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ORIGINAL ARTICLE

Allogeneic transplantation for primary myelofibrosis with BM, peripheral blood or umbilical cord blood: an analysis of the JSHCT

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To determine whether a difference in donor source affects the outcome of transplantation for patients with primary myelofibrosis (PMF), a retrospective study was conducted using the national registry data on patients who received first allogeneic hematopoietic cell transplantation (HCT) with related BM ($n=19$), related PBSCs ($n=25$), unrelated BM ($n=28$) or unrelated umbilical cord blood (UCB; $n=11$). The 5-year OS rates after related BM, related PBSC and unrelated BM transplantation were 63%, 43% and 41%, respectively, and the 2-year OS rate after UCB transplantation was 36%. On multivariate analysis, the donor source was not a significant factor for predicting the OS rate. Instead, performance status (PS) $\geqslant 2$ (vs PS 0–1) predicted a lower OS ($P=0.044$), and RBC transfusion $\geqslant 20$ times before transplantation (vs transfusion $\leqslant 9$ times) showed a trend toward a lower OS ($P=0.053$). No advantage of nonmyeloablative preconditioning regimens in terms of decreasing nonrelapse mortality or increasing OS was found. Allogeneic HCT, and even unrelated BM and UCB transplantation, provides a curative treatment for PMF patients.

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Keywords: idiopathic myelofibrosis; hematopoietic SCT; donor source; engraftment; survival

INTRODUCTION

Primary myelofibrosis (PMF) is a clonal stem cell disorder characterized by anemia, BM fibrosis, progressive splenomegaly, constitutional symptoms and a significant risk of evolution into acute leukemia.[1,2] The median age at diagnosis is ~65 years, with a median survival of ~5 years after diagnosis, depending on the presence or absence of clinically defined prognostic factors, such as those defined by the International Prognostic Scoring System (IPSS), Dynamic IPSS and Dynamic IPSS plus.[3–5] No available conventional drug therapies for PMF have been shown to prolong survival. Palliative therapeutic options include agents such as hydroxyurea, prednisone, EPO, androgens, thalidomide and lenalidomide, and nonpharmacological approaches such as blood transfusion, splenic irradiation and splenectomy.[6,7] The impact of new agents, such as Janus kinase 2 (JAK2) inhibitors, pomalidomide and histone deacetylase inhibitors, on the long-term management of PMF is under investigation.[7,8] The only known curative therapy for PMF is allogeneic hematopoietic cell transplantation (HCT).[9]

The largest retrospective study of PMF patients undergoing allogeneic BM or PBSC transplantation reported OS of 30–40% at 5 years after transplantation with nonrelapse mortality (NRM) of 24–43% at 1 year after transplantation.[10] The prospective study in patients with PMF or secondary myelofibrosis to evaluate a nonmyeloablative preconditioning regimen followed by mainly PBSC transplantation achieved an OS of 51% at 5 years after transplantation with NRM of 16% at 1 year after transplantation.[11] The issues of the choice of stem cell source, the choice of conditioning regimen and the timing of transplantation are currently under debate.[6–9,12,13]

To determine whether a difference in stem cell source affects the outcome of HCT for PMF patients, a retrospective study was conducted using the national registry data on patients who received first allogeneic HCT in Japan with BM, PBSCs or umbilical cord blood (UCB).

PATIENTS AND METHODS

Patients

Clinical data for patients with PMF who received first allogeneic HCT in Japan were extracted from the Transplant Registry Unified Management Program (TRUMP) system, which is a registry of the outcomes of Japanese transplant patients.[14] Patients who had progressed to myelofibrosis from polycythemia vera, essential thrombocythemia, leukemia or other disease were excluded. This study was approved by the Data Management Committee of the Japan Society for Hematopoietic Cell Transplantation (JSHCT) and by the ethics committee of the Nagoya University School of Medicine (no. 2012-0270).

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Definitions

Hematopoietic recovery was defined as time to ANC $\geqslant 0.5 \times 10^9$/L, time to reticulocytes $\geqslant 10‰$ and time to platelets $\geqslant 50 \times 10^9$/L for 3 consecutive days. Engraftment failure was defined as no neutrophil recovery by day 60. Acute and chronic GVHD were diagnosed and graded according to established criteria.[15,16] Based on the report by the Center for International Blood and Marrow Transplant Research (CIBMTR),[17] the conditioning regimens were classified as myeloablative if TBI >8 Gy, oral BU $\geqslant 9$ mg/kg, i.v. BU $\geqslant 7.2$ mg/kg or melphalan >140 mg/m^2 was included in the conditioning regimen, whereas other conditioning regimens were classified as nonmyeloablative.

End points

The primary end point was OS. The secondary end points were engraftment, GVHD, relapse and NRM.

Statistical analysis

The probabilities of hematopoietic recovery, acute and chronic GVHD, relapse and NRM were estimated on the basis of cumulative incidence curves.[18] The probability of OS was estimated according to the Kaplan–Meier method.[19] The groups were compared using the log-rank test. Competing risk regression analysis was used to identify factors associated with NRM. The adjusted probability of OS was estimated using Cox's proportional hazards model, with consideration of other significant clinical variables in the final multivariate models.[20] All variables significant at $P<0.10$ on univariate analysis were included in multivariate stepwise analyses. All tests were two sided, and $P<0.05$ was considered significant. The data were analyzed by STATA version 12 statistical software (StataCorp, College Station, TX, USA).

RESULTS

Patient and transplantation characteristics

A total of 83 patients met the inclusion criteria. Patient and transplantation characteristics are summarized in Table 1. The median age at transplantation was 53 years, and most patients (66%) were male. Transplants were performed between 1993 and 2009, but the majority (90%) of them were performed after 2000. This population consisted of 47 BM transplants, 25 PBSC transplants and 11 UCB transplants. Of the 44 related donor transplants, 40 (91%) were performed from serological HLA-A, B and DR 6/6 matched donor; 28 unrelated BM transplants included 16 (57%) HLA-A, B and DRB1 alleles 6/6 matched donors and 11 (39%) HLA-A, B and DRB1 alleles 5/6 matched donors; all (100%) unrelated UCB transplants were performed from serological HLA-A, B and DR 5/6 or 4/6 matched donors. Most patients (76%) received a nonmyeloablative regimen. The median follow-up for living patients was 40 (range, 0.4–150) months.

Engraftment

Seven patients (8%) died without engraftment within 60 days after transplantation, including heart failure on day 5 after UCB transplant ($n=1$), primary disease on day 7 after related PBSC transplant ($n=1$), infection on day 11 after unrelated BM transplant ($n=1$), multiple organ failure on day 12 after unrelated BM transplant ($n=1$), heart failure on day 18 after unrelated BM transplant ($n=1$), infection on day 30 after unrelated BM transplant ($n=1$) and thrombotic microangiopathy on day 56 after UCB transplant ($n=1$). Another patient (1%) received a second transplant on day 28 because of lack of engraftment signs at that time.

Neutrophil recovery on day 60 occurred in 92% (95% confidence interval (CI), 57–99%) of related BM, 92% (71–98%) of related PBSCs, 79% (58–90%) of unrelated BM and 82% (45–95%) of unrelated UCB (Figure 1a). Unrelated BM and unrelated UCB (vs related BM) transplantations were significantly associated with a lower probability of neutrophil recovery ($P=0.015$ and $P=0.016$, respectively), whereas related PBSC transplantation was

Table 1. Patient and transplantation characteristics ($n=83$)

	N *(%)*
Age at transplant, evaluable n	83
21–39 Years	9 (11)
40–49 Years	22 (27)
50–59 Years	37 (44)
60–79 Years	15 (18)
Median age (range), years	53 (21–79)
Sex, evaluable n	83
Female	28 (34)
Male	55 (66)
Transplant year, evaluable n	83
1993–1999	8 (10)
2000–2004	22 (27)
2005–2009	53 (63)
Performance status at transplant, evaluable n	70
0–1	54 (77)
$\geqslant 2$	16 (23)
Time from diagnosis to transplant, evaluable n	80
<1 Years	33 (41)
1–2 Years	16 (20)
$\geqslant 2$ Years	31 (39)
Median (range), years	1.5 (0.1–21.0)
Frequency of RBC transfusion before transplant, evaluable n	51
$\leqslant 9$	26 (51)
10–19	8 (16)
$\geqslant 20$	17 (33)
Frequency of PLT transfusion before transplant, evaluable n	51
$\leqslant 9$	38 (74)
10–19	4 (8)
$\geqslant 20$	9 (18)
Use of JAK2 inhibitor before transplant, evaluable n	77
Yes	0 (0)
No	77 (100)
Splenectomy before transplant, evaluable n	78
Yes	2 (3)
No	76 (97)
DIPSS at transplant	78
Low	8 (10)
Intermediate-1	17 (22)
Intermediate-2	50 (64)
High	3 (4)
Splenomegaly at transplant	78
Yes	59 (76)
No	19 (24)
CMV serostatus, evaluable n	58
Negative	5 (9)
Positive	53 (91)
Donor source, evaluable n	83
Related BM	19 (23)
Related PBSCs	25 (30)
Unrelated BM	28 (34)
Unrelated umbilical cord blood	11 (13)
Sex matching between patient and donor, evaluable n	71
Match	35 (49)
Female patient and male donor	15 (21)
Male patient and female donor	21 (30)
ABO matching between patient and donor, evaluable n	65
Match	34 (52)
Mismatch	31 (48)
Preconditioning regimen, evaluable n	71
Myeloablative	17 (24)
Nonmyeloablative	54 (76)
Prophylaxis for GVHD, evaluable n	81
CsA based	37 (46)
Tacrolimus based	42 (52)
Others	2 (2)
Use of JAK2 inhibitor after transplant, evaluable n	78
Yes	0 (0)
No	78 (100)

Abbreviations: DIPSS = Dynamic International Prognostic Scoring System; JAK2 = Janus kinase 2.

