さらに効率よく分化したドーパミン神経細胞を得るために、培養条件の検討を行ったところ、分化効率は 1% O_2低酸素培養で向上し安定した。誘導後に中脳やドーパン細胞特異的な遺伝子マーカー：TH, En2, Pitx3, Wnt1, Wnt5a の発現を確認した（Fig.7)。

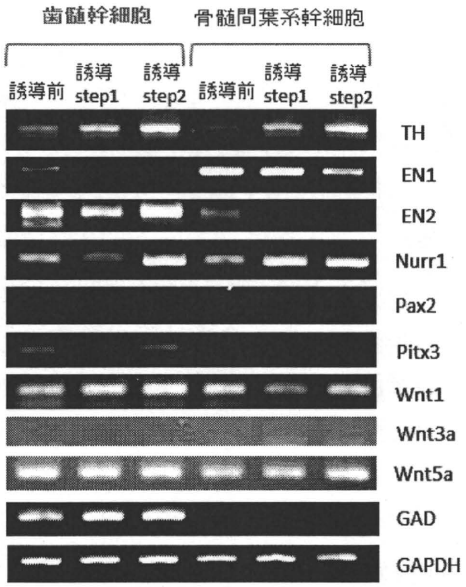

Fig.7　低酸素培養条件下でのドーパミン神経細胞マーカーの発現

今後、Real time PCR にて定量的解析を進める。また preliminary data であるが、ELAISA 法でドーパミンの放出も確認している。

Fig.6、Fig.7 の結果より、我々は歯髄幹細胞から高効率でドーパミン神経細胞を分化誘導する方法を見出したと言える。ただし、マーカー遺伝子での評価が主なので、ドーパミンの放出など機能面での評価も早急に行い、移植実験に移る予定である。

（3）パーキンソン病モデル動物の作成、
（4）パーキンソン病モデルラットにおける歯髄幹細胞移植の有効性・安全性・機能回復効果の検討

当初からの計画通り、平成 23 年 4 月よりパーキンソンモデル動物作成の準備段階に入った。夏以降はパーキンソンモデル動物を用いた研究がスタートできる予定である。歯髄幹細胞移植の有効性・安全性・機能回復効果の検討を行う予定である。

（2－2）脊髄小脳変性症治療に向けて

計画では平成 23 年度よりスタートのテーマである。平成 22 年度の歯髄幹細胞の性状解析において、GABA 作動性神経細胞の分化に必須である転写因子 Ptf1a の発現も解析したところ、特に、乳歯由来歯髄幹細胞 SHED は骨髄間葉系幹細胞と比較して有意に Ptf1a の発現が高いことを確認している。このことから、歯髄幹細胞は GABA 作動性神経細胞であるプルキンエ細胞への分化誘導にも応答能を持つと考えられる。当初からの計画通り、平成 23 年 4 月よりプルキンエ細胞の分化誘導の技術確立のための実験をスタートしており、平成 23 年度中にプルキンエ細胞への高効率な分化誘導技術を確立し、平成 24 年度には移植治療効果の検討を行う予定である。

（3）より治療効果の高い細胞移植方法の検討

この項は今年度の研究成果によって新たに着想を得て追加した研究である。現在、パーキンソン病モデルラットまたはマウスおよび脊髄小脳変性症モデルマウスの準備中であるが、準備が整いしだい、a) 未分化歯髄幹細胞を用いた移植効果の検討、b) 培養上清のみでの再生治療効果の検討、c) 培養上清と分化誘導した細胞の組みあわせでの再生治療効果の検討、を行う予定である。

D．考察

SHED、DPSC とも、９０%以上の歯髄幹細胞が神経幹細胞・幼弱神経細胞・アストロサイト・

未成熟オリゴデンドロサイトのマーカーを共発現していた。その一方で、成熟型神経細胞や成熟オリゴデンドロサイトのマーカーは発現していなかった。このことから、歯髄幹細胞は神経細胞のみならず、アストロサイトやオリゴデンドロサイトへの分化能も持ち合わせた、未成熟状態にある、ユニークな細胞集団であることが見出された。歯髄幹細胞は神経分化誘導にも正常な応答能を示した。他の体性幹細胞である骨髄間葉系幹細胞（BMSCs）や線維芽細胞(FBs)と比較すると、歯髄幹細胞はSHED 、DPSCともGDNF、BDNF、CNTFといった神経栄養因子群を高率に発現していた。GDNF はドーパミンの取り込みと中脳神経細胞の生存および形態学的分化を特異的に促進することが知られており、このことから歯髄幹細胞のほうが骨髄間葉系幹細胞と比較して、パーキンソン病治療応用に有用であると推測される。またSHEDは骨髄間葉系幹細胞と比較して、有意に、GABA 作動性神経細胞への分化に必須である Ptf1a の発現が高かったことから、脊髄小脳変性症治療にも歯髄幹細胞のほうが有用であることが推察される。

パーキンソン病治療のため、高効率でドーパミン神経細胞を分化誘導できる条件を各種検討した結果、塊浮遊培養からスタートし、誘導step1では bFGF と EGF を添加することで 97%がNeuN 陽性成熟型神経細胞に分化し、step2 でさらにshhやBDNF添加することでTH陽性のドーパミン神経細胞に分化誘導できることがわかった。分化効率は 1%O_2低酸素培養で向上し安定した。ドーパミン神経細胞への分化はこれまでのところ遺伝子マーカーの発現で評価しているので、今後はドーパミンの産生など機能面での評価も加え、細胞移植による治療効果の検討と合わせて、歯髄幹細胞の移植治療への実用化の可能性を検討していく予定である。

なお、脊髄損傷モデルを用いた平成 22 年度の我々の研究成果によって新たに着想を得た、未分化歯髄幹細胞移植での治療の可能性や歯髄幹細胞培養上清を用いた神経再生の可能性の検討は、多岐にわたる神経疾患やグリア細胞変性疾患への応用の可能性を秘めていることから、こちらの解析も同時進行で行っていきたいと考えている。

E. 結論

歯髄幹細胞は神経系譜に近い性状を示す細胞集団であり、より効率的な神経分化誘導システムが構築できれば神経疾患治療に有用な細胞源であることが強く示唆された。さらに我々は歯髄幹細胞から高効率でドーパミン神経細胞を分化誘導する方法を見出した。今後は疾患動物モデルでの細胞移植治療の効果を検討し、歯髄幹細胞を用いた神経再生治療の実用化を目指す。

F. 健康危険情報

本研究において、国民の生命・健康に重大な影響を及ぼす事項は発生していない。

G. 研究発表

1. 論文発表　なし
2. 学会発表　なし

H. 知的財産権の出願・登録状況（予定を含む）

1. 特許出願
2. 実用新案登録　なし
3. その他　なし

厚生労働科学研究費補助金（再生医療実用化研究事業）

分担研究報告書

歯髄幹細胞移植による脳室周囲白質軟化症治療法の開発

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名古屋大学大学院医学系研究科顎顔面外科学講座

研究要旨

難治性神経疾患の１つである脳質周囲白質軟化症（periventricular leukomalacia：PVL ）の治療法の開発のため、細胞移植治療の細胞源として ES 細胞、iPS 細胞の研究が進められているが、倫理性や、癌化、免疫拒絶反応などによる安全性に問題があり、実用化にはほど遠いのが現状である。そこで我々は倫理面や安全性の問題を解決しうる移植細胞源として、医療廃棄物であるヒト乳歯から採取可能な乳歯歯髄幹細胞（SHED）に着目した。SHED は自己の乳歯由来の細胞であることから免疫拒絶反応の問題がなく、本来ならば医療廃棄物であることから倫理的な問題もほとんどない。体性幹細胞源としての骨髄や脂肪組織も注目されているが、これらに比べて SHED は採取方法も低侵襲であり、患者への負担も少ない。本研究では、SHED が実用可能な幹細胞源として有用であることを示し、脳質周囲白質軟化症の治療法の確立を目指す。PVL モデルマウスに未分化 SHED を移植し、移植細胞の生着率、機能回復などの移植治療効果の検討と腫瘍形成能などの安全性の検討を行った。SHED を移植した個体では、有意に神経細胞死が抑制されており、脳の委縮・変形も顕著に抑制されていた。細胞移植後 8 週間の段階でも腫瘍形成は見られず、移植細胞の生着が確認された。さらに PVL による運動障害が SHED 移植によって機能回復することも確認された。以上の結果から、PVL に対する移植細胞源として、安全性や倫理面の問題を克服した SHED が有用であることが示された。この結果は今後 PVL に対する細胞移植治療法を確立する上で重要な基礎データとなる。さらに我々は今回の結果を基に、移植した未分化 SHED がどのような機序で PVL の症状の改善に寄与するのか、より詳細なメカニズムの解明を目指している。

