ずしも容易とは言えない。

E. 結論

データマネジメントに関する検討を行った。今後、システムを用いたデータマネジメントを本研究、遺伝子治療臨床研究において実施して行く予定であり、今回の検討により適切な基準に基づいた試験が実施可能と考えられる。

F. 研究発表

なし

Ⅲ. 研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別冊

Platelet-rich plasma inhibits the apoptosis of highly adipogenic homogeneous preadipocytes in an *in vitro* culture system

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Running title: PRP prevents apoptosis of *in vitro* cultured adipocytes.

Abstract

Auto-transplantation of adipose tissue is commonly used for the treatment of tissue defects in plastic surgery. The survival of the transplanted adipose tissue is not always constant, and one of reasons is the accelerated apoptosis of the implanted preadipocytes. We have recently established highly homogeneous preadipocytes, named ccdPAs. The aim of the current study was to evaluate the regulation of the potency of platelet-rich plasma (PRP) on the apoptosis of ccdPAs *in vitro*. PRP stimulated the proliferation of the preadipocytes in a dose-dependent manner, and the stimulatory activity of 2% PRP was significantly higher than that of 2% FBS or 2% platelet-poor plasma (PPP). The presence of 2% PRP significantly inhibited serum starvation- or TNF-α/cycloheximide-induced apoptosis in comparison to 2% FBS or 2% PPP. DAPK1 and BIM mRNAs were reduced in the preadipocytes cultured with 2% PRP in comparison to those cultured in 2% FBS. The gene expression levels were significantly higher in cells cultured without serum in comparison to cells cultured with 2% FBS, and the levels in the cells with 2% PRP were reduced to 5-10% of those in the cells without serum. These results indicated that ccdPAs exhibit anti-apoptotic activities, in addition to increased proliferation, when cultured in 2% PRP in comparison to the same concentration of FBS, and that this was accompanied with reduced levels of DAPK1 and BIM mRNA expression in *in vitro* culture. PRP may improve the outcome of transplantation of adipose tissue by enhancing the anti-apoptotic activities of the implanted preadipocytes.

Key words: preadipocytes; platelet-rich plasma (PRP); apoptosis; transplantation; DAPK1; BIM

Introduction

Aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery (Billings and May, 1989; Patrick, 2000; Patrick, 2001). Aspirated fat contains multipotential preadipocytes and progenitor cells, which have been utilized as a source of cell-based regenerative medicine (Stashower *et al.*, 1999; Zuk *et al.*, 2001; Gimble *et al.*, 2007; Yoshimura *et al.*, 2009; Bauer-Kreisel *et al.*, 2010; Sterodimas *et al.*, 2010). Although several different techniques of fat grafting have been developed, the outcomes of the transplantation vary widely. The most important factor required for successful grafting is to optimize the survival of the transplanted preadipocytes and other cells in the graft. In previous studies, we and others have shown that various cytokines are involved in the efficient cell survival of the implants (Kimura *et al.*, 2003; Yamaguchi *et al.*, 2005; Cho *et al.*, 2006; Torio-Padron *et al.*, 2007; Kuramochi *et al.*, 2008; Ning *et al.*, 2009).

Platelet rich plasma (PRP) (Eppley *et al.*, 2006; Foster *et al.*, 2009; Redler *et al.*, 2011) has been widely applied for practical medicine, such as aesthetic plastic surgery and the treatment of soft-tissue ulcers (Welsh, 2000; Man *et al.*, 2001; Margolis *et al.*, 2001; Bhanot and Alex, 2002; Martinez-Zapata *et al.*, 2009; Sclafani, 2009). Once activated, platelets secrete various bioactive cytokines, including platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF-β1), which increase angiogenesis and cell proliferation relevant to soft tissue regeneration. PRP has been applied for fat grafting, and in fact, has been shown to improve the survival of implanted adipose tissue in patients (Abuzeni and Alexander, 2001; Sadati *et al.*, 2006; Cervelli *et al.*, 2009). Thus, the use of PRP has been broadened to the tissue-engineering field using adipose tissue-derived multi-potential cells (Anitua *et al.*, 2006; Muller *et al.*, 2009). PRP is also expected to function as an autologous fibrin-based scaffold for transplanted cells (Anitua *et al.*, 2006; Wu *et al.*, 2009; Kang *et al.*, 2011). In fact, recent our study showed that fibrin-based scaffold decreased the apoptotic cell death of murine ccdPAs in mice transplantation model (Aoyagi *et al.*, 2011).

