the samples were incubated with rabbti anti-goat IgG (H+L)-Alexa 594 (A-11080, Molecular Probe) diluted in 5% rabbit serum PBST (1:500) for 1 hour at RT, and then mounted with VECTASHIELD HardSet Mounting Medium with DAPI, after washing.

Fluorescence Immunohistochemistry to detect BM derived EPC incorporated into vessel wall in Uterus of Tie-2/EGFP/BMT/OVX Mice

The frozen sections were treated as described above, using rat anti-mouse CD31 antibody (BD Pharmingen)(1:50) at 4 C° for overnight followed by rabbti anti-goat IgG (H+L)-Alexa 594 (1:1000) at RT for 1 hour in 5% rabbit serum PBST.

SM-X;

Flow Cytometry in Menstrual Cycle of Premenopausal Women

The mouse/anti-human monoclonal antibodies for FACS were as follows; CD133 conjugated APC (130-090-826, Militenyi Biotec.), CD34 conjugated PE (555822, BD Pharmingen), VEGFR-2, i.e. KDR (V9134, Sigma), P1H12 (MAB16985, Chemicon), or VE-cadherin (MAB1989, Chemicon). For the detection of CD133 or CD34 positivitiy, the cells were double-stained with CD45 conjugated FITC (555482, BD Pharmingen). The secondary rat anti mouse antibodies for KDR, P1H12, or VE cadherin were conjugated FITC (BD Pharmingen). Using a FACStar flow cytometer, accumulated cells were segregated into lymphocyte-size (LS) and monocyte-size (MS) cells by gating in light scatter analysis. Gating of the MS fraction was arbitrarily programmed so that PB-MNCs would be accumulated until a total of 5,000 MS cells had been counted; at the point when accumulation was completed, the number of cells in the LS fraction was noted as well. The number of MS cells together with the number of LS cells comprised the total number of PB-MNCs that served as the denominator for calculation of percent antigen-positive cells. An identical immunoglobulin

control for each antibody was employed to detect non-specific binding of immunoglobulin. The absolute number of antigen-positive cells per ml of whole PB was calculated by multiplying the percent antigen-positive cells by the total number of MNCs per ml of PB.

SM-XI;

EPC colony Forming Assay of PB-MNCs in Menstrual Cycle of Premenopausal Women

Semi-solid culture medium was adjusted, using serum free 1.0% methylcellulose in Iscove Modified Dalbecco's Medium (IMDM) (MethoCult™ SF^{BIT} H4236, Stem Cell Tech.), contaning 30% FBS (JRH Bioscience), 50 ng/ml recombinant human (rh)-VEGF (Peprotec), 50 ng/ml rh-bFGF (Peprotec), 100 ng/ml rh-SCF (Peprotec), 50 ng/ml rh-IGF-1 (Peprotec), 20 ng/ml rh-IL3 (Peprotec), 50 ng/ml rh-EGF (Peprotec), and 2 IU/ml heparin (Shimizu Pharmaceutical Co.). The isolated PB-MNCs ($2x10^5$ cells per dish) were seeded into a 35 mm tissue culture dish, Primaria. Eighteen days later, the adherent colonies were counted as EPC colony forming units (CFUs-EPCs), following confirmation of UEA-1 lectin-FITC binding and acLDL-DiI uptake.

SM-XII;

FITC conjugated UEA-1 lectin binding, and acLDL-DiI uptake of CFU-EPCs

To confirm the adherent colonies of EPC colonies in a dish, double staining of acLDL-DiI and UEA-1-FITC was performed. Following the addition of 0.5 ml IMDM containing 30% FBS with 10 μl of acLDL-DiI, the colonies were incubated at 37C° for 3 hours. After the removal of methylcellulose with gently washing ice-cold PBS twice, the colonies were fixed with 1ml of 4% paraformaldehyde at 4C° for 3 hours. After gently washing with PBS twice, 1 ml of PBS containing UEA-1 lectin-FITC (20 μg/ml) was added to the dish and incubated at

RT for 2 hours. And then, after the renewal of PBS, the colonies were observed by fluorescence microscopy after mounting the colonies in VECTASHIELD HardSet Mounting Medium with DAPI.