A.　研究目的

本研究の目的は、難治性神経疾患の１つである脳質周囲白質軟化症（periventricular leukomalacia：PVL）に対する細胞移植による治療法を確立することである。

PVL は、在胎 32 週未満で出生した早産児に多くみられる疾患 であり、側脳室周囲の深部白質の神経細胞に壊死を生じ、のちに脳性麻痺や知的障害、てんかん、視空間認知障害、学習障害などの神経学的後障害を合併する。現在 PVL に対する治療法はなく、神経学的後傷害に対する理学療法が行われるのみである。PVL の発生機序や修復過程に関する

免疫組織化学的研究では、PVL病巣周囲における、グリア細胞の免疫活性や、大脳白質におけるMyT1(myelin transcription factor 1)など特異的蛋白の発現が、損傷された組織の修復や神経系の可塑性・再生を示唆する報告があるが、失われた神経細胞を再生し症状を改善するまでには至っていない。PVLの治療法としてES細胞やiPS細胞を用いた移植治療の研究が進められているが、倫理性、安全性の問題から未だに確立されたものはない。われわれは、乳歯から採取、培養した乳歯歯髄幹細胞（SHED）が神経系に特化した性質を有することを発見し、PVLで最も障害をうけやすいオリゴデンドロサイトへの分化が可能であることを確認した。そこで本研究では、PVLモデルマウスを作製後、SHEDを脳虚血部位へ移植し、生着率、腫瘍形成能、組織学的所見での移植治療効果、行動解析からの機能の改善効果などについて検討し、PVLに対する細胞移植治療の確立を目指すものとする。

B. 研究方法

1）脳質周囲白質軟化症（PVL）モデルマウスへの乳歯歯髄幹細胞（SHED）移植

名古屋市立大学再生医学分野　澤本和延教授の指導のもと、臨床所見に基づいたPVLモデルマウスを作製した(図1)。右総頸動脈焼灼術を施し虚血障害を起こしてから24時間後に、SHED、hFb、PBSをStereotaxic Injector (Muromachikikai Co.)にてガラスニードルを用いて虚血部位へ移植した。ヒト線維芽細胞（hFb、セルライン）、溶媒（PBS）の移植個体の作成も同様に行い、比較群とした。SHED、hFbは$2x10^5$ cells/2 μl PBSに調整して、虚血部位へ移植を行った。異種移植であるため、移植細胞の宿主の免疫拒絶反応による影響を排除するため、移植時から屠殺時まで免疫抑制剤（シクロスポリン）を10 μg/BWg/day腹腔内投与を行った。移植に用いるSHEDについては、（財）実験動物中央研究所ICLASモニタリングセンターへ微生物検査を依頼し、マイコプラズマなどの感染の危険がないことを確認済である。

2）SHED移植によるPVL治療効果の組織学的検討

PVLモデルマウスへのSHED／hFb／PBS移植48時間後に脳を取り出し、4%パラホルムアルデヒド固定後、60 μmに薄切した。その切片をヘマトキシリン・エオジン染色を行い形態変化による傷害の程度の比較を行った。また細胞死マーカーとして抗Cleaved Caspase3抗体を用いた免疫染色を行い、神経細胞死数の比較検討を行った。さらに、神経細胞マーカーとして抗NeuN抗体、オリゴデンドロサイトのマーカーとして抗APC抗体、アストロサイトのマーカーとして抗GFAP抗体を用い、どのような機序でPVLの傷害改善に介入したのか検討した。

3）SHED移植による安全性の検討

PVLモデルマウスへSHED移植後、48時間（短期）、4週間（中期）、8週間（長期）において、その脳を取り出し、同様に切片を作製した後、ヒト抗核抗体にて染色し、移植細胞の拡散の程度や腫瘍化について検討を行った。

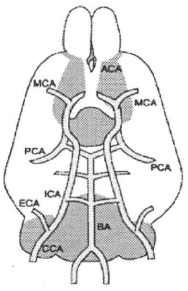
4）SHED移植によるPVLモデルマウスの機能障害改善の評価

PVLモデルマウスでは、四肢の神経学的機能障害が生じることが知られている。SHED／hFb／PBSを移植したPVLモデルマウスに対し、移植4週間後、6週間後、8週間後にFoot Fault-Testを行い、

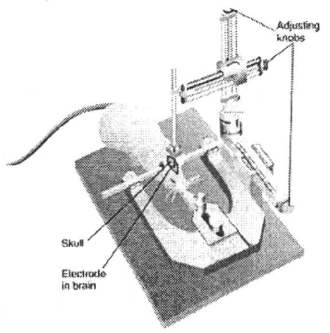
神経学的機能障害に対する治療効果の検討を行った。

（倫理面への配慮）

本研究で用いる乳歯歯髄幹細胞（SHED）は、名古屋大学医学部付属病院歯科口腔外科において、該当患者に書面および口頭で十分に説明し、本研究への理解と同意のもと提供された乳歯を使用した。同意書については、名古屋大学倫理委員会にて承認を受けたものを用いた。また、SHED は完全に匿名化されており、プライバシー保護のためドナー情報が不明な状態で移植実験に用いた。



(図 1)右総頸動脈永久焼灼術　麻酔下にて電気メスを用い、右総頸動脈(CCA)を永久焼灼する。



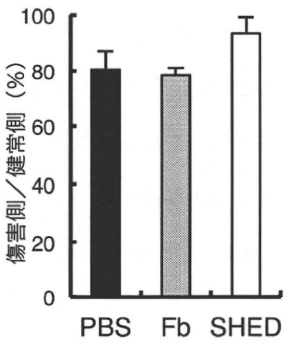
(図 2) Stereotaxic Injector　PVL モデルマウスを固定し、脳虚血部へ細胞移植を行う。

C. 研究結果

1）SHED 移植による PVL 治療効果の組織学的検討

はじめに、移植細胞が虚血部位に生着していることを、ヒト抗核抗体を用いて免疫染色を行い確認した。移植後 48 時間後に脳を取り出しパラホルムアルデヒド固定後、60 μm 切片を作製し、ヘマトキシリン・エオジン染色を用いて脳傷害の改善の程度を定量した(図 3)。その結果、hFb、PBS 移植群と比較して、SHED 移植群では有意に脳の萎縮、変形が少なかった(図 4)。

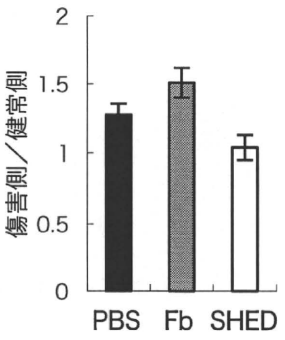
(図 3) ヘマトキシリン・エオジン染色　左：PBS 移植群／右： SHED 移植群



(図 4) 健常側に対する傷害側の脳の大きさの割合を定量
SHED 移植群が有意に脳の萎縮や変形が少なかった

次に傷害脳における細胞死の割合を検討したところ、hFb、PBS 移植群と比較して、SHED 移植群では有意に細胞死が減少していることがわかった(図 5)。このことから、SHED は低酸素虚血傷害による細胞死に対し、神経保護的に作用することが示唆された。この細胞死が抑制された現象について詳細に解析するため、脳を皮質、脳梁、線条体、海馬の４つの部位に分け、さらにこの神経保護作用が脳内に存在する神経系細胞のうち、どのポピ

ュレーションに対して効果があるのか、細胞死マーカーである抗 Cleaved Caspase3 抗体と、神経細胞マーカーNeuN、オリゴデンドロサイトマーカーAPC、アストロサイトマーカーGFAP との二重染色を行い、解析を行った。その結果、SHED 移植群では皮質ではオリゴデンドロサイト、線条体と海馬では神経細胞の細胞死が特に減少していることがわかった。