We have recently identified proliferative preadipocytes, ceiling culture-derived proliferative adipocytes (ccdPAs), as homogeneous cells suitable for *ex vivo* gene therapy applications via autologous transplantation (Asada *et al.*, 2011; Kuroda *et al.*, 2011). The ccdPAs are characterized by their high proliferative capacity with spontaneous adipogenic potential in scaffold fibrin gel culture (Aoyagi *et al.*, 2012). The establishment of a highly homogenous preadipocyte line made it possible to perform examinations to identify the optimal scaffolds and cytokines that can be used to improve the survival of transplanted preadipocytes. We herein studied the effects of PRP, and an autologous cytokine cocktail, on the apoptotic properties of preadipocytes using the ccdPAs.

Results

PRP inhibits fibrin scaffold gel shrinkage and improves the viability of ccdPAs in 3-dimensional culture

We have recently established a 3-dimensional (3-D) culture system for ccdPAs using fibrin gel (FG) (Aoyagi *et al.*, 2012). Using the 3-D culture system, the effects of PRP on the gel shrinkage and cell viability were analyzed in comparison to FBS. The FG/ccdPAs were formed and maintained in culture medium containing 10% FBS for 16 hr. The culture medium was replaced with fresh medium containing 2% PRP, 2% FBS or 10% FBS, or with medium without serum, and cells were subsequently incubated for an additional 24 hr.

The resulting gel sizes varied among the cultures grown in each type of medium. The gels without serum or with 2% FBS showed a drastic volume reduction, while the volumes of the gels cultured with 10% FBS or 2% PRP were not obviously reduced (Fig. 1A). The culture supernatants were collected from each well and LDH activity was measured to evaluate the viability of cells. The LDH activity significantly decreased in the culture medium with 2% PRP in comparison to the medium with 2% or 10% FBS (Fig. 1B). TUNEL staining of the gel sections showed the number of apoptotic cells to significantly decrease in the medium with 2% PRP in comparison to the medium with 2% FBS (1.5 ± 1.1 % vs 9.8 ± 1.9 %, $p<0.05$). These results suggested that 2% PRP inhibits the shrinkage of FG/ccdPAs gels, and improves the cell viability in comparison to the same concentration of FBS.

PRP has a high proliferation-inducing potential for ccdPAs in plate culture

In order to evaluate the function of PRP on cell survival in the gel, we next examined the effects of PRP on the proliferation of ccdPAs in comparison to FBS. The cells (2.5×10^5 cells) were seeded and incubated with DMEM/HAM containing 20% FBS in 10 cm dishes for 16 hr. The media was replaced with medium containing 2% PRP, 2% FBS, or 10% FBS, and the cells were then cultured for 3 days. The cell appearance was not apparently changed among the ccdPAs cultured for 3 days in plates with media containing 2% PRP, 2% FBS, or 10% FBS (Fig. 2A). To examine the cell proliferation, 2×10^3 cells of ccdPAs were seeded onto 96 well plates and incubated at 37°C for 24 hr. The media was replaced with medium with or without 2% PRP, 2% FBS, or 10% FBS (Day 0), and the cells were cultured for 3 days. The number of cells in each well was evaluated by measuring the DNA content. In contrast to the observation that the cell numbers on Day 3 were not significantly changed in comparison to those at Day 0 in the cultures incubated in medium containing 2% FBS, the cell numbers were significantly increased in cells cultured in the medium with 10% FBS or 2% PRP, and notably, the number of cells on Day 3 in the medium containing 2% PRP was significantly increased in comparison to the cells cultured with 10% FBS (Fig. 1B). The cell numbers in the media with various concentrations of PRP showed a dose-dependent increase up to 5% PRP; the number of cells present in the media with 0.5-1% PRP was almost equivalent to that of the cells cultured with 5-10% FBS (Fig. 2C). These results indicated that the proliferation-inducing potential of PRP for ccdPAs was higher in comparison to that of FBS.