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ORIGINAL ARTICLE

Risk factors and organ involvement of chronic GVHD in Japan

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Few studies have evaluated the risk factors for chronic GVHD and organ involvement associated with different graft types, including unrelated cord blood (U-CB). We retrospectively studied 4818 adult patients who received their first allogeneic transplantation and survived for at least 100 days. The incidence of chronic GVHD at 2 years was 37%. The following factors were associated with the development of chronic GVHD: female donor/male recipient, CMV-Ab seropositivity, matched related peripheral blood grafts vs matched related BM grafts, no *in vivo* T-cell depletion and the occurrence of grade II–IV acute GVHD. Among these factors, the association with acute GVHD occurrence was consistently significant across donor subtypes. The use of U-CB was not associated with chronic GVHD, but was associated with a low incidence of extensive chronic GVHD. Chronic GVHD patients who had received U-CB transplants showed less frequent involvement of the oral cavity (28% vs 55%), eye (12% vs 26%), liver (20% vs 44%), lung (11% vs 25%) and joint (0% vs 6%) than those with matched related BM grafts. In conclusion, we found that U-CB transplants were associated with a low incidence of extensive chronic GVHD and less frequent involvement of the oral cavity, eye, liver, lung and joints.

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Keywords: chronic GVHD; unrelated cord blood; acute GVHD; risk factors

INTRODUCTION

Chronic GVHD is a serious complication that affects the survival and quality of life of long-term survivors after allogeneic hematopoietic SCT.[1–3] Various pre- and post-transplant risk factors associated with chronic GVHD have been identified, mostly in transplantations using BM and PBSC grafts from related or unrelated donors.[2,3] Several studies have reported a history of acute GVHD to be a strong risk factor that is consistently associated with chronic GVHD development.[4–8] Other identified risk factors include the following: female donor and male recipient,[4,6] use of PBSC grafts,[6,9–13] older patient,[4,6–8] older donor,[6,7] transplantation from a mismatched or unrelated donor,[5,6,14] diagnosis of CML[4,7,8] and absence of anti-thymocyte globulin (ATG) use.[15]

The number of unrelated cord blood (U-CB) transplantations performed has rapidly increased during the past decade. However, few studies have compared the incidences and risk factors of chronic GVHD and its organ-specific symptoms in adult patients receiving U-CB and other available grafts, including related or unrelated BM/PBSC grafts.[16,17] Therefore, we conducted a retrospective study using national registry data involving 4818 patients who underwent allogeneic transplantation. This study aimed to evaluate the incidence and risk factors of chronic GVHD, and the prevalence of chronic GVHD organ involvement in patients who received transplantation using various types of graft, including U-CB.

MATERIALS AND METHODS

Data collection

Data for 54072 patients who had received auto-SCT or allo-SCT by December 31, 2009 were provided by the Transplant Registry Unified Management Program (TRUMP).[18] We included 4993 adult patients who had: (1) received allogeneic transplantation for hematologic malignancies; (2) received their first SCT; (3) used the same questionnaire form involving chronic GVHD organ involvement (skin, oral cavity, eye, liver, lung, joint, intestine/genitals and other manifestations; 2006–2009 for transplantations using BM or PBSC grafts and 2007–2009 for transplantations using U-CB units); (4) achieved neutrophil engraftment; (5) survived for at least 100 days; and (6) received the following: (a) a related BM or PBSC graft (R-BM/PB), (b) an unrelated BM (U-BM) or (c) a single U-CB unit. Donation of peripheral blood by unrelated volunteers was permitted for the first time in Japan in 2011. The following patients were excluded: (1) patients who received *ex vivo* T-cell-depleted grafts ($n = 26$) and (2) patients who lacked data on acute or chronic GVHD ($n = 149$). Thus, 4818 patients were included in this study, which was approved by the TRUMP Data Management Committees and by the institutional review board of the Nagoya University Graduate School of Medicine, where this study was performed.

Histocompatibility

Histocompatibility data for the HLA-A, HLA-B and HLA-DR loci were obtained through reports acquired from the institution where the transplantation was performed or from the cord blood bank. HLA

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matching was assessed using serological data for the HLA-A, HLA-B and HLA-DR loci in R-BM/PB or U-CB transplantations, and using allelic data for the HLA-A, HLA-B and HLA-DRB1 loci in U-BM transplantations.

Statistical analysis

The physicians who performed the transplantations at each center diagnosed and classified acute and chronic GVHD according to traditional criteria.[1,19] The reported type of chronic GVHD was reclassified according to the information on its organ involvement. 'Progressive onset' of chronic GVHD was defined as chronic GVHD transitioned from active acute GVHD, 'quiescent onset' as chronic GVHD after remission of acute GVHD and '*de novo* onset' as chronic GVHD without history or acute GVHD. The intensity of conditioning regimen was classified as myeloablative or reduced intensity on the basis of the Center for International Blood and Marrow Transplant Research report and the information from the questionnaire, as previously described.[20–23] We defined the following as standard-risk diseases: AML and ALL in first or second remission; CML in the first or

Table 1. Patient characteristics

Variable	*R-BM/PB*		*U-BM*		*U-CB*		P-*value*
	n = *1859*	%	n = *2215*	%	n = *744*	%	
Recipient age, years, median (range)	46 (16–74)		47 (16–73)		51 (16–82)		<0.001
Donor age, years, median (range)	43 (10–79)		35 (20–55)[a]		—	—	—
Recipient sex							
Female	789	42	916	41	334	45	0.238
Male	1070	58	1299	59	410	55	
Sex match between recipient and donor							
Match	965	52	1251	56	227	31	<0.001
Male to female	398	21	573	26	109	15	
Female to male	496	27	389	18	131	18	
Missing	0	0	2	0	277	37	
Disease							
AML	799	43	986	45	395	53	0.004
MDS	210	11	276	12	76	10	
CML	60	3	73	3	25	3	
ALL	385	21	439	20	123	17	
ATL	110	6	131	6	29	4	
NHL	206	11	214	10	70	9	
Other diseases	89	5	96	4	26	3	
Disease risk							
Standard	1058	57	1351	61	331	44	<0.001
High	724	39	780	35	390	52	
Missing	77	4	84	4	23	3	
Source of stem cells							
BM	842	45	2215	100	—	—	—
Peripheral blood	1017	55	—	—	—	—	
Cord blood	—	—	—	—	744	100	
HLA compatibility[b]							
Matched	1486	80	1507	68	53	7	<0.001
Mismatched	373	20	708	32	691	93	
Conditioning regimen							
Myeloablative	1202	65	1505	68	436	59	<0.001
Reduced intensity	649	35	696	31	308	41	
Missing	8	1	14	1	0	0	
GVHD prophylaxis							
CsA based	1367	74	469	21	311	42	<0.001
Tac based	449	24	1737	78	425	57	
Others/missing	43	2	9	1	8	1	
Use of in vivo *T-cell depletion*							
No	1741	94	2143	97	730	98	<0.001
Yes	118	6	72	3	14	2	
CMV Ab (recipient and donor)							
Both negative	127	7	150	7	151	20	<0.001
Either positive	1561	84	2003	90	535	72	
Unknown	171	9	62	3	58	8	
Acute GVHD							
Grade II–IV	665	36	897	41	338	45	<0.001
Grade III–IV	217	12	236	11	81	11	0.578
Follow-up of survivors (years), median (range)	2.0 (0.3–4.7)		1.9 (0.3–4.8)		1.7 (0.3–3.9)		<0.001

Abbreviations: ATL = adult T-cell leukemia; MDS = myelodysplastic syndrome; NHL = non-Hodgkin's lymphoma; R-BM/PB = related BM or PBSC; Tac = tacrolimus; U-BM = unrelated BM; U-CB = unrelated cord blood. [a]Data are missing in 20 patients [b]HLA matching was assessed by serological data for HLA-A, HLA-B and HLA-DR loci in transplantation using R-BM/PB or U-CB grafts, whereas it was assessed by allelic data for HLA-A, HLA-B and HLA-DRB1 loci in transplantation using U-BM grafts.

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Continuing increased risk of oral/esophageal cancer after allogeneic hematopoietic stem cell transplantation in adults in association with chronic graft-versus-host disease

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Background: The number of long-term survivors after hematopoietic stem cell transplantation (HSCT) showed steady increase in the past two decades. Second malignancies after HSCT are a devastating late complication. We analyzed the incidence of, risk compared with that in the general population, and risk factors for secondary solid cancers.

Patients and methods: Patients were 17 545 adult recipients of a first allogeneic stem cell transplantation between 1990 and 2007 in Japan. Risks of developing secondary solid tumors were compared with general population by using standard incidence ratios (SIRs).

Results: Two-hundred sixty-nine secondary solid cancers were identified. The cumulative incidence was 0.7% [95% confidence interval (CI), 0.6%–0.9%] at 5 years and 1.7% (95% CI, 1.4%–1.9%) at 10 years after transplant. The risk was significantly higher than that in the general population (SIR = 1.8, 95% CI, 1.5–2.0). Risk was higher for oral cancer (SIR = 15.7, 95% CI, 12.1–20.1), esophageal cancer (SIR = 8.5, 95% CI, 6.1–11.5), colon cancer (SIR = 1.9, 95% CI, 1.2–2.7), skin cancer (SIR = 7.2, 95% CI, 3.9–12.4), and brain/nervous system cancer (SIR = 4.1, 95% CI, 1.6–8.4). The risk of developing oral, esophageal, or skin cancer was higher at all times after 1-year post-transplant. Extensive-type chronic graft-versus-host disease (GVHD) was a significant risk factor for the development of all solid tumors (RR = 1.8, $P < 0.001$), as well as for oral (RR = 2.9, $P < 0.001$) and esophageal (RR = 5.3, $P < 0.001$) cancers. Limited-type chronic GVHD was an independent risk factor for skin cancers (RR = 5.8, $P = 0.016$).