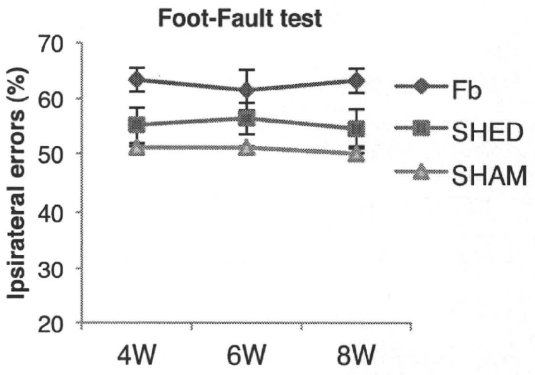
(図 5)健常側に対する傷害側の Caspase3 陽性細胞数の割合
SHED 移植群では有意に細胞死が減少していた

2）SHED 移植による安全性の検討

PVL モデルマウスへの SHED 移植 48 時間後、4 週間後、8 週間後に脳を取り出し、パラホルムアルデヒド固定後に 60 μm に薄切して切片を作製したのち、抗ヒト核抗体を用いて免疫染色を行い、移植細胞の局在を確認した。また、その周囲組織の腫瘍化、形態変化を検討した。その結果、移植細胞は移植部位に腫瘍化することなく生着しており、形態的にも著変は認めなかった。

3）SHED 移植による PVL モデルマウスの機能障害改善の評価

SHED、hFb を移植した PVL モデルマウスに対し、移植 4 週間後、6 週間後、8 週間後に Foot Fault-Test を行い、神経学的機能障害に対する治療効果の検討を行ったところ、SHED 移植群において有意に改善がみとめられた(図 7)。



(図 7)Foot Fault – Test　SHED 移植群は Fb 移植群に比較して、有意に神経学的機能障害の回復が認められた

D.考察

その起源を神経堤にもつ乳歯歯髄幹細胞(SHED)は、低侵襲で採取可能であり、移植における安全性も高く、かつ倫理的な問題を伴う可能性も低い、非常に有用な幹細胞源である。神経堤由来であることから、幹細胞の中でも神経系に特化した性質を有するため、中枢神経系の治療応用に適した細胞集団であると考える。本研究において、PVL モデルにおける SHED 移植による治療効果を検討したところ、組織学的、機能的な解析において有益な結果を得ることができた。このことから、SHED は PVL の移植治療において実用化可能な移植細胞源であることが示された。

今後はどのようなメカニズムで SHED が細胞死を抑制したのか、詳細に解析することが次なる研究課題である。そのメカニズムを明らかにすることが出来れば、その他の脳傷害モデルへの応用も可能になると思われる。

E.結論

本研究では、医療廃棄物である乳歯より採取したSHEDは、PVLモデルに移植することによって虚血傷害脳の細胞死を減少させ、運動機能の回復にも寄与することがわかった。このSHEDの神経保護的な作用の詳細なメカニズムを解明することができれば、臨床応用へ大きな一歩となるだろう。SHEDが有する細胞死を抑制するメカニズムを明らかにすること、そしてさらなる安全性について検討を重ねることを今後の課題とする。

F.健康危険情報

本研究において国民の生命、健康に重大な影響を及ぼす事項は発生していない。

G.研究発表

該当なし

H.知的財産権の出願・登録状況（予定を含む）

該当なし

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Methods Article

Viable Cryopreserving Tissue-Engineered Cell-Biomaterial for Cell Banking Therapy in an Effective Cryoprotectant

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The application of cell-biomaterial systems in tissue engineering and regenerative medicine is an important challenge in biomedicine, which preserves not only cells, but also tissue-engineered constructs. In this study, the constructs and cryoprotectant parameters were optimized, and it was evaluated whether the characteristics of dental pulp stem cells (DPSCs), which have high proliferation ability as stem cells, were maintained during encapsulation and cryopreservation. The optimal cell-biomaterial gel constructs with the gelation rate of 2% alginate: 100 mM $CaCO_3$: 200 mM glucono-δ-lactone (GDL) = 4:1:1 and suitable cryoprotectants (CPAs) used for cryopreservation were Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% ethylene glycol (EG), 1.0 M sucrose and 0.00075 M polyvinylpyrrolidone (PVP). Optimality was confirmed by cell viability (trypan blue, live/dead analysis), the proliferation of DPSCs, and the microstructure using scanning electron microscopy (SEM) in the constructs, and surface epitope by flow cytometric analysis before and after cryopreservation. There were no visible differences in the structure. In conclusion, this study indicates that the optimal cell-biomaterial gel constructs and the cryoprotectant are promising biomaterials. The defined encapsulation/thawing system offers an excellent option for cell-banking therapy to be developed with ready-to-use viable biomaterials and patient-specific products as drug delivery systems.

Introduction

TISSUE ENGINEERING and regenerative medicine (TERM) has been recognized as a promising method to restore damaged tissues and maintain biological functions.[1,2] Among the therapies used in TERM, cell-based therapies have been one of the recent highlights. These therapies have been noted to regenerate lost tissues and provide a sustained source of many beneficial factors. In particular, stem cells seeded in scaffolds are used to enable cell function adaptation by stem cells[3] Numerous studies have also been developed with the goal of producing clinically useful scaffolds (e.g., scaffold design and material selection).[3–5] Further, challenges in scaffold fabrication are to encapsulate cells into scaffolds to create synthesis/living composite biomaterials; however, the process of sourcing, growing, and encapsulation is labor intensive and difficult to apply for acute diseases. As a result of this limitation, an attractive system to cryopreserve the cell-biomaterial construct has emerged.[6–8]

The biomaterials used as scaffolds are natural or synthetic polymers such as polysaccharides, hydrogels, or thermoplastic elastomers. In particular, hydrogel provides a three-dimensional environment similar to that *in vivo* and, therefore, allows cells to maintain their characteristics and function well in the body. The hydrogels used most frequently in TERM are agarose, alginate, collagen, fibrin, gelatin, and hyaluronic acid. Among these, the sources of collagen, fibrin, and gelatin are animal/human proteins; and the source of hyaluronic acid is animal/human polysaccharide, but the source of agarose and alginate is seaweed. The alginate gelation method uses ionotrophic cross linking and is degradable by ion exchange, whereas the agarose gelation method uses thermal change and is nondegradable.[9] In this study, alginate, known for its safe, low-cost, biodegradable, and easy-to-manipulate properties, was examined as a scaffold.

The role of cryoprotectants (CPAs) is to replace water in cells/tissue and to form an amorphous state at low temperature. Ideal CPAs can be achieved using penetrating cryoprotectants with the additional usage of nonpenetrating cryoprotectants.[10] The problem with conventional CPAs is that they contain dimethyl sulfoxide (DMSO) and animal substances. DMSO has long been used as a cryoprotectant for its high penetrating property; however, due to its cytotoxicity, an alternative agent is required. Serum or protein additives have also been used in CPAs to protect cells, but the possibility of contamination remained.[11–14] CPAs that do

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not contain DMSO or protein additives need to be developed but are highly expensive.

Next, as a cell source of important TERM elements, we focused on dental pulp stem cells (DPSCs), which have high proliferation and differentiation ability and have been considered appropriate candidates not only for dental tissue regeneration but also for the treatment of general diseases.[15–18] We encapsulated the DPSCs in alginate gel and optimized the gelation rate containing $CaCO_3$ and glucono-δ-lactone (GDL).[19] After fabricating the cell-biomaterial constructs, we cryopreserved them in CPAs. For medical approval, we examined the optimized condition of ethylene glycol (EG) as a penetrating cryoprotectant and sucrose and polyvinylpyrrolidone (PVP) as nonpenetrating cryoprotectants without DMSO and animal substances. EG, sucrose, and PVP have the merit of being cost-effective CPAs.

Taken together, the approach in this study contributes to the development of effective cryopreservation methods and novel cell banking therapy with ready-to-use viable biomaterials, which reduce the time to fabricate cell-biomaterial constructs and are easy packaging treatments in tissue engineering and medicine technology.