PRP inhibits the apoptosis of ccdPAs

The high proliferation-inducing property of PRP for ccdPAs in culture prompted us to further analyze PRP for protective effects against apoptosis in ccdPAs, since PRP is rich in cytokines and proteinases involved not only in proliferation, but also in regulating apoptosis (Eppley *et al.*, 2004; Eppley *et al.*, 2006; Foster *et al.*, 2009; Redler *et al.*, 2011). The protection of the cells from death may contribute to their longer survival after transplantation, together with a high potential for proliferation. To investigate the possibility, the ccdPAs (1×10^6 cells) were seeded and incubated in a 10 cm dish with DMEM/HAM medium containing 20% FBS for 16 hr, and subsequently incubated with the medium with or without 2% FBS, 2% PRP, or 2% PPP. After incubation for 8 hr, the cells were collected and stained with Annexin V-FITC. The flow cytometric analysis showed that 5.5% of cells were identified as Annexin V positive in the medium without serum (Fig. 3A). The number of apoptotic cells was significantly decreased in the media with 2% FBS, 2% PRP, or 2% PPP in comparison to that in the cells cultured in serum-free medium. Among the various supplements, 2% PRP drastically reduced the number of apoptotic cells in comparison to 2% FBS or 2% PPP. ERK1/2 phosphorylation was examined to further analyze the protective effect of PRP against apoptosis, because the activation of the cascade is important for apoptosis via various intracellular signals including TNF-α (Cawthorn and Sethi, 2008; Mebratu and Tesfaigzi, 2009; Cagnol and Chambard, 2010). The cells were incubated with 2% PRP, FBS, or PPP for 2 hr, and apoptosis was induced by TNF-α and cycloheximide. Phosphorylation was detected following treatment with TNF-α and cycloheximide for 5 min in the cells cultured with 2% FBS. A densitometric analysis showed the amount of phosphorylated ERK2, and not phosphorylated ERK1, to significantly decrease in the medium with 2% PRP in comparison to the medium with 2% FBS (Fig. 3B). The caspase-3 activity induced by TNF-α and cycloheximide for 3 hr were also significantly decreased in ccdPAs in the medium with 2% PRP in comparison to the cells cultured with 2% FBS or 2% PPP (Fig. 3C). Thus, the apoptosis of ccdPAs was inhibited by culturing them in the medium with 2% PRP *in vitro*.

PRP almost completely inhibits the expression of the pro-apoptotic genes, DAPK1 and BIM, in ccdPAs after serum starvation

In order to identify the molecules involved in the anti-apoptotic effects of PRP on ccdPAs in culture, the expression profiles of representative apoptosis-related genes were examined using a PCR array profiler. The cells (2.5×10^5 cells) were seeded into 10 cm dishes and incubated in DMEM/HAM containing 20% FBS for 16 hr. The media was replaced with DMEM/HAM containing 2% PRP or FBS, the cells were incubated for 3 days, and the total RNA was isolated from the cultured cells to analyze the expression of apoptosis-related genes. Two independent experiments showed that, among the 84 genes examined, there were 8 genes with a more than 2-fold increase in expression, and 9 genes with a more than 2-fold decrease in expression in the cells cultured in the medium with 2% PRP compared with those cultured in the medium with 2% FBS (Table 1). We focused our interest on two genes, DAPK1 (reduced to 7.4% of the expression level observed with FBS) and BCL2L11 (also called BIM, reduced to 18.9% of the level observed with FBS), as representative genes with the obvious downregulation in the medium with 2% PRP (Fig. 4). DAPK1 and BIM have been shown to be one of master regulators of cell death (Gozuacik and Kimchi, 2006), and is essential for BAX-dependent cell death (Kim *et al.*, 2009; Ren *et al.*, 2010), respectively.