Conclusion: Recipients of allogeneic HSCT had a significantly higher ~2-fold risk of developing secondary solid cancers than the general population. Lifelong screening for high-risk organ sites, especially oral or esophageal cancers, is important for recipients with active, or a history of, chronic GVHD.

Key words: secondary solid cancers, late effect, hematopoietic stem cell transplantation

introduction

Hematopoietic stem cell transplantation (HSCT) is a curative treatment of choice for malignant and non-malignant hematological disorders [1]. The annual number of allogeneic HSCT has increased steadily over the past three decades worldwide [2–6]. Progress in transplant procedures in addition to this steady increase in the number of HSCT procedures worldwide has contributed to an increase in the number of long-term survivors.

Secondary malignancies, including new solid cancers, are an important cause of late mortality. Several studies have reported that survivors of HSCT have a 2–3-fold increased risk of

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developing new solid cancers compared with an age-, sex-, region-, and calendar-year-adjusted population and the risk among long-term survivors ranges from 1% to 6% at 10 years after transplantation [7–14]. Identified risk factors include exposure to radiation as a part of the conditioning regimen and chronic graft-versus-host disease (GVHD), and the latter has been shown to be strongly correlated with the development of squamous cell carcinoma [8, 10, 12, 15–17]. However, a recent long-term follow-up analysis of patients who were transplanted after myeloablative doses of busulfan and cyclophosphamide without total body irradiation (TBI) found a similar increased incidence of 0.6% at 5 years and 1.2% at 10 years after transplantation [13]. We conducted a nationwide, retrospective cohort study with a large and different cohort from those used in previous reports from North America and Europe, to determine the incidence and risks of developing secondary solid cancers.

methods

data source and collection of data

The recipient clinical data were collected by the Japan Society for Hematopoietic Cell Transplantation (JSHCT) using the Transplant Registry Unified Management Program, as described previously [18]. The JSHCT collect recipients' baseline, disease, transplant, and transplant outcome information who received HSCT in the previous year. Patient information regarding survival, disease status, and long-term complications including chronic GVHD and second malignancies are renewed annually. This study was approved by the data management committee of the JSHCT, as well as the institutional review board of Nagoya University Graduate School of Medicine.

patients

Adult patients (at least 16 years of age) who received a first HSCT between 1990 and 2007 were considered as subjects for the present study. Those who were inherently susceptible to developing cancer [Fanconi anemia ($N = 3$) and congenital immunodeficiency ($N = 12$)] were excluded. Three-hundred five recipients (1.7%) were excluded because of insufficient follow-up data. The study included 17 545 recipients; 5358 recipients of related bone marrow, 3587 recipients of related peripheral blood stem cells (including 134 bone marrow and peripheral blood stem cells combined), 6508 recipients of unrelated bone marrow, and 2092 recipients of unrelated cord blood.

statistical analysis

Standard incidence ratios (SIRs) were calculated to determine whether the number of recipients in the present cohort who developed secondary solid tumor after receiving a HSCT was different than that in the general population (supplementary method, available at *Annals of Oncology* online). Cumulative incidences of solid cancer or GVHD were estimated by taking into account the competing risk of death among patients who did not develop a second malignancy or GVHD [19]. The influence of potential risk factors was estimated by using the Cox proportional hazard model [20]. A stepwise multivariate approach was used to identify the most important predictor with respect to the development of secondary solid cancers. The variables considered were age at transplant, patient sex, donor-type (related versus unrelated), graft source, TBI as part of the conditioning regimen, reduced-intensity conditioning, grade 2–4 acute GVHD, and chronic GVHD. The model was stratified into four categories according to the primary disease; acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, and others. Acute and chronic GVHD were considered as time-dependent covariates. TBI and chronic GVHD were frequent risk factors and were always kept in the model. Risk factors for high-risk cancer sites with adequate numbers of events for analyses were also analyzed: oral cavity/pharynx, esophagus, colon, and skin. The models for high-risk cancer sites were stratified according to the primary disease as described, and patient age at transplantation (<19, 20–29, 30–39, 40–49, 50–59, and >60), and also adjusted by patient age as a continuous variable. All P-values were two-sided.

results

patient and transplant characteristics

Table 1 shows the patient characteristics, their disease, and transplant regimens for 17 545 recipients of a first HSCT. The cumulative incidences of grade 2–4 acute GVHD at 150 days and chronic GVHD at 2 years post-transplant were 35% [95% confidence interval (CI), 35%–36%] and 41% (95% CI, 40%–41%), respectively. The observation period reached 69 465 person-years among the subjects for analyses. Of the 17 545 recipients, 5864 had survived for 5 or more years, and 2192 recipients had survived 10 or more years at the time of analysis (Table 2).

incidence and types of secondary solid cancers

The cumulative incidence of solid cancers was 0.7% (95% CI, 0.6–0.9) at 5 years, 1.7% (95% CI, 1.4–1.9) at 10 years, and 2.9% (95% CI, 2.5–3.4) at 15 years after transplantation (Figure 1). Two-hundred sixty-nine solid cancers were identified. Multiple solid cancers were observed in 11 patients. Nineteen recipients were diagnosed within 1-year post-transplantation (Table 2).

risk compared with the general population

HSCT recipients had a 1.8-fold higher risk of invasive solid cancers compared with the general population (95% CI, 1.5–2.0). SIR was significantly higher for cancers of the oral cavity/pharynx (SIR = 15.7), esophagus (SIR = 8.5), colon (SIR = 1.9), skin (SIR = 7.2), and brain/nervous system (SIR = 4.1; Table 2). The risks of developing secondary cancers of the oral cavity/pharynx, esophagus, and skin were significantly higher than those in the general population throughout all periods after 1 year (Figure 2). The risk for developing colon cancer was elevated during the period of 1–4 years (SIR = 2.7), whereas the risks for developing cancer of the pancreas (SIR = 4.5) were elevated during the period of 5–9 years. Recipients were at higher risk of developing cancers of the rectum (SIR = 3.6) and the brain/nervous system (SIR = 19.1) after 10 years post-transplantation. The risk of developing secondary solid cancers of all types compared with the general population increased with the time since transplantation. This trend was observed for oral/pharynx and esophageal cancer (Table 2; Figure 2).

recipients' age at transplantation and risks for developing secondary solid cancers

SIRs were also analyzed according to the recipient's age at transplantation (Table 3). Compared with the general population in Japan, the SIRs were significantly increased for all solid cancers, oral/pharynx, esophagus, liver, bronchus/lung, and brain/nervous system for recipients who were 16–19 years of age at transplant, all solid cancers, oral/pharynx, and esophagus for recipients who

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Histology and Histopathology

Cellular and Molecular Biology

Review

Ischemic culture of dental pulp-derived cells is a useful model in which to investigate mechanisms of post-ischemic tissue recovery

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Summary. Dental pulp is a soft tissue characterized by unique regenerative properties. It is located in the center of each tooth, and is surrounded by hard tissue (dentin). Vascular access is limited to a small foramen at the root apex. Because of this anatomical limitation, dental pulp can easily lose its blood supply, causing the tissue to become ischemic. This occurs, for example, when a tooth is dislocated by traumatic injury or is subjected to inflammation. Since ischemia is caused by a critical shortage of oxygen and nutrients, ischemic damage is usually irreversible, even when the ischemic event is transient. However, unlike ischemia-sensitive organs such as the brain and heart, dental pulp is relatively ischemia-resistant, and recovers from ischemic injury by regenerating damaged tissue. The mechanisms by which this regeneration occurs are poorly understood, but are being investigated in cell culture models that mimic *in vivo* ischemic conditions using a combination of hypoxia and nutrient deprivation. Here, we review the use of ischemic cell culture to investigate the mechanisms of post-ischemic dental pulp tissue recovery.

Key words: Ischemia, Dental pulp, Stem cells, Odontoblasts, Regeneration

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Introduction

Ischemia occurs when arterial blood flow to a tissue is restricted. Because arterial blood supplies oxygen and nutrients, ischemia is characterized by a lack of these essential elements (Shinzawa and Tsujimoto, 2003). The intensity of tissue damage depends on the length and severity of the ischemic event. Although direct comparison is difficult, tolerance to ischemia appears to differ among various tissues and organs. For example, brain tissue is highly vulnerable to ischemia, and often becomes necrotic even when only transiently exposed to ischemic conditions (Sugiyama et al., 2011). In contrast, dental pulp is relatively tolerant to ischemia and is able to survive transient ischemic events such as tooth extraction and replantation (Tsukamoto-Tanaka et al., 2006). Dental pulp recovers its functions by regenerating damaged tissue after ischemia exposure. This unique response suggests that dental pulp contains ischemia-tolerant cells that play important roles in post-ischemic tissue regeneration.

Dental pulp contains a postnatal stem cell population named dental pulp stem cells (DPSCs) (Gronthos et al., 2000). DPSCs possess multi-lineage differentiation abilities (odontogenic, osteogenic, chondrogenic, adipogenic and neurogenic lineage), and are considered potent stem cells for use in tissue engineering and regenerative medicine (Gronthos et al., 2002; Iohara et al., 2006). Although the characteristics and functions of DPSCs within the pulp remain largely unknown, these cells appear to play an important role in tissue development, homeostasis and regeneration and are a

particularly interesting target for investigations into the mechanisms of post-ischemic tissue regeneration.