Materials and Methods

Culture of DPSCs and formation of the cell-biomaterial gel encapsulation

Isolation and culture of DPSCs. Human dental pulp tissues were obtained from clinically healthy extracted deciduous teeth. The experimental protocols were approved by the ethics committee of Nagoya University. DPSCs were isolated and cultured as previously described.[15,17] Briefly, the pulp was gently removed and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 h at 37°C. After filtration using 70-mm cell strainers (Falcon; BD Labware, Franklin Lakes, NJ), cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Rockville, MD) containing 20% mesenchymal cell growth supplement (Lonza, Inc., Walkersville, MD) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/

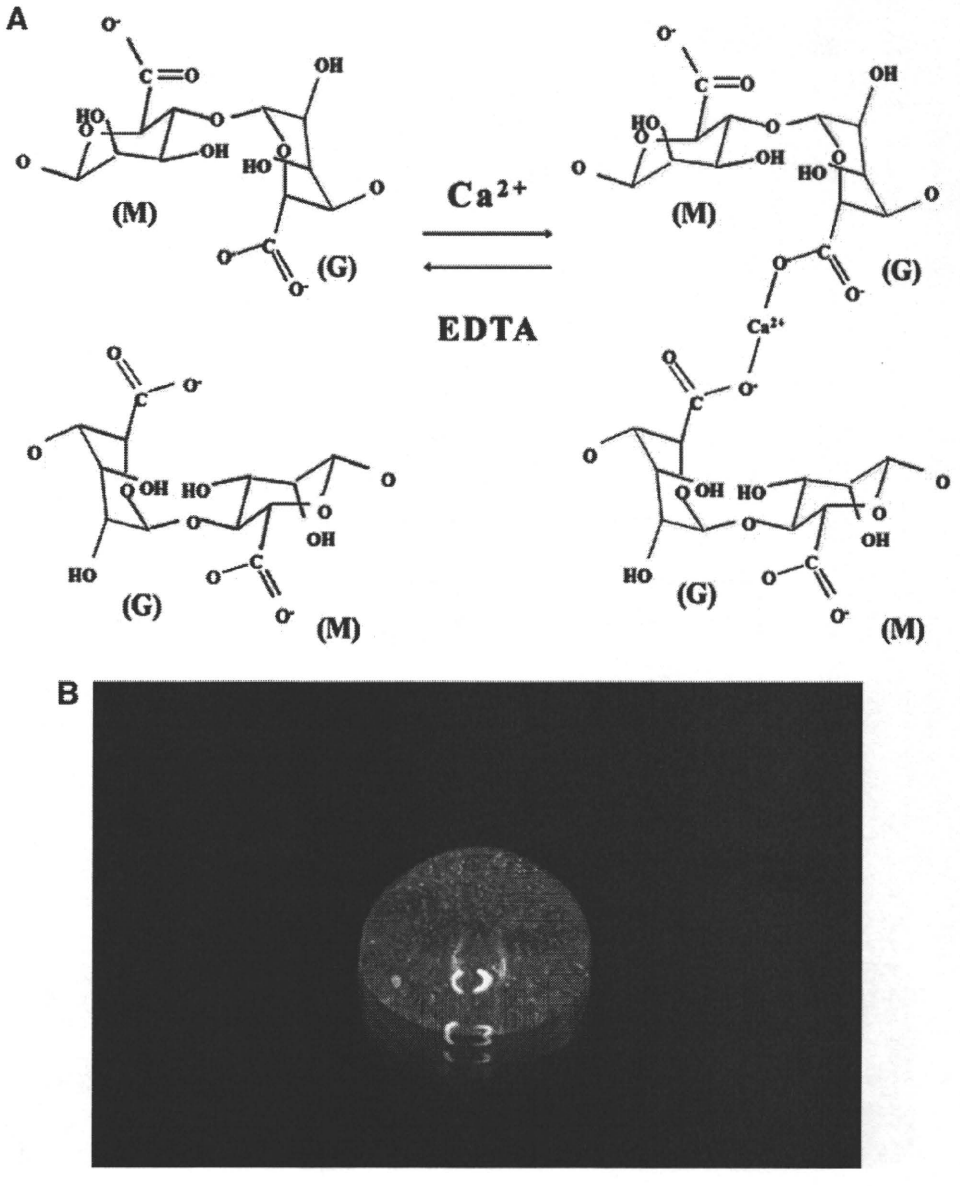


FIG. 1. **(A)** Alginate polysaccharide consisting of one α-L-guluronic acid (G) and one β-D-mannuronic (M) residue with (1,4)-linkages. G-block regions can dimerize to form hydrogels in the presence of aqueous divalent cations, such as calcium. **(B)** Macro view of cell-biomaterial gel construct formation.

mL amphotericin B; GIBCO) at 37°C under 5% CO_2. After primary culture, cells were subcultured at about 1×10^4 cells/cm^2. The cells were used in the experiment from one to five passages.

Optimization of cell encapsulation with alginate gel (cell-biomaterial gel constructs). DPSCs were encapsulated in alginate gel, the gelation rate of which was controlled using $CaCO_3$ (Sigma-Aldrich, Tokyo, Japan) and GDL (Sigma-Aldrich, St Louis, MO). The $CaCO_3$ to GDL molar ratio of 0.5 was maintained to achieve a neutral pH. The DPSCs were mixed with the 5 w/v sodium alginate solution (Kaigen, Hokkaido, Japan) to obtain a final density of 5.0×10^6 cells/mL before the gelation process. Cell-biomaterial gel constructs were fabricated by pipetting $CaCO_3$ solution and GDL solution into the cell-alginate solution (Fig. 1). The gelation rate of experimental groups was as follows: 2% alginate: 100 mM $CaCO_3$: 200 mM GDL: (1) 1:1:1, (2) 2:1:1, (3) 3:1:1, (4) 4:1:1, (5) 5:1:1, (6) 6:1:1, respectively, and the formation and viability of the encapsulated DPSCs were investigated. The average gel diameter was 5 mm. After fabrication, cell-biomaterial gel constructs were cultured for up to 7 days at 37°C under 5% CO_2.

Cryopreservation

Optimization of CPAs. The optimal CPAs were determined by investigating CAPs of various mixture rates (Fig. 2). The agents tested were (1) DMEM + 10% EG (Wako, Osaka, Japan), (2) DMEM + 10% EG + 1.0 M sucrose (Wako), (3) DMEM + 10% EG + 1.0 M sucrose + 0.00025 M PVP (Sigma-Aldrich, St. Louis, MO), (4) DMEM + 10% EG + 1.0 M sucrose + 0.0005 M PVP, (5) DMEM + 10% EG + 1.0 M sucrose + 0.00075 M PVP, (6) DMEM + 40% EG + 0.6 M sucrose, (7) DMEM + 10% fetal bovine serum (FBS) + 12% DMSO (Wako), and (8) Banbanker (Genetics, Tokyo, Japan). Among these agents, the most optimal CPAs were determined by