The role of DAPK1 and BIM genes in apoptosis of preadipocytes was investigated by examining the effect of serum starvation of cells incubated with PRP on the expressions of these genes. The ccdPAs (2.5×10^5 cells) were seeded into 10 cm dishes and incubated in DMEM/HAM containing 20% FBS for 16 hr. The medium was replaced by medium containing 10% FBS (control), 2% FBS, 2% PRP, or 2% PPP, the cells were cultured for 3 days and the expression of DAPK1 and BIM were analyzed. The DAPK1 mRNA level was increased 10.6-fold by serum starvation in comparison to the level in the cells cultured with 10% FBS, and the mRNA level was decreased to that of 10% FBS by the presence of 2% PRP. It is worth noting that, although the mRNA levels of DAPK1 in the cells cultured with 2% FBS and 2% PPP significantly decreased in comparison to those cultured without serum, the reductions were by 20.4% and 11.9%, respectively, which were less than that (93.6%) induced by culture in 2% PRP. The BIM mRNA levels were also drastically increased in the cells cultured in serum free medium in comparison to those cultured in the medium with 10% FBS (Fig. 2C). The mRNA levels were reduced to those observed in the cells cultured with 10% FBS by the addition of 2% PRP. Again, the potential of 2% PRP to inhibit the mRNA expression of the target gene (by 87.2%) was significantly higher than that of the 2% FBS (54.1%) or 2% PPP (72.2%). Thus, PRP almost completely inhibited the expression of apoptosis-related genes induced by serum starvation.

Discussion

PRP inhibited the volume reduction of the 3D gels embedded with ccdPAs, the homogeneous preadipocytes, in comparison to the same concentration of FBS, and this was accompanied by increased cell viability in the gel. These observations prompted us to analyze the effects of PRP on the apoptosis and proliferation of the ccdPAs. The results showed that 2% PRP had a higher inhibitory effect on the apoptotic cell death of ccdPAs than 2% FBS or 2% PPP (see Fig. 3). A comparison between 2% PRP and 2% FBS by a gene expression profile analysis revealed that PRP downregulated 11% of the 84 representative apoptosis-related genes and upregulated 10% of the 84 representative apoptosis-related genes (see Fig. 4 and Table 1). The most drastically reduced genes were DAPK1, the protein product of which plays important roles in a wide range of signal transduction pathways with diverse outcomes, such as apoptosis, autophagy and immune responses (Lin *et al.*, 2010), and BIM, encoding one of the BH3-only proteins, which is a critical regulator of apoptosis in many cell types (Ramesh *et al.*, 2009). The induction of these genes by apoptotic stimuli was almost completely prevented in the presence of PRP (see Fig. 5).

PRP, a concentrate of physiological cytokines, has been widely utilized as an injectable material in the clinic since the 1970s to enhance soft and hard tissue healing (Andia *et al.*, 2010; Lopez-Vidriero *et al.*, 2010; Redler *et al.*, 2011; Yu *et al.*, 2011), mainly by stimulating cell proliferation and angiogenesis in the injured tissues. PRP promotes the growth of various cells, including tissue-derived progenitor cells (Liu *et al.*, 2002; Lucarelli *et al.*, 2003; Doucet *et al.*, 2005; Frechette *et al.*, 2005; Vogel *et al.*, 2006; Kakudo *et al.*, 2008; Kurita *et al.*, 2008; Cervelli *et al.*, 2009; Chieregato *et al.*, 2011), and increases the revascularization of the transplanted tissues (Bir *et al.*, 2009). Based on these findings, the clinical applications of PRP have been broadened to recommend its use as an additive to tissue/cell transplantation therapies in plastic and reconstructive surgeries, and more recently in regenerative medicine. In fact, PRP has been shown to improve the fat graft survival (Abuzeni and Alexander, 2001; Sadati *et al.*, 2006; Cervelli *et al.*, 2009; Nakamura *et al.*, 2010; Pires Fraga *et al.*, 2010; Oh *et al.*, 2011) and bone and periodontal regenerations via cell transplantation (Tobita *et al.*, 2008; Chen *et al.*, 2010; Yamada *et al.*, 2010; Arvidson *et al.*, 2011).