Evaluating dental pulp tissue reactions during and after ischemia would ideally be undertaken *in vivo*, but this is experimentally difficult due to the anatomic location of the tissue and its complex cellular composition (Liu et al., 2006). Thus, *in vitro* ischemic culture of dental pulp-derived cells has been developed as an alternative experimental model, though studies differ in the culture conditions utilized (Agata et al., 2008; Wang et al., 2010). In this review, we discuss *in vitro* cell culture conditions that best approximate *in vivo* ischemia. Next, we evaluate the relevance of these conditions in dental pulp-derived cell culture. Finally, we examine the characteristics of dental pulp-derived cells that survive ischemic culture conditions and explore possible mechanisms of post-ischemic pulp tissue regeneration.

Approximating *in vivo* ischemic conditions in experimental cell culture systems

Under ischemic conditions, cells experience both low oxygen tension and nutrient deprivation. Hence, ischemia can be mimicked *in vitro* by exposing cells to both hypoxia and a low-glucose environment (Jones et al., 2011). These appear to be the two most influential factors for tissue survival (Acosta et al., 1978). When PC12 cells (derived from a pheochromocytoma and able to differentiate into neurons) are cultured under hypoxic conditions and in a low-glucose environment, they are severely damaged, often to the point of necrosis (Shinzawa and Tsujimoto, 2003). Unfortunately, most of the current literature on ischemic culture of dental pulp-derived cell or DPSCs is limited to investigation of the effect of low oxygen tension alone; the number of studies using both low oxygen tension and nutrient deprivation is limited (Agata et al., 2008; Wang et al., 2010) (Table 1). In fact, low nutrient supply may enhance the effect of low oxygen tension. It has been shown that caspase-independent cell death, which is commonly seen under ischemic conditions, is significantly upregulated when cells are deprived of both oxygen and glucose (Agata et al., 2008).

Another important consideration when developing *in vitro* models of ischemia is the level of hypoxia used in the experiments. Conventional cell culture experiments use approximately 20% oxygen, with a partial pressure of oxygen (pO_2) of 140 mmHg (Rodrigues et al., 2010). However, pO_2 in the arterial blood of normal human subjects ranges from 60-90 mmHg and pO_2 in bone marrow is even lower (47-49 mmHg). The discrepancy between *in vitro* and *in vivo* conditions suggests that conventional cell culture may occur in a relatively hyperoxic environment, while traditional "hypoxic" culture environments actually reproduce normal physiologic conditions. This may explain why mesechymal stem cells grow and survive better in low oxygen cell culture environments. Mesenchymal stem cells cultured under low oxygen tension (5%) have a greater number of colonies as primary isolates, proliferate more rapidly and produce more bone (Lennon et al., 2001). In fact, "hypoxic" cell culture conditions can increase proliferation rates and enhance differentiation along multiple mesenchymal lineages (Das et al., 2010), providing further evidence that the

Table 1. Effect of hypoxia on DPSC culture.

Species	Oxygen tension	culture conditions	proliferation (compared to 20% O2)	cell properties (compared to 20% O2)	References
Human	1%	Monolayer culture for 24 hours	Cell proliferaton ↑	HIF-1α↑, CXCR4↑, SDF1↓	Gong et al. 2010
Human	1%	Monolayer culture for 24 hours /Endothelial cells culture in conditioned medium from hypoxic pulp cells for 72 hours	Endothelial cell proliferation ↑	HIF-1α↑, VEGF↑, bFGF→	Aranha et al. 2010
Human	2%	Monolayer culture for 24 or 48 hours	Cell proliferation ↓	SP cells↑, ABCG2↑, Oct4↑	Wang et al. 2010
Human	2%	Monolayer culture for 24 hours after80% confluent in normoxic conditions	none	Erythropoietin↑, Erythropoinetinreceptor↑	Gong et al. 2010
Human	3%	Monolayer culture for 14 days	Cell proliferation ↑	CD133↓, STRO-1↑	Sakdee et al. 2009
Human	3%	Monolayer culture with or without osteogenic supplements for 14 days	Cell proliferation ↑	STRO-1↑, osteogenic differetiation ↓	Iida et al. 2010
Human	5%	Monolayer culture with or without osteogenic supplements for 21 days	viability ↑	OCN↑, DMP1↑, BSP↑, DSPP↑ von kossa, alizarin↑(at 21 days of culture)	Li et al. 2011
Porcine	0.1% or 5%	Monolayer culture with or without osteogenic supplements under various glucose concentrations for 24 hours /Re-oxygenation after 24 hours ano/hypoxic culture + 3 day normoxic culture	Cell proliferation; Hypoxia ↑, Anoxia ↓	Oct4 ↑, Sox2↑ (at 6 hours in non-induced cells), ALP activity↑ (at 7 days after re-oxygen)	Agata et al. 2008

HIF-1α, hypoxia-inducible factor-1α; CXCR4, CXC chemokine receptor 4; SDF1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; bFGF, basicfibroblast growth factor; SP cells, side population cells; ABCG2, ATP-binding cassette sub-family G member 2; Oct4, octamer binding transcription factor 4; OCN,osteocalcin; DMP1, dentin matrix acidic phosphoprotein 1; BSP, bone sialoprotein; DSPP, dentin sialoprotein; Sox2, SRY-box 2; ↑, increas; ↓, decrease; →, unchanged

oxygen levels in traditional hypoxic cell culture experiments may not accurately reflect *in vitro* ischemic environments. The oxygen tension within dental pulp tissue *in vivo* is difficult to measure directly. However, it is possible that the oxygen range commonly used in "hypoxic" cell culture experiments (2-5%) may not be an accurate approximation of *in vivo* conditions, and very low oxygen tension (less than 1%) might be necessary (Agata et al., 2008).

Experimental ischemia in dental pulp-derived cell culture

Several studies have demonstrated that low oxygen tension (2-5%) promotes proliferation of dental pulp-derived cells (Amemiya et al., 2003; Sakdee et al., 2007; Iida et al., 2010; Li et al., 2011). This finding may reflect the fact that dental pulp-derived cells are exposed to relatively low oxygen tension within their normal physiologic environment. When these cells are exposed to both hypoxia (2%) and serum deprivation for 24 or 48 hours, a condition that mimics *in vivo* ischemia, proliferation rates decline (Wang et al., 2010). Cells survive in this environment even though proliferation rates decline, suggesting a complex cellular defensive response to ischemia. This response includes suppression of cell growth and induction of cellular defense systems, including upregulation of hypoxia-inducible factor 1α (HIF-1α), heat shock protein 70 (HSP 70), and AMP-activated protein kinase (AMPK) (Amemiya et al., 2003; Fukuyama et al., 2007; Agata et al., 2008; Aranha et al., 2010). These factors may even activate cell growth after the ischemic event has resolved (Ueno et al., 2006; Fukuyama et al., 2007). This complex response to hypoxia and nutrient deprivation reflects a balance between damage due to noxious stimuli and activation of cellular defense systems.

The effect of low oxygen tension on the differentiation capacity of dental pulp-derived cells is unclear. After being cultured for 14 days in 5% O_2, dental pulp-derived cells increase expression of osteonectin (ON), dentin matrix protein-1 (DMP-1),

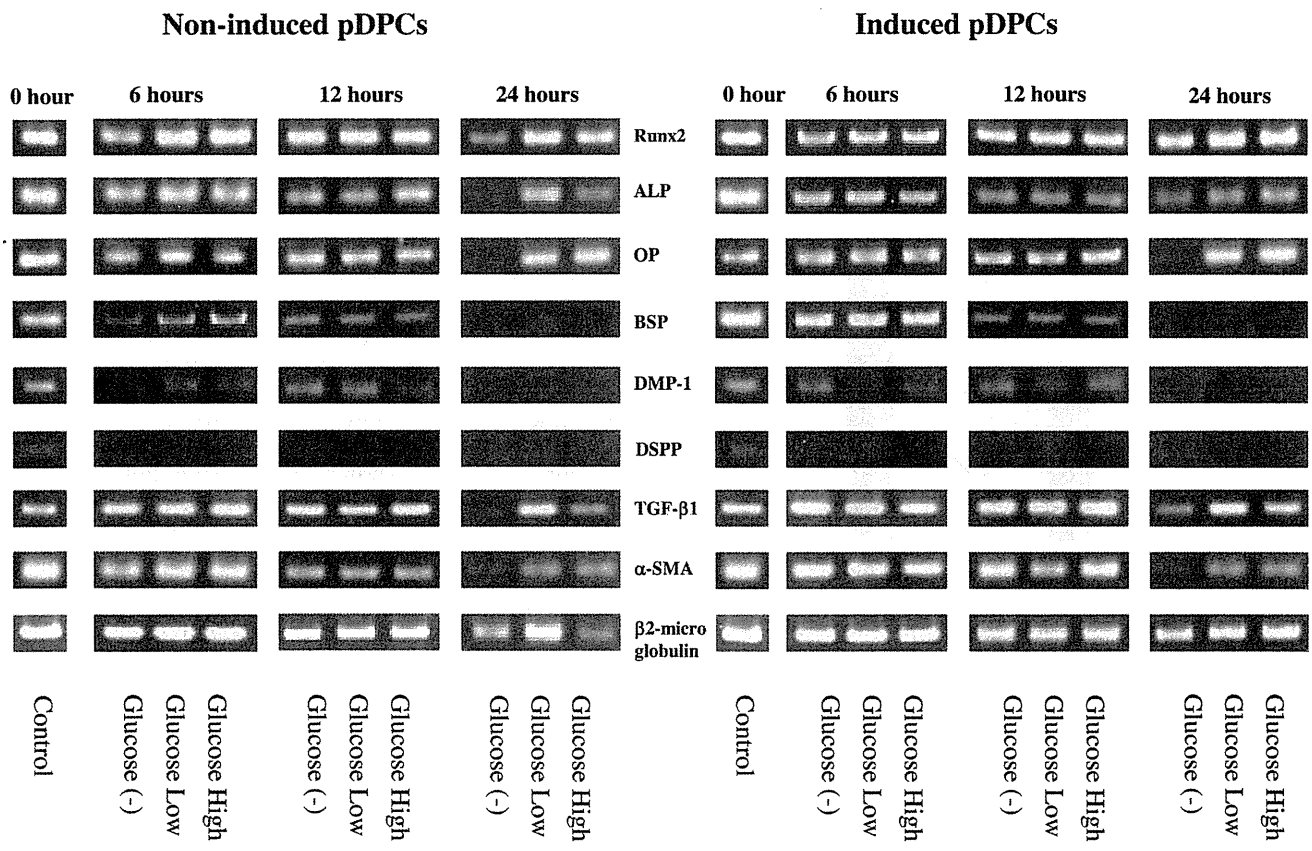

Fig. 1. Changes in the expression of odontogenic/osteogenic genes in non-induced and induced pDPCs cultured under severe-hypoxic conditions. Induced and non-induced pDPCs were cultured under severe hypoxic conditions in either low or high glucose concentration. Gene expression was analyzed by RT-PCR at 0, 6, 12 and 24 hours. Expression of Runx2, ALP, OP, TGFß1, and α-SMA was slightly inhibited in both non-induced and induced cells. The expression of BSP, DMP-1, and DSPP was strongly inhibited in both populations. Non-induced and induced pDPCs underwent de-differentiation in a time-dependent manner. (From Agata et al., 2008 with modification).

bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP), and after 21 days significantly greater calcified nodule formation is observed. Both of these factors appear to promote differentiation (Li et al., 2011). Canine dental pulp-derived cells show decreased alkaline phosphatase activity (ALP, an early marker of osteogenic (odontogenic) differentiation) after 4 days in both hypoxic (2% O_2) and normoxic culture conditions, though ALP activity in the hypoxic cells remained higher than in the normoxic cells (Amemiya et al., 2004). Other studies have reported that hypoxia has an inhibitory effect on differentiation of dental pulp-derived cells. For example, we have demonstrated that porcine dental pulp-derived cells have significantly lower expression of BSP, DMP-1, and DSPP in both differentiation-induced and non-induced cells regardless of the severity of ischemia , though expression of other marker genes is not significantly different when cells are grown in glucose-containing medium (Fig. 1). Additionally, when human dental pulp cells are cultured under 3% O_2 for 14 days, ALP activity and the expression of DMP1, DSPP and osteocalcin are suppressed (Iida et al., 2010).

Investigations into the effect of low oxygen tension on the ability of dental pulp-derived cells to differentiate differ in terms of the species from which the cells were isolated and the conditions under which the cells were cultured (i.e. level and duration of hypoxia, and the status of cell differentiation), making direct comparisons difficult. However, accumulating evidence indicates that ischemic conditions do affect the differentiation of dental pulp-derived cells, and the type and magnitude of the effect may correlate with the severity of ischemia. Detailed cellular analyses are required to understand the mechanisms underlying this phenomenon.

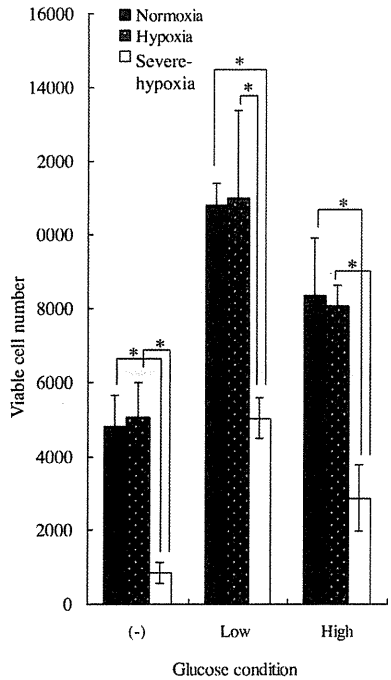

Fig. 2. Viability of porcine dental pulp-derived cells (pDPCs) in cell culture depends on ischemic conditions. To investigate the effect of differential ischemic conditions, pDPCs were cultured under a range of O_2 concentrations (normoxia, hypoxia, or severe-hypoxia) and glucose concentrations (high glucose, low glucose, or glucose (-)) in serum-free media. Viable cell number was calculated as the percentage of surviving cells. Significantly more cells survived under normoxic and hypoxic conditions than under severe-hypoxic conditions. No significant difference was observed between normoxic and hypoxic conditions. pDPCs cultured in low glucose media were significantly more viable than cells cultured in high glucose or glucose (-) media. Error bars represent the mean ± standard deviation for six separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

Characteristics of dental pulp-derived cells that survive under ischemic cell culture conditions.

Dental pulp tissue often recovers its function after an ischemic event, and ischemia-surviving cells likely contribute to tissue recovery. As previously discussed, traditional low oxygen culture conditions may not accurately reflect hypoxic environments *in vivo*. Accordingly, very low oxygen tension (<0.1% O_2, or even anoxia) is required for culture conditions to truly approximate physiologic environments (Fig. 2) (Agata et al., 2008). Furthermore, these cultures may require "ischemic conditions", which involves not only very low oxygen tension but also nutrient deprivation (Agata et al., 2008; Wang et al., 2010).

Investigations into the mechanisms by which dental pulp-derived cells recover from ischemia have focused on determining whether cellular differentiation affects survival. However, the number of experiments performed with very low oxygen tension (hypoxia) or ischemic conditions (hypoxia plus nutrient deprivation) is limited. We have shown that differentiation-induced and non-induced cells (possibly stem/progenitor cells) survive at equivalent rates under a range of ischemic conditions. Hence ischemia-tolerance is comparable between differentiated and non-differentiated cells (Agata et al., 2008). Despite these similarities, differences between the two cell populations do exist. Upregulation of the pluripotent stem cell markers octamer-binding transcription factor 4 (Oct4) and Sox2 is observed only in non-induced cells under ischemic conditions (Fig. 3). This finding suggests that ischemia-surviving non-odontogenic cells (undifferentiated cells) may be able to de-differentiate, acquiring greater growth and differentiation potential for post-ischemia tissue regeneration. In contrast, de-differentiation of ischemia-surviving odontogenic (differentiated) cells may allow them to re-acquire mitotic potential (pulp-resident odontoblasts are post-mitotic cells that are not able to divide or repair damaged dentin) (Liu et al., 2006).

Hence cells that survive ischemic insult may de-differentiate under ischemic conditions and obtain more stem cell-like characteristics, thereby contributing to the regeneration of damaged tissue.

Insights into the mechanisms of dental pulp tissue recovery after ischemic damage

Cellular damage caused by ischemia is generally irreversible even when the ischemic insult is transient. Dental pulp tissue can recover its functions after transient ischemia, suggesting that this tissue may undergo cellular regeneration rather than cellular recovery. The physiologic function of dental pulp (protection, nutrition, and tooth sensation) are supported by multiple cell types, hence post-ischemic pulp recovery has previously been attributed to multiple cell populations (Sloan and Smith, 2007). However, with the discovery of dental pulp stem cells (DPSCs) which are able to differentiate into multiple cell lineages (angiogenic, vasculogenic, neurogenic, chondrogenic, and osteo/odontogenic) a new paradigm has emerged in which a single stem cell population may be all that is required for pulp tissue recovery. Thus, it is of interest to investigate whether the post-ischemic recovery of pulp function is mediated solely by DPSCs.

One of the most important functions of dental pulp is to protect the tooth from noxious stimuli through dentin formation. Reparative dentin formation is frequently observed in teeth that survive ischemic insults (Spahr et al., 2002). The cell types responsible for this restorative process are unknown. Dental pulp contains terminally differentiated odontoblasts, but these cells are considered post-mitotic and thus not able to divide and form dentin (Liu et al., 2006). Additionally, odontoblasts may become necrotic under ischemic conditions. This has led to the assumption that reparative dentin formation following an ischemic event might be solely mediated by DPSCs (though other functions appear to be recovered by multiple cell populations) (About and Mitsiadis, 2001; Liu et al., 2006). However, as stated above, results from ischemic culture of induced dental pulp cells