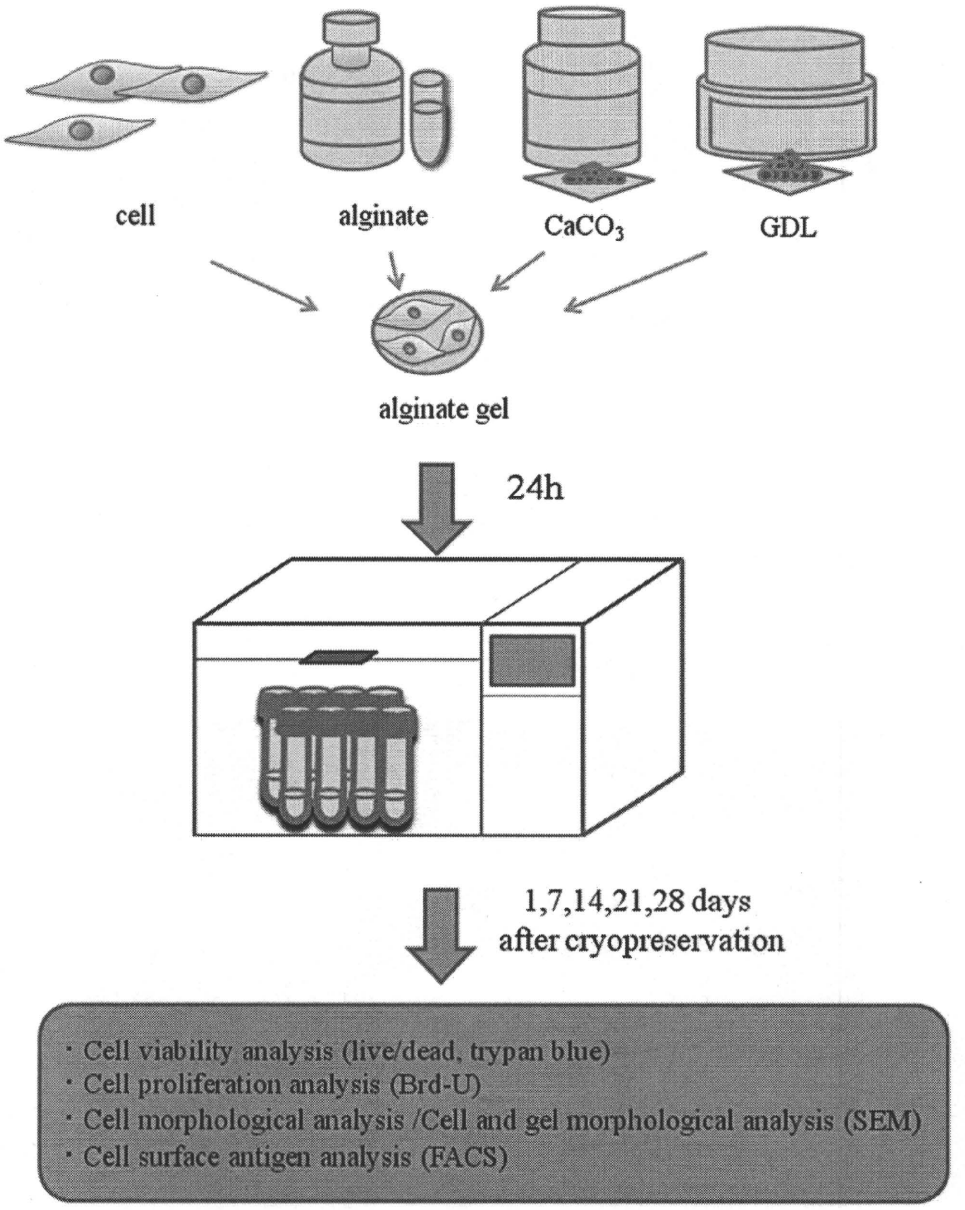


FIG. 2. Schematic illustration of the preparation of cell-biomaterial gel constructs and experimental protocol.

cell viability. All materials used in this study were analytical grade.

Cryopreservation procedures and subsequent culture of cell-biomaterial gel constructs. Cell-biomaterial gel constructs were divided into two groups: (1) control: cell-biomaterial gel constructs without undergoing cryopreservation-thawing process; (2) cryopreservation: cell-biomaterial gel constructs treated with cryopreservation-thawing process. The cell-biomaterial gel constructs were cultured for 24 h at 37°C under 5% CO_2, and then 1 gel was added to each 1.8 mL cryotube (Nunc, Rochester, NY). For the cryopreservation process, cell-biomaterial gel constructs were left at 4°C for 5 min, −30°C for 30 min, and finally stored at −80°C for 1, 7, 14, 21, and 28 days. For the warming process, cell-biomaterial gel constructs were thawed directly in a water bath at 37°C. The cell-biomaterial gel constructs were lysed using 0.5 M ethylene diamine tetraacetic acid (EDTA) (Gibco, Auckland, NZ), and the resulting cell suspension was cultured at 37°C under 5% CO_2 or was analyzed without it. The cells were cultured for 7 days after thawing and before analyzing.

Analysis of cell viability with trypan blue and live/dead assay

Cells in the gel suspension were stained with 0.4% trypan blue (Invitrogen, Tokyo, Japan) to investigate the dead cells. The number of surviving and dead cells was counted, and the survival rate was calculated using Vi-CELL XR (Beckman Coulter, Tokyo, Japan). Noncryopreserved cell-biomaterial gel suspension was used as a control. The cell viability of cell-biomaterial gel constructs in both control and cryopreserved groups was also assayed by confocal laser microscopy using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Eugene, OR). Images were obtained with a Nikon confocal razor microscope A1Rsi (Nikon, Tokyo, Japan) with excitation wavelengths of 488 nm and 543 nm for calcein-AM and ethidium homodimer, respectively. Z stacks of images composed of 17–24 optical slices with z axis steps of 5 μm were obtained for each cell-biomaterial gel construct, and maximum projection images were made with the aid of NIS-Elements AR 3.0 software.

Analysis of cell proliferation

The proliferation rates of cultured DPSCs in cell-biomaterial gel construct suspensions of both control and cryopreserved groups were assessed by bromodeoxyuridine (BrdU) incorporation for 24 h using a BrdU staining kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Assessment of microstructure using scanning electron microscopy

Cell-biomaterial gel constructs from both control and cryopreserved groups were rinsed three times with phosphate-buffered saline (PBS) and fixed in 10% formalin solution (Wako) for 30 min. The constructs were dehydrated with an increasing gradient of ethanol solutions (70%, 80%, 90%, 95%, and 100%), treated with t-butyl alcohol, and freeze dried with liquid nitrogen. Each sample was mounted, sputter-coated with osmium, and examined using scanning electron microscopy (SEM; JEOL JSM-7600; JEOL Ltd, Tokyo, Japan) with 2 kV accelerating voltage.

Analysis of surface epitope by flow cytometric analysis

Cultured DPSCs from both control and cryopreserved groups were analyzed by flow cytometric analysis according to a previous method.[20] Fluorescein isothiocyanate (FITC)-conjugated mouse antibodies against human CD13, CD14, CD29, CD31, CD34, biotin-conjugated mouse antibody against human CD44, CD45, CD73, CD146 (BD Biosciences, San Jose, CA), and CD105 (Ancell Corporation, Bayport, MN) were used for analysis of specific surface antigens. PerCP-conjugated streptavidin (BD Biosciences) was used as

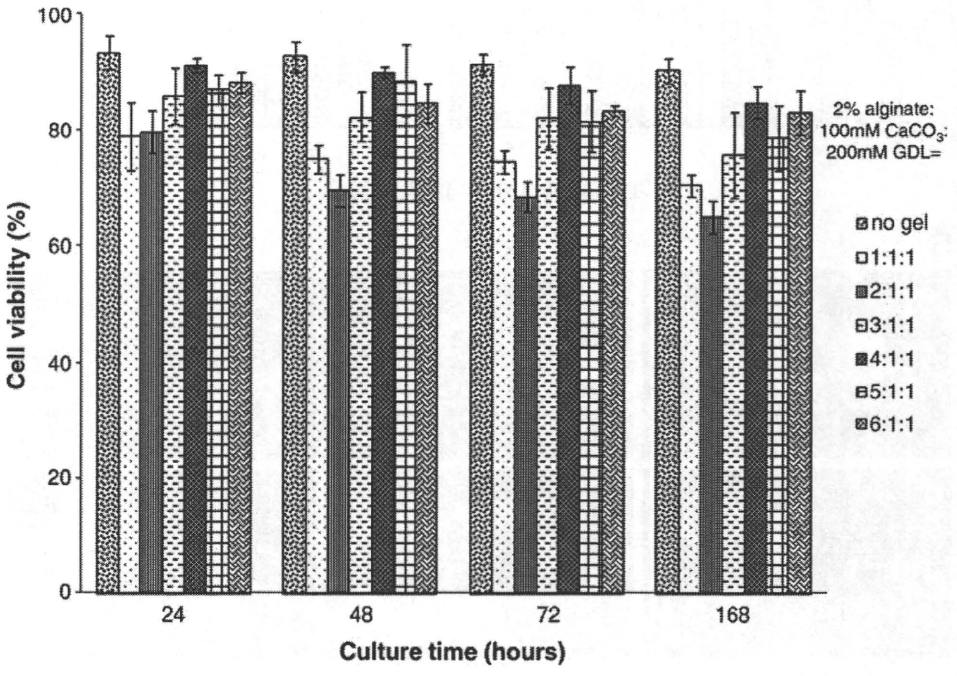


FIG. 3. Cell viability at different gelation rates of cell-biomaterial gel constructs using 2% alginate solution, $CaCO_3$ solution, and GDL solution after culture (h). Bar: standard deviation ($n=3$). GDL, glucono-δ-lactone.

the secondary antibody to detect biotin-conjugated mouse antibody against human CD44.

Statistical analysis

Statistical differences were evaluated using the Tukey-Kramer test after one-way analysis of variance. $p<0.05$ was considered significant.