In order to apply PRP for clinical transplantation therapy using preadipocytes cultured *in vitro*, it is necessary to elucidate the effects of PRP on cell survival in the grafts. However, the mechanisms by which PRP increases graft survival have not been well-characterized so far. The current study showed that PRP strongly induces the proliferation of ccdPAs, preadipocytes which were previously shown to be more adipogenic than ASCs (Asada *et al.*, 2011), compared with FBS at an equivalent concentration. Vogel *et al.* (Vogel *et al.*, 2006) described that, because the addition of 2% PRP did not result in sufficient thrombocyte-clot formation to maintain a clot in the medium, a higher concentration of PRP, 3%, was evaluated for the stimulation of the MSC proliferation. In this study, to evaluate the efficacy of lower concentrations of PRP, the PRP was activated by thrombin to release cytokines (Aiba-Kojima *et al.*, 2007) prior to the experiments. As a result, 2% PRP showed almost the same effects on proliferation as 10% PRP, indicating its usability as a substitute for FBS in the expansion of preadipocytes for clinical applications. Finally, 2% PRP showed anti-apoptotic activities on the preadipocytes, providing evidence that it can be used as an efficacious additive in the cell transplantation cocktail.

We observed that the expression of the DAPK1 and BIM genes was substantially upregulated by serum starvation in ccdPAs. However, the addition of PRP in the growth media effectively inhibited the apoptosis and downregulated the expression of these genes. TGF-β has been shown to induce the expression of DAPK1 and BIM, and to lead to subsequent apoptosis in other cell types (Jang *et al.*, 2002; Wildey *et al.*, 2003; Ramjaun *et al.*, 2007; Yu *et al.*, 2008). PRP may therefore inhibit the TGF-β-induced apoptosis cascade(s) during serum starvation in ccdPAs. Further analyses are needed to elucidate the mechanism(s) underlying the inhibitory potential of PRP on the expression levels of the representative apoptotic genes. The gene expression analysis also showed that PRP regulated the expression levels of genes involved in TNF signaling (TNFRSF10A, TNFRSF25, TNFSF10, TNFRSF9, and TRAF2), and of the Bcl protein superfamily, with its related proteins (HRK, BNIP1, and BAG4) (see Table 1). The changes in the expression of these genes may also improve the survival of ccdPAs by modulating the apoptotic stimuli, considering that TNF-α signaling plays an important role in the regulation of the adipose tissue mass (Warne, 2003).

In conclusion, PRP inhibitions cell apoptosis as well as or better than FBS, and also promotes the proliferation of the ccdPAs. The gene expression analyses identified that the DAPK1 and BIM genes were the most highly downregulated apoptosis-related genes by PRP treatment in the preadipocytes. The identified characteristics of PRP with regard to the preadipocytes have advantages including increases in the cell number and improved cell survival in the transplanted grafts. Together with our findings for the efficacies of fibrin scaffold in transplantation of ccdPAs (Aoyagi *et al.*, 2011), the use of PRP for cell preparation and implantation of fat tissues and/or propagated cells may provide the graft with stable long-term survival after auto-transplantation.

Methods

Cell culture

Subcutaneous adipose tissues were obtained from healthy donors after informed consent was obtained, with approval from the ethics committee of Chiba University School of Medicine, and all studies were performed according to the guidelines of the Declaration of Helsinki. The preparation of the ceiling culture-derived proliferative adipocytes (ccdPAs) was performed as described previously (Kuroda *et al.*, 2011). Dulbecco's modified Eagle's medium/F12-HAM (DMEM/HAM, Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ) was used as the culture media, unless otherwise noted in the text.