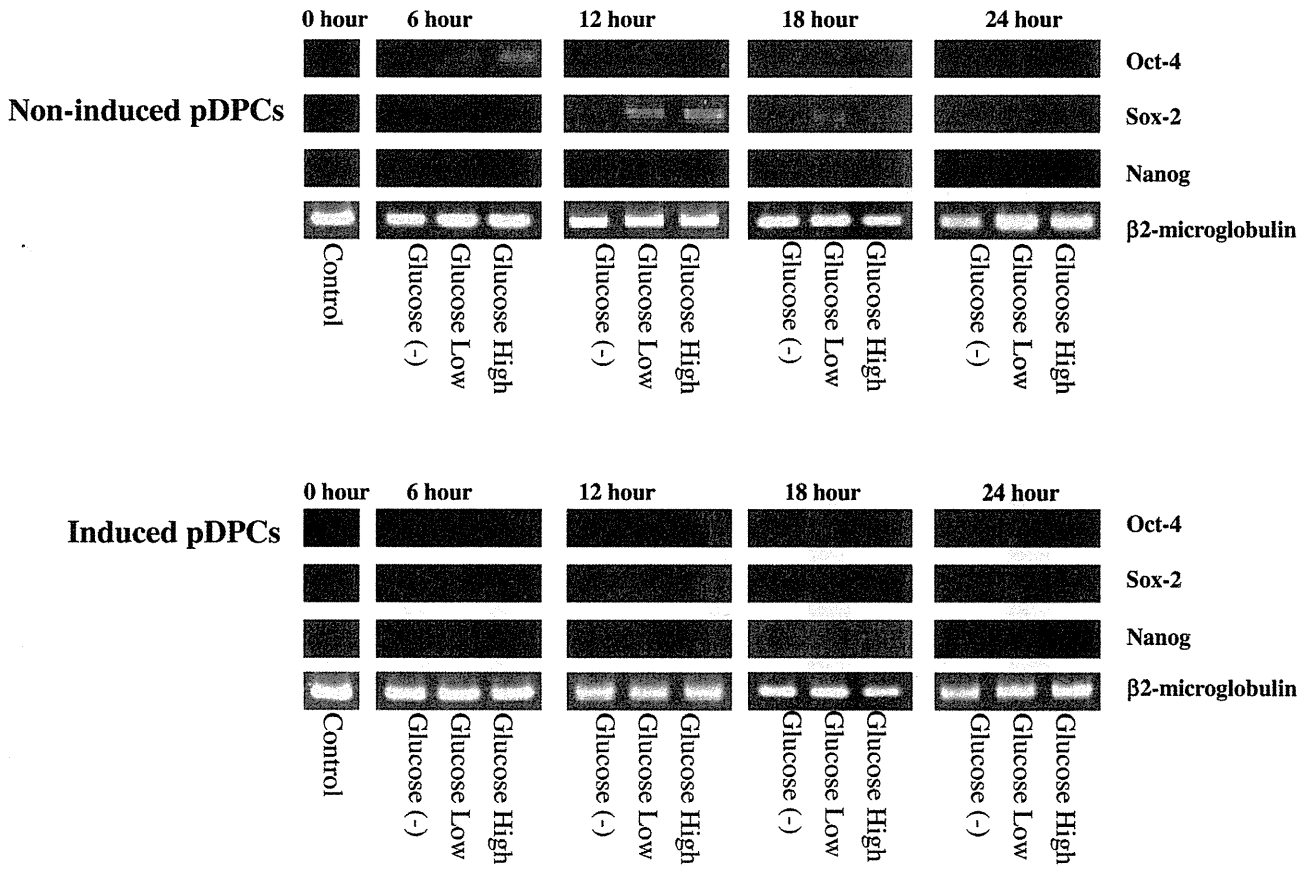

Fig. 3. Expression of pluripotent stem cell markers in induced and non-induced pDPCs cultured under severe-hypoxic conditions. RNA from induced and non-induced pDPCs cultured under severe-hypoxic conditions was extracted at 0, 6, 12, 18, 24 hours. Upregulation of the pluripotent stem cell markers Oct-4 and Sox-2 was observed only in non-induced pDPCs. Oct-4 was transiently activated at 6 hours, followed by the activation of Sox-2 from 12 to 18 hours. Nanog expression was not observed under any conditions. (From Agata et al., 2008 with modification).

(odontoblast-like cells) indicate that resident committed odontoblasts/precursors may de-differentiate and re-acquire mitotic potential during ischemia (Fig. 1). Therefore, post-ischemic reparative dentin formation may in fact be mediated by both DPSCs and de-differentiated odontoblasts, though further investigation is required to confirm this. If pulp-resident odontoblasts do contribute to post-ischemic reparative dentin formation, their ability to form dentin may be lower than that of DPSCs, because ALP activity of de-differentiated cells is lower in induced cells than in non-induced cells (which possibly contain DPSCs) (Fig. 4A,B).

Investigations into the post-ischemic recovery of other physiologic functions of dental pulp tissue are rare (e.g. nutrition and tooth sensation), but studies in other tissues demonstrate reparative roles for multiple cell types, including endothelial and neural cells (Sheridan and Bonventre, 2000; Bernert et al., 2003). Thus a more complete understanding of the mechanism of post-ischemic pulp tissue recovery should include analysis of endothelial and neural cell populations residing in dental pulp tissue. Finally, angiogenic factors, such as vascular endothelial growth factor (VEGF), are induced under hypoxic conditions, which may support the durability of dental pulp during hypoxia *in vivo* (Amemiya et al., 2003; Aranha et al., 2010).

Conclusion

It has long been known that dental pulp recovers its function after transient ischemia, but the mechanisms underlying this phenomenon have not been fully investigated, in part because *in vivo* monitoring of this tissue is anatomically difficult. Cell culture is emerging as a useful model for examination of cellular responses to ischemia and may become a valuable approach for investigation into the mechanisms of post-ischemic pulp tissue recovery.

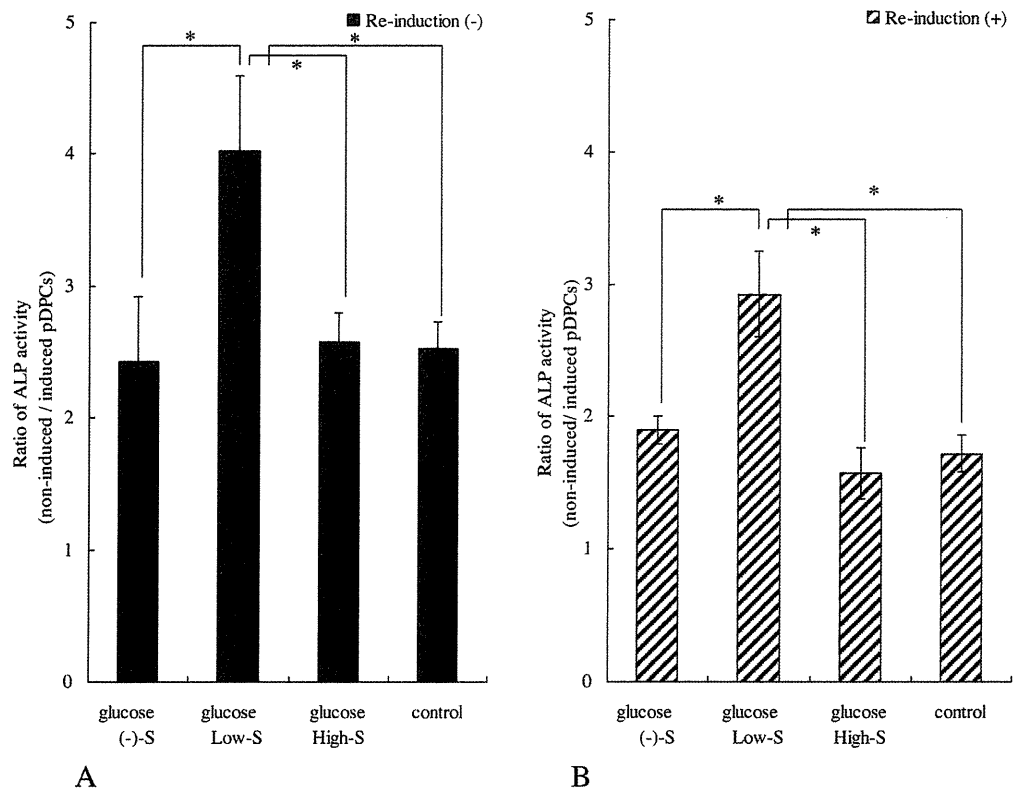

Fig. 4. Differentiation potential of odontogenic cells after ischemic de-differentiation. Induced and non-induced pDPCs were cultured in serum-free D-MEM at various glucose concentrations and exposed to severe-hypoxia for 24 hours. Surviving cells were then "re"-differentiated. Controls consisted of induced and non-induced pDPCs, respectively, cultured in serum containing D-MEM with high glucose under normoxia. ALP activity after "re"-differentiation was measured and compared with that before "re"-differentiation in both non-induced pDPCs groups (re-induction (-), **A**) and induced pDPCs groups (re-induction (+), **B**). Although ALP activity was increased after "re"-differentiation in both groups, the differentiation plasticity was observed to be higher among non-induced pDPCs groups (re-induction (-), **A**) than that of induced pDPCs groups (re-induction (+), **B**). S: Severe-hypoxia. Error bars represent the mean ± standard deviation for three separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

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ORIGINAL ARTICLE

GDFs promote tenogenic characteristics on human periodontal ligament-derived cells in culture at late passages

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Abstract

Tendon/ligament injures are leading disabilities worldwide. The periodontal ligament (PDL) connects teeth to bone, and is comparable to a tendon/ligament-to-bone insertion. PDL-derived cells (PDLCs) express both osteo/cementogenesis and teno/ligamentogenesis genes. However, an efficient method to induce a tenogenic differentiation of PDLCs has not been thoroughly examined. Therefore, this study tested if growth/differentiation factors (GDFs) enhanced tenogenic characteristics of human PDLCs, as a potential cell source for tendon/ligament engineering. Results demonstrated recombinant GDF-5/GDF-7 inhibited alkaline phosphatase (ALP) activity of PDLCs from passage 3 to 6, while GDF-5 enhanced ALP in dental pulp-derived cells and mesenchymal stem cells. GDF-5 (particularly at 10 ng/ml concentration) induced high expression of both early (*scleraxis*) and mature (*tenomodulin, aggrecan, collagen3*) tenogenic genes in P4-6 PDLCs, while inhibiting expression of specific transcription-factors for osteogenic, chondrogenic and adipogenic differentiation. Exogenous GDFs might lead PDLCs being expanded in culture during several passages to highly useful cell source for tendon/ligament engineering.

Keywords

Growth differential factors, periodontal ligament cells, tenocyte differentiation, tissue engineering

History

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Introduction

Tendon and ligament injuries are leading disabilities for athletes and the active working population (Rodrigues et al., 2013). There is a sizeable economical burden associated with these medical injuries. Researchers in tissue engineering strive to cure these injuries by testing three critical components, which are the cell source, carrier vehicle (scaffold), and bioactive molecules (growth factors) for the repair/regeneration of tendons and ligaments (Gott et al., 2011). Our group has been working on obtaining an easily accessible autologous cell source for the engineering of tendon/ligament (Inoue et al., 2012). The periodontal ligament (PDL) is the tissue that connects teeth to bone, and can be compared to a tendon/ligament-to-bone insertion (Smith et al., 2012). Native periodontal ligament-derived cells (PDLCs) express both hard tissues (osteo- and cemento-genesis) and soft tissues (teno- and ligamento-genesis) genes. These cells can be easily obtained from common teeth removal procedures (such as wisdom teeth or for orthodontic purposes) and can be expanded *in vitro*. in large quantities like tenocytes and dermal fibroblasts (the currently preferred and accessible cell source for engineering tendons). Current research on using PDLCs as a cell source has mainly focused on bone tissue engineering (Cabral et al., 2007; Chung et al., 2009; Hiraga et al., 2009; Ikeda et al., 2011; Saito et al., 2002; Seo et al., 2004; Shi et al., 2005) because PDLCs can be coaxed into mineralized tissues like the cementum and alveolar bone, with growth factors such as bone morphogenetic protein-2 (BMP-2) (Lin et al., 2013; Zhao et al., 2002). However, little is known about the bioactive molecules that can induce/enhance the tenogenic characteristics of PDLCs. In other words, while many studies induced the ostegogenic characteristics of PDLCs *in vitro* and *in vivo*, their induction into a tenogenic phenotype has not been well examined.