Results

Optimization of cell-biomaterial gel constructs

The optimal gel constructs were determined by the homogeneity of the gel and the viability of the encapsulated cells. The gelation rate of the alginate gels that would be easy to manipulate was investigated (Fig. 1). Homogenous alginate gels were investigated by controlling the gelation rate to 2% alginate: 100 mM $CaCO_3$: 200 mM GDL=1–6: 1:1. At a rate higher than 5:1:1, full gelation did not occur, and the gel obtained was not homogeneous with the rate lower than 1:1:1. Next, the optimal gelation rate was investigated by analyzing the survival rate of DPSCs cultured in the gels. The optimal cell-biomaterial gel constructs were determined with a gelation rate of 2% alginate: 100 mM $CaCO_3$: 200 mM GDL=4:1:1 (Fig. 3). The percentage of surviving cells at a gelation rate of 4:1:1 was 92.3±5.4, 90.9±6.2, 85.3±5.5, and 82.5±5.4 at 24, 48, 72, and 168 h, respectively (Fig. 3). Cell survival at this gelation rate showed statistically significant differences between 4:1:1 and 1:1:1 or 2:1:1 at 24, 48, 72, and 168 h; between 5:1:1 and 1, 2, 3:1:1, or between 6:1:1 and 2:1:1 or between 3:1:1 and 2:1:1 at 48 h; between 6:1:1 and 1, 2, 3:1:1 at 72 h; or 5, 6:1:1 and 2:1:1 at 168 h. However, there were no significant differences between 4, 5, 6:1:1 and the positive control (only the cells without gelation) at all times (Fig. 3).

Optimization of CPAs

The optimal CPAs were determined by the survival rate of cells (trypan blue staining, live/dead analysis) after the cryopreservation-thawing process. CPAs containing EG,

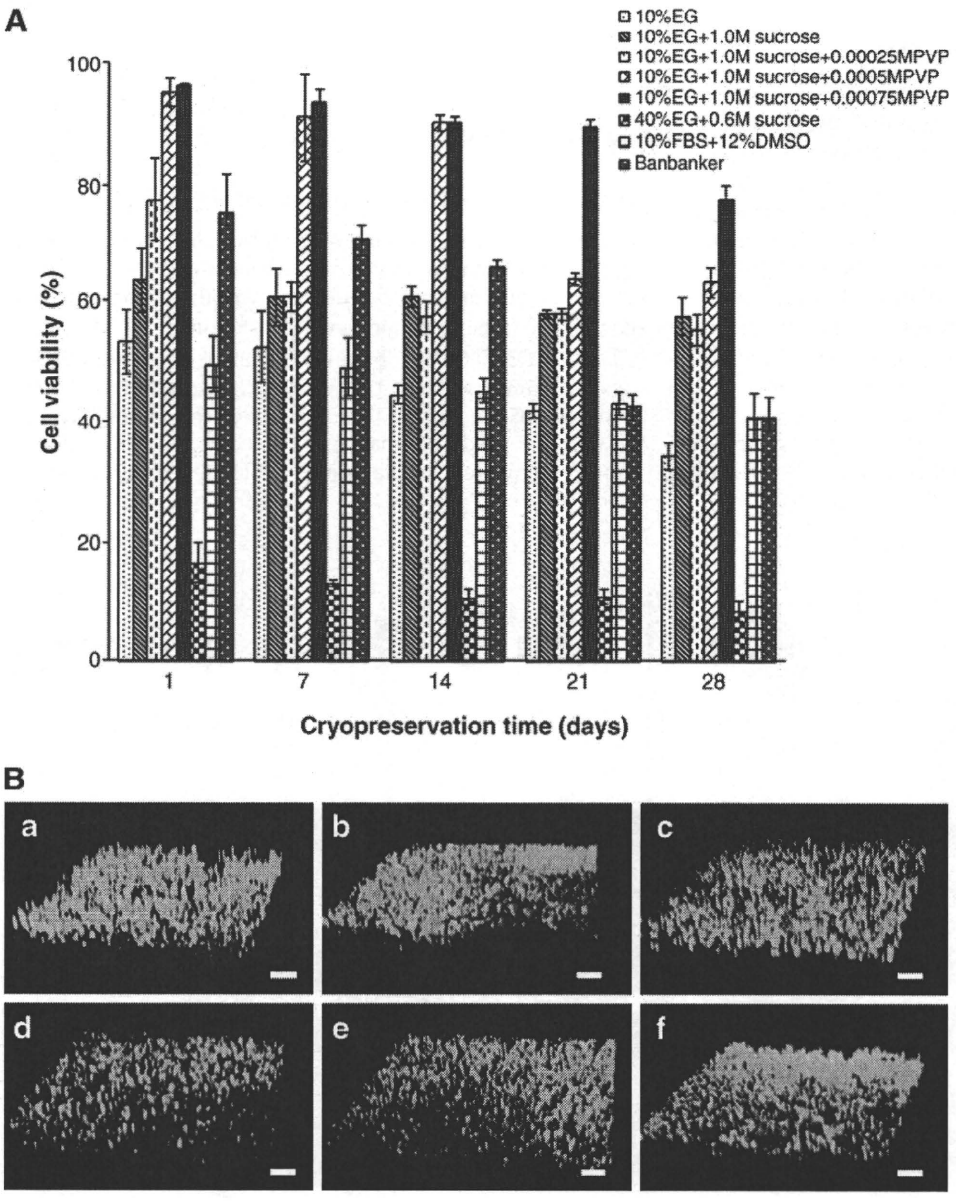
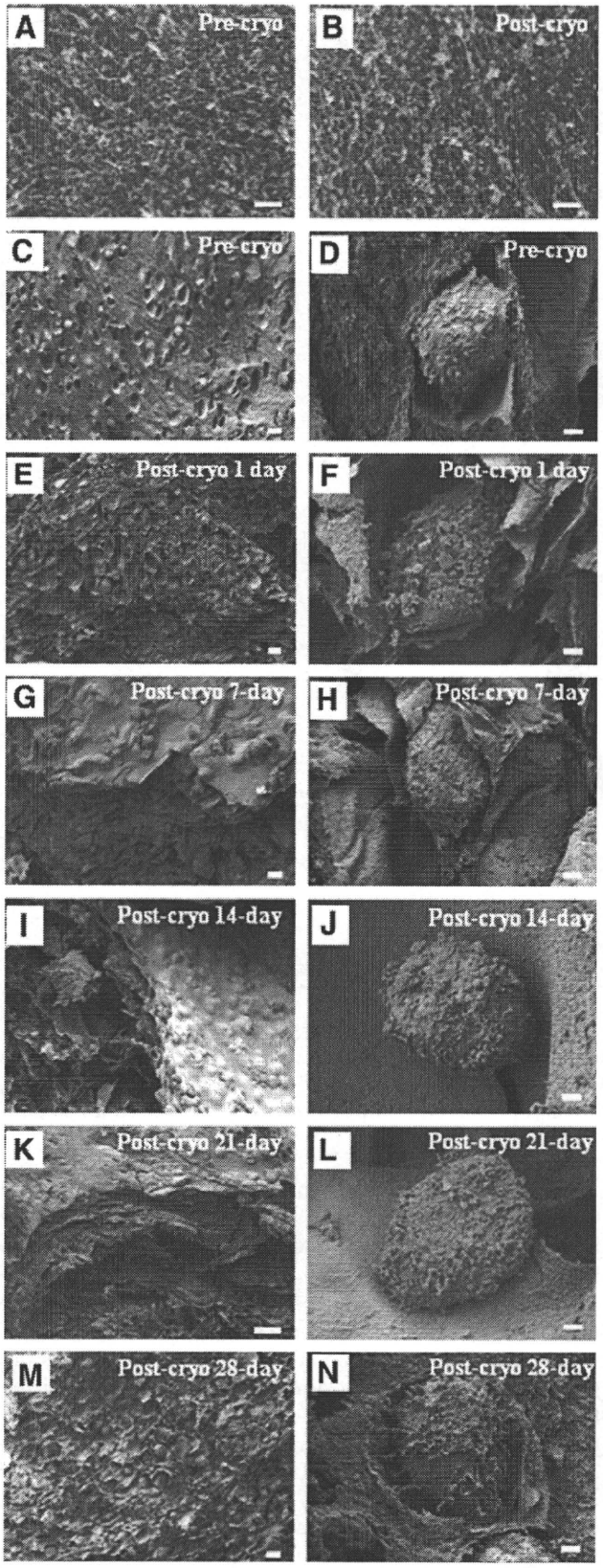


FIG. 4. **(A)** Cell viability after 1, 7, 14, 21, and 28 days of cryopreservation. Bar: standard deviation ($n=3$). **(B)** Confocal lazer scanning microscopy images of control (noncryopreserved) and cryopreserved groups. Viability of encapsulated DPSCs at different time points, **(a)** control, and cryopreservation at 1 day **(b)**, 7 days **(c)**, 14 days **(d)**, 21 days **(e)**, and 28 days **(f)**. Viable cells stained green with ethidium homodimer, whereas nonviable cells stained red with calcein-AM. DPSC, dental pulp stem cell.

sucrose, and PVP were revealed to have better cell viability than the existing commercial product (Banbanker). Further analysis was performed by controlling the mixture rate of EG, sucrose, and PVP. The percentage of surviving cells (cell viability) using CPAs containing DMEM+10% EG+1.0 M sucrose+0.00075 M PVP group was 92.1±3.4, 91.8±1.4, 90.3±0.9, 89.4±1.3, and 77.8±2.5 at 1, 7, 14, 21, and 28 days, respectively. The cell viability of CPAs showed statistically significant differences between DMEM+10% EG+1.0 M sucrose+0.00075 M PVP group and DMEM+10% EG group, DMEM+10% EG+1.0 M sucrose group, DMEM+10% EG+1.0 M sucrose+0.00025 M PVP group, DMEM+40% EG+0.6 M sucrose group, DMEM+10% FBS+12% DMSO group, and Banbanker group at 1, 7, and 14 days, and all groups at 21 and 28 days; therefore, DMEM supplemented with 10% EG, 1.0 M sucrose, and 0.00075 M PVP was the most optimal CPA (Fig. 4). There were no significant differences among these days, and they corresponded with the data from trypan blue staining.