Preparation of PRP

Human PRP and PPP were prepared from healthy donors as follows; 52 ml of blood was obtained from the donors and mixed with 8 ml of Anticoagulant Citrate Dextrose Solution Formula A (ACD-A, TERUMO, Tokyo, Japan) solution, and transferred to 15 ml tubes. The tubes were centrifuged at 300×g for 15 min at 20°C. The plasma and the buffy coat below the plasma were collected and transferred to new tubes. Secondary centrifugation was performed at 2000×g for 15 min at 20°C. The clear supernatant (plasma) was decanted off until 6 ml was left and the middle portion of supernatant (plasma) was taken to be used as PPP. Finally, the remaining supernatant including the buffy coat was taken to be used as PRP. The platelet number of each product was automatically measured (XS 800i, sysmex Japan). The PRP utilized in this study contained 8.6 x 10^6 platelets/μl, which was approximately 7-fold concentrated from the original concentration in whole human plasma. Preparations of serum lysates containing platelet-released growth factors were essentially performed according to the method described by Aiba-Kojima *et al.* (2007). In brief, 2U/ml of thrombin (Astellas Pharma Inc. Tokyo, Japan) was added to PRP and PPP, and the samples were agitated for 1 hr at 37°C and then incubated overnight at 4°C. Platelet bodies and any remaining fibrin were eliminated by centrifugation (2000×g for 10 min), and the supernatants were obtained for the PRP and PPP. The serum samples were frozen at -20°C and thawed at 37°C before use. The growth medium was supplemented with 2 U/ml of heparin (Novo-Heparin, 5,000 units/5 ml for Injection,

Mochida Pharm. Co. Tokyo, Japan).

Culture on fibrin scaffolds

Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as a clinically available material to generate the fibrin gel. Fibrinogen and thrombin solutions were diluted with Ringer's Solution (Fuso Pharmaceutical Industries, Osaka, Japan) containing 0.5% human serum albumin (Mitsubishi Tanabe Pharma., Tokyo, Japan). The ccdPAs were suspended at 1 x 10^7 cells/ml by the diluted fibrinogen and thrombin solution. The final concentration of fibrinogen was 4 mg/ml and the thrombin solution was used at 1 U/ml. To form fibrin clots, 50 μl of the cell-fibrinogen suspension was added to each cell culture insert (Falcon 3104; Becton Dickinson, Franklin Lakes, NJ), then shortly thereafter, 50 μl of the cell-thrombin suspension was added into the insert, mixed by pipetting, and incubated at room temperature for 2 hr. The inserts with fibrin clots were put on 12 well culture plates, and culture media were added to the inserts and wells. The plates were incubated at 37°C for 12 hr in a 5% CO_2 incubator, and the media were replaced by fresh media containing FBS or PRP.

LDH assay

LDH released into the culture supernatant from the FG/ccdPA was measured using the CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI). A 100 μl sample of each culture supernatant was collected and added into to a 96-well plate. An equal volume of CytoTox-One Reagent was added and incubated for 10 min. Fifty μl of Stop Solution was added and the sample fluorescence was measured on a fluorescence microplate reader (SPECTRA max GEMINI XPS, Molecular Devices, Carlsbad, CA) using a wavelength of 560 nm/590 nm for excitation/emission. The original culture medium before the serum concentration was changed served as a pre-treatment control sample and the control value was subtracted from the value obtained after incubation with the medium containing different concentrations of serum.

Cell proliferation assay

The cell proliferation was examined using the CyQUANT^R Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA). Cells were seeded into 96 well plates at a density of 2 × 10^3 cells per well with DMEM/HAM/20%FBS. After 24 hrs, the culture medium was removed and changed to fresh DMEM/HAM without serum, or with FBS, PRP or PPP. After 3 days, the microplates were gently inverted and blotted onto paper towels to remove the medium from the wells. The microplates were then frozen and stored at -80°C and thawed at room temperature prior to analysis. CyQUANT GR dye/cell-lysis buffer was added to each well. Cells were incubated at room temperature for 5 minutes and the sample fluorescence was measured on a fluorescence microplate reader (SPECTRA max GEMINI XPS, Molecular Devices) using wavelength of 480 nm/520 nm for excitation/emission.