Growth/differentiation factors (GDFs) are a subfamily of the BMPs involved in skeletal and extra-skeletal tissues development (Chan et al., 1994; Merino et al., 1999; Storm et al., 1994; Storm & Kingsley, 1999; Thomas et al., 1996). GDFs, such as GDF-5, -6 and -7, are essential for normal skeletal development of the limb, including cartilage and joints (Erlacher et al., 1998; Faiyaz-Ul-Haque et al., 2002; Nakase et al., 2002; Settle et al., 2003; Storm & Kingsley, 1996). In addition to the osteo-inductive ability of traditional BMPs, the GDFs induce neo-tendon/ligament formation when implanted at ectopic sites *in vivo* (Wolfman et al., 1997).

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Therefore, GDFs have been considered as a marker for tendon/ligament tissues (Yoki et al., 2007). Indeed, numerous studies have demonstrated that GDFs can be used to enhance tendon and ligament regeneration in several animal models using different cell types and delivery materials (Bolt et al., 2007; Dines et al., 2007; Forslund et al., 2003; Park et al., 2010; Tan et al., 2012; Wolfman et al., 1997).

Expression of GDF-5, -6 and -7 mRNAs was detected during periodontal tissue formation (Morotome et al., 1998). Cells (expressing GDFs) were localized along the insertion sites of the periodontal ligament to the alveolar bone or cementum surfaces during root formation. GDFs expression was down-regulated in these cells after completion of root formation (Sena et al., 2003). Recently, our group demonstrated that GDF-5 also regulated differentiation of other cell types during (porcine) tooth development (such as dental papilla and dental follicle cells) and might affect differentiation co-operatively with other growth factors such as BMP-2 (Sumita et al., 2010). Meanwhile, rhGDF-7 was shown to form a functionally oriented periodontal ligament between newly formed bone and cementum (Miyoshi, 2002). In addition, recombinant human (rh) GDF-5 was demonstrated to enhance the proliferation of cultured human PDLCs and reduced alkaline phosphatase activity (ALP is a marker of osteoblastic activity) (Nakamura et al., 2003). Then, our group reported that recombinant mouse (rm) GDF-5 induced the expression of *scleraxis*, a crucial transcription factor of tenocytic differentiation, in cultured human PDLCs (Inoue et al., 2012). Taken together, these data suggest that GDFs can regulate periodontal ligament formation and that PDLCs could be a potential cell source for tendon engineering. However, there are selected studies reporting that GDFs induced osteo/cementogenesis in periodontal defects (Kim et al., 2009; Kwon et al., 2010; Lee et al., 2010; Stavropoulos et al., 2009).

Thus, more data are required to characterize GDFs' action on cultured PDLCs. During our work on bone engineering using PDLCs, we unexpectedly discovered that the expressions of osteogenic- and mature tenogenic-genes in PDLCs were severely decreased after passage 3 (P3) (Itaya et al., 2009). However, expression of *scleraxis* mRNA (a marker for early tendon formation/genesis) persisted in cultured PDLCs even after passage 6 (P6). This finding led us to believe that PDLCs could be induced toward a tenogenic differentiation at this specific "time window" (i.e. at passage 3 and higher). However, an efficient method to induce tenogenic differentiation on cultured PDLCs for the goal of tendon/ligament tissue regeneration has not been reported yet. Therefore, the aim of this study was to make progress towards establishing suitable cell culture conditions for the induction of PDLCs into tenocyte-like cells. Here, we analyzed the capacity of recombinant GDFs to enhance or to maintain tenogenic characteristics of human PDLCs being expanded in culture during several passages (P3 to P6) for potential use as a cell source for tendon/ligament engineering.

Materials and methods

Preparation of PDLCs

This study conformed to the tenets of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Capital Medical University School of Stomatology, Nagasaki University, and McGill University. Informed consent was obtained from each subject prior to donation of the tissue.

Normal healthy erupted third molars or premolars extracted for orthodontic reasons were collected from five patients (ages 18, 19, 21, 24 and 30 years; two males and three females). Each tooth sample was rinsed twice in a phosphate-buffered saline (PBS) solution containing 1000 units/ml of penicillin G sodium, 1 mg/ml streptomycin sulfate, and 2.5 μg/ml of amphotericin B (Invitrogen, Carlsbad, CA) for 5 min at room temperature (RT). Periodontal ligament (PDL) tissue was mechanically removed from the root surface by a scalpel. To avoid contamination by the gingival and apical regions, this coronal and apical PDL tissue was discarded. Tissue was digested with 2 mg/ml collagenase (Worthington Biochem, Freehold, NJ) for 1 h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Thermo Trace Ltd, Melbourne, Australia) and antibiotic-antimycotic solution on a shaker at 37 °C. A single cell suspension was obtained by filtering the cell suspension through a 70 μm strainer (Falcon, BD labware, Franklin Lakes, NJ). Then, after centrifugation for 5 min at 440 *g*, the cell pellet was re-suspended in culture medium (DMEM containing 10% FBS and antibiotic-antimycotic solution). Isolated cells were seeded into 10-cm culture dishes, and then incubated at 37 °C in 5% CO_2. When cultured cells reached 80–90% confluence, they were trypsinized and sub-cultured (1×10^5 cells in a 10-cm dish) until passage 2. Experiments in this study used cells at passage 3 (P3, referred previously by our group as cells in "early passages", P1 to P3; Itaya et al., 2009) and passages 4–6 (P4-6; referred as "late passages"; Itaya et al., 2009). Similarly, human dental pulp-derived cells (DPCs) and mesenchymal stem-cells (MSCs) were cultured until passage 3 as controls. DPCs and PDLCs were harvested from the same teeth of patients, and MSCs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD).

Characteristic analysis of PDLCs

Flow-cytometry experiments used a FACscan argon laser cytometer (BD, San Jose, CA). Cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in ice-cold 2% formaldehyde. The fixed cells were washed in flow-cytometry buffer (PBS, 2% FBS, 0.2% Tween-20) and incubated for 30 min in flow-cytometry buffer containing fluorescein isothiocyanate-conjugated monoclonal antibodies to Stro-1 (R&D, Minneapolis, MN) and antigens CD34, CD45, CD90, CD105, CD146 and CD271 (Becton, Mountain View, CA).

To confirm the presence of Vimentin and Stro-1 proteins in PDLCs, immunofluorescence-cytostaining was performed. P5 PDLCs were cultured in a chamber slide until 70% confluent. The slides were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min. After incubation for 30 min with PBS containing 5% donkey (Jackson ImmunoResearch, Baltimore Pike, PA) or 5% goat (Vector Laboratories, Burlingame, CA) serum albumin as a blocking reagent and 0.25% Triton X-100, cells were incubated with mouse monoclonal anti-human Vimentin (1:50) (Dako, Via Real

Carpinteria, CA) or mouse monoclonal anti-human Stro-1 (1:400) (Millipore, Temecula, CA) for 2 h at RT. The slides were then washed with PBS and incubated for 30 min at RT with FITC-conjugated donkey anti-mouse secondary antibody (1:200) (Jackson ImmunoResearch, Baltimore Pike, PA) and RHODAMINE-conjugated goat anti-mouse secondary antibody (1:100) (Millipore, Temecula, CA). After washing three times with PBS, slides were covered with a mounting reagent (Vector Laboratories, Burlingame, CA).

Treatments with GDF-5 and GDF-7

PDLCs at P3-P6 were seeded at concentration of 1×10^5 cells/dish into 10-cm dishes and were cultured in DMEM culture medium for 24 h. Then, the medium was replaced with DMEM supplemented with 1% FBS in the presence of recombinant mouse (rm) GDF-5 (R&D Systems, Minneapolis, MN) and/or recombinant human (rh) GDF-7 (BioVision, Mountain View, CA) at different concentrations (0, 10, 100 or 1000 ng/ml). These concentrations were chosen based on our preliminary experiments aiming to determine a range of GDFs concentrations that would reduce ALP activity of PDLCs in a dose-dependent manner (data not shown). Data reported in this paper were from GDFs at 10 or 100 ng/ml concentrations, and these concentrations were also chosen based on previous works by Park et al. (2010) and Inoue et al. (2012).

Total cell number and ALP activity analyses

Cell proliferation was measured using WST-8 kit (Dojindo, Kumamoto, Japan) according to manufacturer's protocol. Briefly, cells were incubated with medium containing 100 µl/ml of WST-8 for 1 h. The absorbance was read by a spectrophotometer at 450 nm (SmartSpeck™3000, BIO-RAD, Hercules, CA). ALP activities were measured according to the method of Lowry (1955). An aliquot of supernatant was added to p-nitrophenylphosphate containing $MgCl_2$ (Sigma-Aldrich, St. Louis, MO) and the mixture was incubated at 37 °C for 15 min. 0.2 N NaOH was added to stop the enzymatic reaction and absorbance was read at 415 nm with a spectrophotometer. ALP activity was expressed as µmol p-nitrophenol/cell. Cell proliferation and ALP activity were evaluated at 7 and 14 d after incubation with 0 and 100 ng/ml GDF-5 and/or GDF-7. Each experiment was performed in triplicate for three samples.