Characterization of DPSCs before and after cryopreservation-thawing process

Microstructure of the cell-biomaterial gel constructs after thawing process by SEM images. In the thawing process, the microstructure of cell-biomaterial gel constructs with and without cells (DPSCs) was examined. Low-magnification SEM images before and after cryopreservation showed no visible differences in the structure (Fig. 5A, B). Even at low magnification, DPSCs were found in cell-biomaterial gel constructs before cryopreservation and after 1, 7, 14, 21, and 28 days (Fig. 5C, E, G, I, K, M). The pore structures of the constructs were retained. At higher magnification, the cell morphology was intact in the constructs with no differences (Fig. 5D, F, H, J, L, N). Representative images revealed that the integrity of constructs was maintained during the process (cooling and warming), and the cells survived.

Effects of DPSCs proliferation ability and cell-surface antigen in the constructs on cryopreservation time. The proliferation rate of DPSCs cultured in the optimized cell-biomaterial gel constructs in both control and cryopreserved groups was assessed using BrdU staining. The percentage of BrdU-positive cells in control and cryopreserved groups at 1, 7, 14, 21, and 28 days was 82.0±5.0, 80.7±3.9, 78.2±6.3, 77.8±2.1, 76.2±4.0, and 76.0±6.3, respectively (Fig. 6A). DPSCs proliferation showed no significant difference between control and cryopreservation groups. Consequently, there was no cryopreservation impact on cell proliferation.

We have previously confirmed that DPSCs exhibit the characteristics of mesenchymal stem cells (MSCs).[18,20] In the present study, the expression pattern of DPSCs was

FIG. 5. Scanning electron microscopy images of alginate and cell-biomaterial gel constructs showing the impact of cryopreservation. Sections of alginate gels without cells: precryopreserved constructs **(A)**, cryopreserved constructs **(B)**. Sections of cell-biomaterial gel constructs: precryopreserved constructs **(C, D)**, cryopreserved constructs 1 **(E, F)**, 7 **(G, H)**, 14 **(I, J)**, 21 **(K, L)**, and 28 **(M, N)** days after thawing process. Wide view images **(A, B, C, E, G, I, K, M)** show structure integrity, magnified cells **(D, F, H, J, L, N)** illustrate clear view of microstructure. Scale bars represent 10 μm **(A, B, C, E, G, I, K, M)**, 1 μm **(D, F, H, J, L, N)**, respectively. pre-cryo, precryopreservation; post-cryo, post-cryopreservation.

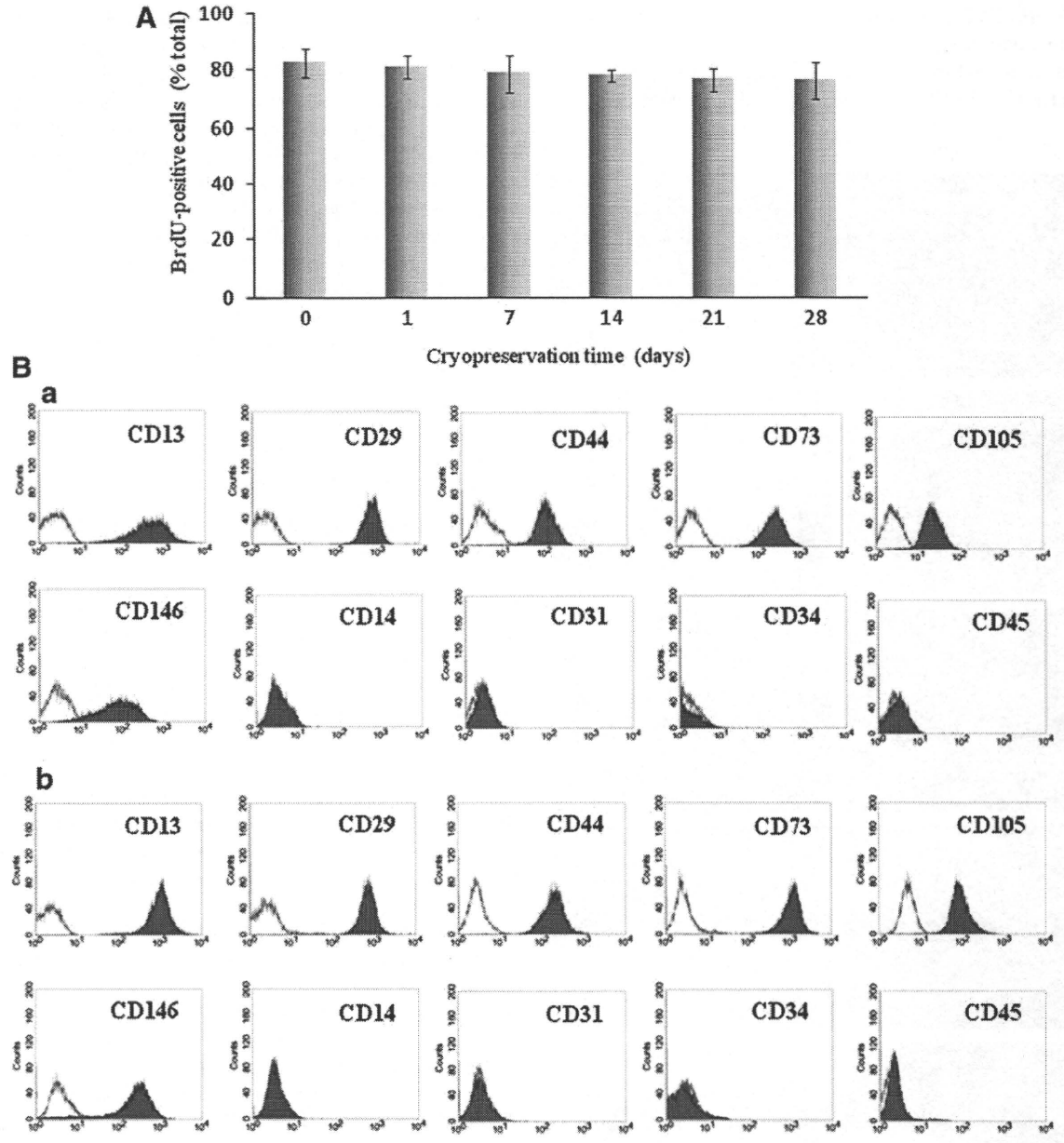


FIG. 6. **(A)** Cell proliferation rates of control and cryopreserved groups (1, 7, 14, 21, and 28 days after cryopreservation-warming process) were assessed using BrdU. Bar: standard deviation ($n=3$). **(B)** Typical flow cytometric analysis diagrams on the expression of MSC markers CD13, CD29, CD44, CD73, CD105, and CD146, as well as hematopoietic markers CD34, and CD45, and monocytic markers CD14, CD31. **(a)** Control and **(b)** cryopreserved group. The cells cryopreserved for 28 days were used in this study. BrdU, bromodeoxyuridine; MSC, meschymal stem cell.

investigated and was found to be comparable to that of MSCs. The expression pattern of DPSCs encapsulated in cell-biomaterial gel constructs and cryopreserved in CPAs was also investigated. It was found that encapsulated and cryopreserved DPSCs also preserved the expression pattern of markers even after cryopreservation (Fig. 6B).