Induction of apoptosis, and the annexin V binding and caspase 3 activity assays

The cells were seeded into 10 cm dishes at a density of 1 × 10^6 cells per well with DMEM/HAM/20%FBS. After 24 hrs, the culture medium was removed and changed to fresh DMEM/HAM with 2% FBS, 2% PRP or 2%PPP. After 2 hrs, apoptosis was induced by the addition of 100 ng/ml TNF-α (Peprotech, Rocky Hill, NJ) and 100 μg/ml cycloheximide (CHX, Sigma-Aldrich). After 3 hrs, the culture supernatant was collected, and the cells were detached by TrypZean treatment. The detached cells were suspended in the collected culture supernatant. Subsequently, the cells were stained with Annexin V-FITC using a Tali^{TM} Apoptosis kit (Life Technologies). Stained cells were analyzed by a Tali^{TM} Image Based Cytometer (Life technologies). Cell lysates at the concentration of 1 x 10^4 cells/μl were prepared from the cells treated to induce apoptosis, and the caspase 3 activity levels were measured by a caspase-3/CPP32 Fluorometric Assay Kit (Biovision, Mountain View, CA). To examine the phosphorylation status of ERK1/2, the cells were scraped off at each time point in PBS and washed. The cells were pelleted and lysed by RIPA buffer (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and the protein concentration was determined by Quant-iT Protein Assay Kit (Life technologies), and 5 μg of protein were analyzed by Western blotting using anti-ERK1 and anti-ERK1/2 (pT202/pY204) as primary antibodies (BD Biosciences, Franklin Lakes, NJ). Mouse TrueBlot ULTRA HRP-conjugated Anti-Mouse IgG (eBioscience, Inc. San Diego, CA) was used as a secondary antibody, and the signals were detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) with LAS1000 apparatus (FUJI film, Tokyo, Japan). ERK signals were normalized using Actin signals detected by Anti-Actin monoclonal antibody (clone AC-40, Sigma-Aldrich).

Gene expression analysis

Cells (2.5 × 10^5 cells) were seeded into 10 cm dish with DMEM/HAM/20% FBS and cultured for 16 hr. The culture medium was changed to fresh DMEM/HAM supplemented with 2% FBS or 2% PRP, and the cells were further incubated for 72 hrs. The total RNA from cultured cells was extracted using a RT^2 RNA Isolation Kit (SA Bioscience, Frederick, MD). Complementary DNA was generated from 1 μg of total RNA using the RT^2 First Strand Kit. An Apoptosis Reverse Transcriptase RT^2 profiler PCR array and RT^2 Real-Time SYBR Green/ROX PCR Mix (SA Bioscience, Frederick, MD) were used to identify the genes affected by PRP according to manufacturer's instruction. The data were analyzed by web-based data analysis software provided by the manufacturer. The probe and primer sets used to quantify the mRNA for the DAPK1 and BIM genes were purchased from Applied Biosystems (Life Technologies). The quantification of given genes was expressed as the relative mRNA level compared with a control after normalization to 18S RNA. All the real-time PCR were performed using an ABI 7500 real-time PCR apparatus.

Statistical analysis

The data are presented as the means ± S.D. Statistical comparisons were made by Student's *t*-test or an ANOVA followed by the post-hoc Tukey test using the SPSS software program. In all cases, P values < 0.05 were considered to be significant.

Acknowledgements

We thank Dr. Fumiaki Matsumoto, Mr. Shunichi Konno, Dr. Shigeaki Tanaka, and Mr. Masayuki Aso for helpful and valuable suggestions about our research. This study was supported by Health and Labour Sciences Research Grants for Translational Research, Japan (H. B.), and in part by the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, Japan (Y.O., Y.N., and H.B.).