For ALP staining, GDF-5 treated PDLCs at day 14 of culture were fixed with 4% paraformaldehyde and stained with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue in an ALP kit, according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Signals were normalized based on protein concentrations (Fan et al., 2009).

Reverse transcription-polymerase chain reaction and quantitative PCR

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine the expression of tenogenic marker genes in P4 PDLCs cultured with GDF-5 and/or -7 (at 0, 10 and 100 ng/ml) for 7 d. Total cellular RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 µg of total RNA by SuperScript First-strand Synthesis (Invitrogen) according to the manufacturer's protocol. cDNA was amplified with Takara Taq (Takara Bio Inc., Shiga, Japan) and human-specific primer (*scleraxis, tenomodulin biglycan, collagen1, collagen3, gapdh*) sets are shown in the Appendix Table (online version only). Samples were incubated in a Thermal Cycler GP (Takara Bio Inc.) at 95 °C/(2 min) for 1 cycle and then 95 °C/(60 s), 56 °C/(60 s), and 72 °C/(60 s) for 20 cycles, with a final 5 min extension at 72 °C. After amplification, 10 µl of each product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Furthermore, the effect of GDF-5 on the expression of tenogenic, osteogenic, chondrogenic and adipogenic mRNAs in P4-P6 PDLCs at 7, 14 and 21 d was evaluated by real-time fluorescent quantitative PCR. Real-time PCR reactions were performed with the QuantiTect SYBER Green PCR kit (Quagen, Hilden, Germany) and Icycler iQ Multi-color Real-time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA).The PCR consisted of an initial enzyme activation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Human-specific primer (*scleraxis, aggrecan, collagen3, tenomodulin, runx2, sox9, pparγ*) sets used in this experiment are shown in the Appendix Table (online version only).

Statistics

One-way repeated measures ANOVA were used for the measurements of cell proliferation, ALP activity and quantitative gene expressions to detect any significant difference within each group. When a significant difference was detected, the differences among any selected groups were confirmed using Dunnett's test. Experimental values were presented as mean ± s.d. A p value of <0.05 was considered to be statistically significant.

Results

Characteristics of cultured PDLCs

PDLCs cultured at passage 4 had a characteristic spindle shape and formed a monolayer (Figure 1A). Immunofluorescence analysis revealed that human anti-Vimentin (mesenchymal cell marker) antibody reacted with cultured PDLCs (Figure 1B), and anti-Stro-1 (multi-lineage stem/progenitor marker) antibody reacted with some cells (Figure 1C). Cell surface markers on P4 PDLCs were characterized using immunofluorescence combined with flow-cytometric analysis. Many cells were positive for CD90 (86.28%) (mesenchymal cell marker), and there was a low level of CD105 (23.67%), CD146 (0.94%), CD271 (0.02%), and Stro-1 (2.18%) expressions (mesenchymal stem/progenitor cell markers). In contrast, expressions of CD34 and 45 (hematopoietic cell marker) was almost absent (less than 0.01%) (Figure 1D).

Effects of exogenous GDF-5 on cultured PDLCs

When P3 PDLCs were cultured with 100 ng/ml of GDF-5 for 14 d, their ALP activities were significantly lowered than that of cells cultured without GDF-5 (Figure 2A). In contrast,

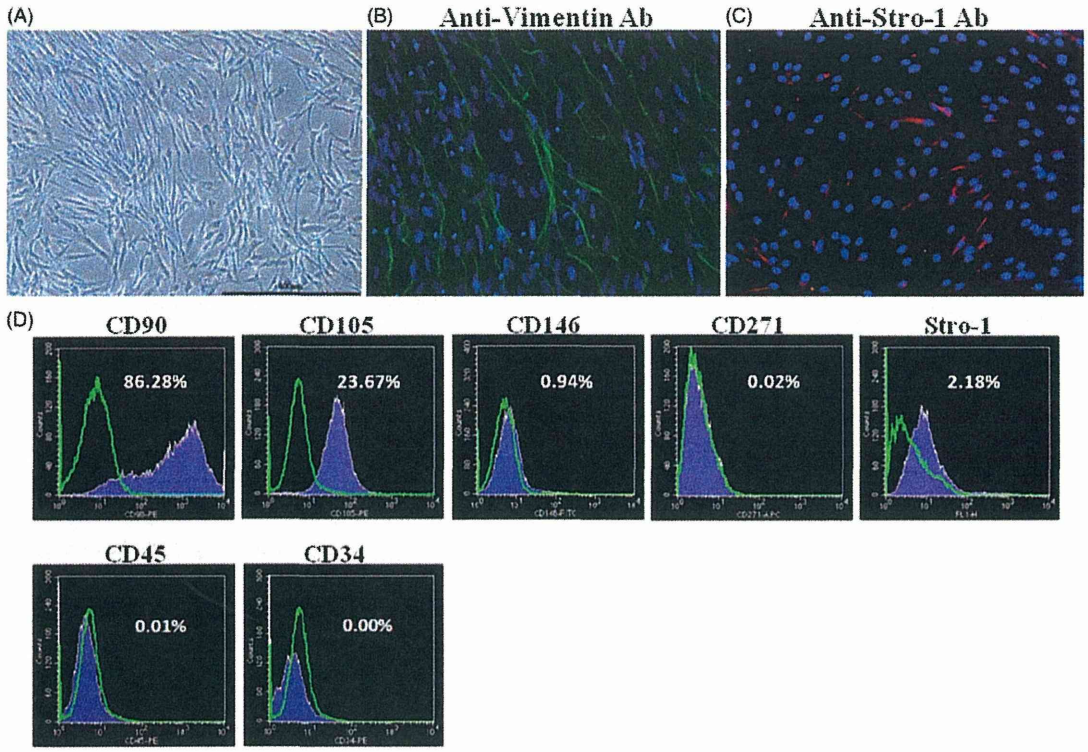

Figure 1. Cell characterization of cultured PDLCs at passage 4. (A) P4 PDLCs showed a characteristic spindle shape in DMEM with 10% FBS (40×). (B) Immunofluorescence staining of anti-Vimentin antibody. This mesenchymal cell marker is labeled by FITC (green); nuclei are labeled with DAPI (blue) (100×). (C) Immunofluorescence staining of anti-Stro-1 antibody. This stem cell marker (labeled by PE) (red) is detected in some cells; nuclei are labeled with DAPI (blue) (100×). (D) Flow cytometric analysis of mesenchymal stem cell markers (CD90, CD105, CD146, CD271, Stro-1) and hematopoietic cell markers (CD34, CD45) for P4 PDLCs.

GDF-5 increased ALP activities of P3 DPCs (derived from same teeth with PDLCs) and MSCs, and this difference was significant between non-treated and GDF-5 treated DPCs (Figure 2A). To further confirm this inhibitory effect of GDF-5 on ALP activity of PDLCs, we performed cell staining and demonstrated that a dose-dependent increase in GDF-5 concentrations resulted in a decrease of ALP-positive P3 PDLCs cultured for 14 d (Figure 2B).

Then, we examined the effects of 100 ng/ml GDF-5 on P3 PDLCs and P4-6 PDLCs. Total cell number did not show a significant difference across all concentrations and passages at day 7 and 14 in culture (Figure 2C and E). However, GDF-5 exhibited a trend of inhibitory effect on ALP activity for all passages between P3-6 PDLCs at both day 7 and 14, with most significant differences on day 14 (Figure 2D and F); and in particular on P3 PDLCs cultured for 14 d.

Effects of GDF-5 and GDF-7 on ALP activity and tenogenic gene expressions

Next, the effect of GDF-7 on PDLCs was evaluated to confirm its induction potential for tenogenic differentiation. GDF-5 and/or GDF-7, at concentration of 100 ng/ml, were added to cultures of P4 PDLCs for 14 d. Again, total cell number did not significantly change across all groups at both day 7 and 14 (data not shown). On the other hand, GDF-7 reduced the ALP activity of P4 PDLCs as compared with non-treatment, but its inhibitory effect was less than that of GDF-5 (Figure 3A). When GDF-7 was combined with GDF-5, their synergistic effect exhibited the lowest level of ALP activity at 14 d in culture.

To further investigate the effect of exogenous GDF-5 and -7 on cultures, the expression of tenogenic marker genes was analyzed in P4 PDLCs treated with these proteins for 7 days. Expression of *scleraxis* (a transcription factor of early tendon formation), *biglycan*, *collagen 3* and *collagen 1* (extracellular matrix related genes in tendon) was observed in cells across all groups (Figure 3B). *Tenomodulin* mRNA (a mature tenocytic gene; a late marker for tendon formation) was not detectable in cells treated with GDF-7 alone. However, tenomodulin expression seemed up-regulated with GDF-5, and slightly up-regulated with both GDF-5 and -7 treatments.

Characteristic changes on PDLCs treated with GDF-5

Since GDF-7 did not demonstrate a significant effect on tenogenic expression (Figure 3B), we focused the remaining experiments on characterizing the effects of GDF-5 on the expression of several genes in P4-6 PDLCs cultured for 3 weeks. Results indicated a ~2-fold increase in the expression of *scleraxis* could be maintained by GDF-5 treatment at both 10 and 100 ng/ml for up to 21 d of culture (Figure 4A). The expressions of *aggrecan* and *collagen 3* (extracellular matrix proteins; used here as markers for mature tenocytes) were increased significantly in GDF-5 treated cells after 14 d of culture (Figure 4B and C). In particular, this tendency was remarkable (4- to 8-fold increase) in cells treated with 10 ng/ml of GDF-5. Moreover, PDLCs cultured with 10 ng/ml