Discussion

Alginate gels are easy to manipulate, and the gels used in this study, which are used clinically to protect cells and tissues, are safe. They were solvated using EDTA as a chelating agent and GDL as a pH reducing agent and formed gels in the presence of aqueous divalent cations, such as Ca^{2+}.[19] The alginate gel with a gelation rate of 2% alginate: 100 mM $CaCO_3$: 200 mM GDL=4:1:1 was found to be optimal by investigating the survival rate of cells cultured in the cell-biomaterial gel constructs (Fig. 3). The viability of the encapsulated cells might have been maintained because of the mild internal environment of the gel.

An ideal CPA is a solution that is nontoxic to cells/tissues even after prolonged exposure. Strategies to avoid toxic effects of the solutions require component selection and adjustment of the solute concentration. This minimizes damage caused by ice formation and encourages the formation of an amorphous state in cells/tissues.[21] Currently, effective CPAs commonly consist of a minimum of two (penetrating cryoprotectant + sugar/polymer) or three (penetrating

cryoprotectant + sugar + polymer) components.[16] The most traditional penetrating cryoprotectants, including DMSO, glycerol, and EG, have been tested alone and in combination.[22] EG is known for its detrimental effects on developmental potential, membrane integrity and cytoskeletal structure, which has made it the principal penetrating cryoprotectant.[22] As a nonpenetrating cryoprotectant, sugar is used as an important component of osmotic buffers. In this study, we used sucrose, a monosaccharide that can be dissolved more efficiently in solutions of penetrating CPAs, than either the disaccharides or polysaccharides. In terms of polymers, we used PVP for its ability to cryopreserve delicate tissues.[23] This is used in a wide variety of applications in medicine, pharmacy, cosmetics, and industrial production and is also known to be a more effective CPA than other polymers, such as dextran and Ficoll.[24] In this study, the cell viability of DPSCs was investigated in various CPAs. The viability of CPAs in DMEM supplemented with 12% DMSO and 10% FBS, commercial CPAs (Banbanker), DMEM supplemented with 10% EG, and 1.0 M sucrose and 0.00075 M PVP, which were the optimized CPAs, was 49.8% ± 4.5%, 77.5% ± 6.5%, and 96.1% ± 0.4% on day 1, respectively (Fig. 4A). No visual difference between the cell viability of control and cryopreserved gel was found when observed through a confocal microscope with trypan blue staining after 1, 7, 14, 21, and 28 days of cryopreservation (Fig. 4B). Moreover, the maintenance of structure integrity is a prerequisite in the cryopreservation of cell-biomaterial constructs, and the cryopreserved constructs were undamaged, such as the cell surface of SEM images showed no significant difference in microstructure between the control and cryopreserved groups (Fig. 5). The cryopreserved structure of the constructs appeared to have more pores and wrinkles on the surface (Fig. 5). This optimized CPA is DMSO, serum, and animal substance free; and the cryopreserved-thawed DPSCs had normal morphology (data not shown), maintained the properties of multipotent cells with high proliferation ability, and expressed the corresponding marker of MSCs (Fig. 6). Therefore, the matricellular environment of the DPSCs in our cell-biomaterial gel and CPAs constructs also remained unaltered. These results imply that the constructs were well preserved during the cooling-warming cycle and the DPSCs would be well protected.

On the other hand, culturing and expanding cells *ex vivo* is lengthy and costly work, and obtaining a sufficient cell number for tissue engineering applications would take some months[25]; therefore, if well-preserved viable cell-biomaterial constructs could be prepared, it would eliminate the lengthy waiting period and bring down the medical fees. An attractive aspect of applying cell-biomaterial gel constructs and their successful long-term storage lies in the potential of providing immediate solutions to patients with acute diseases. Moreover, cell-biomaterial constructs and the application of CPAs are a step toward the application of stem cells such as DPSCs in TERM and provide novel tissue-engineering products, such as ready-to-use and patient-specific products, for cell-based services or drug delivery systems.

Conclusion

In this study, we optimized cell-biomaterial gel constructs and cryoprotectant parameters, and it was found that the characteristics of DPSCs were maintained during encapsulation and cryopreservation. In conclusion, this study indicates that the optimal cell-biomaterial gel constructs and the cryoprotectant could be promising biomaterials. The defined encapsulation/thawing system offers an excellent simple option for cell-banking therapy.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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Human Deciduous Teeth Dental Pulp Cells (hDPC) with Basic Fibroblast Growth Factor (b-FGF) Enhance Wound Healing of Skin Defect

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Abstract: In this research, we examined the effect on wound healing applying basic fibroblast growth factor (b-FGF) that is approved for clinical use to enhance wound healing, and human deciduous teeth dental pulp cells (hDPC) in clinics, but that have been attracting attention as a novel stem cell source in recent years. Human deciduous teeth were harvested from healthy volunteers and hDPC were isolated. We used a nude mouse full thickness skin defect model and evaluated wound healing by macroscopic view, histological and histomorphometric analysis. The mice were randomly divided into four groups: phosphate buffered saline (PBS) treated group (control group), b-FGF treated group (b-FGF group), hDPC treated group (hDPC group), and hDPC and b-FGF treated group (hDPC/b-FGF group). b-FGF and hDPC groups accelerated wound healing compared to the control group. There was no statistically significant difference in wound healing observed between hDPC and b-FGF group. hDPC/b-FGF group demonstrated accelerated wound healing compared to other groups. At day 14, PKH 26-positive cells were surrounded by human Type I collagen in hDPC and hDPC/b-FGF groups in immunohistological evaluation. Significantly increased collagen fibril areas in wound tissues were observed in b-FGF, hDPC and hDPC/b-FGF groups as compared with control group at day 7 and 14. Our results showed that hDPC/b-FGF group significantly promotes wound healing compared with other groups. This study implies that deciduous teeth that are currently considered as medical spare parts might offer a unique stem cell resource for potential of new cell therapies for wound healing in combination with b-FGF.

Key Words: human deciduous teeth dental pulp cells (hDPC), basic fibroblast growth factor (b-FGF), wound healing, cell transplantation

INTRODUCTION

Wound healing is a complex phenomenon that involves sequential phases that overlap in time and space, interact, and affect each other dynamically both at the gene and protein levels. It is difficult to control wound healing after tumor excision, cleft lip and trauma in craniofacial area. In case of intractable ulcers, keloids and hypertrophic scars, wound healing is extremely stressful on patients.[1] A lot of treatment methods have been examined; however, there is no one established, acceptable method. A new treatment modality needs to be established.

Regenerative medicine is a promising tool in a new clinical platform for a whole spectrum of intractable diseases. Various stem cells have been reported, especially mesenchymal stem cell (MSC) isolated from various tissues including bone marrow, adipose tissue, skin, umbilical cord, and placenta, and used in clinical applications in skin regeneration.[2-5] They promote wound healing and may reduce scars.[6] There are several lines of evidence reported that MSC have been applied to accelerate wound healing through differentiation and paracrine effects.[6,7] MSC, referred to as stromal progenitor cells, are self-renewing and expandable stem cells. However, bone marrow aspiration is an invasive procedure for the donor. In addition, the number, proliferation, and differentiation potential of MSC decline with increasing age.[8]

Dental pulp appears to be an alternate and more readily available source of stem cells in the craniofacial area. Stem cells from the deciduous teeth dental pulp have been identified as a novel population of stem cells that have the capacity of self-renewal and multi-lineage differentiation similar to MSC.[9,10] They have also been reported to have the

potential for use in cell-based therapy for systemic disease, such as neurologic disease and cardiac disease, and to ameliorate ischemic disease.[11-13] To date, there is no report of them having been used in wound healing.

Basic fibroblast growth factor (b-FGF) was approved for clinical use in Japan in 2001, and has been used clinically in recent years. FGF have been shown to have profound effects on various cell types, influencing their proliferation, differentiation and other functions.[14-16] b-FGF is one of the FGF families of single-chain polypeptides (14±18 kDa) that regulates the development and maintenance of the cellular derivatives of mesoderm and neuroectoderm. Of all known growth factors, b-FGF probably has the broadest range of target cells, including essentially all of the diverse cells involved in wound healing.[17] b-FGF is noted for its non-invasive approach.

In this research, we examined the effect on wound healing applying b-FGF, which has already been used in clinical applications, and human deciduous teeth dental pulp cells (hDPC). These results may provide us with new information of cell therapies for wound healing in conjunction with b-FGF.