SM-XIII;

Notice on Experiments in Animal and Human Subjects

The human protocols were approved by the Tokai University School of Medicine ethical committee, while all animal experiments were performed under the approval of animal experimentation committee of the Isehara Campus, Tokai University School of Medicine. The mice were adequately anesthetized before collecting blood or sampling uteri by intraperitoneal pentobarbital injection (50 mg/kg). The volume of aspirated blood from right ventricle of a heart was approximately 0.8~1.0 ml per mouse. After blood aspiration, the mice were terminated without any physical charge or pain under anesthetization. Each in vitro assay, i.e. differentiation, proliferation, migration, or apoptosis was repeated three times as individual experiments. EPC culture assay and EPC colony forming assay in the mouse and human samples were assessed in triplicate, by double blinded investigators.

Supplementary Figure (SF) Legends

SF-I;

Receptor Mediated E2 Effects on EPC Activity

(A) E2 promotes EPC differentiation through ERs. (a) control, (b) ICI, (c) E2, (d) E2+ ICI, *p < 0.05. (B) Upregulation of proliferative activity of cultured human EPCs via ERs. V; 50ng/ml of rh-VEGF, **p< 0.01, *p< 0.05. (C) EPC migration is induced by E2 via ERs. The migrated cells were counted in 6 HPFs per well. x200 HPF in x200 magnification, *p < 0.05. (D) Antiapoptotic effect of E2 evaluated by quantifying pyknotic nuclei. E2 treatment reduced pyknotic nuclei of cultured EPCs. (a) control, (b) ICI, (c) E2, (d) E2+ ICI , *p < 0.05. (E) Antiapoptotic effect of E2 evaluated by a cell death detection kit. E2 decreased the value of cultured EPCs at OD405 nm. The effect of E2 was blocked by ICI, *p < 0.05.

Differentiation; The number of EPC colonies increased significantly at a physiological E2 concentration (10^{-9} M) (233.7±4.1 cells/well) vs control (191.7±4.6 cells/well). The increased EPC colony number by E2 decreased to the control level in the presence of ICI (189.3±7.0 cells/well), whereas the EPC colony number was not affected by ICI alone (10^{-6} M) (187.0±8.5 cells/well)(Supplementary figure (SF) 1A).

Proliferation; The physiological concentrations of E2 stimulated the proliferation of EPC significantly, as compared to control (OD450 =0.178±0.006 for 10^{-9} M, 0.173±0.005 for 10^{-8} M vs 0.099±0.009 for control). The stimulated proliferative activity was inhibited by ICI (10^{-6} M) at each dose of E2, although the activity was not inhibited by ICI in the absence of E2 (OD450 ratio= 0.124±0.011 for 10^{-9} M+ICI, 0.113±0.017 for 10^{-8} M+ICI, 0.114±0.005 for ICI alone). In the presence of 50 ng/ml rh-VEGF, the activity was significantly upregulated, as a positive control (OD450 = 0.143±0.005)(SF1B).

Migration; E2 also increased the migration of EPCs (66.9±4.8 cells/high power field= HPF at 10^{-8} M) vs control (48.9.7±3.0 cells/HPF), or ICI (10^{-6}M) alone (49.5.0±4.0 cells/HPF). The

increase in EPC migration induced by E2 significantly returned to the control level by ICI (51.0±1.8 cells/HPF)(SF1C).

Apoptosis; The percentage of pyknotic EPC nuclei was decreased by E2 (19.7±3.49% at 10^{-8} M) vs control (39.6±4.89%), and the E2 effect was depleted in the presence of ICI (10^{-7} M) (36.1±2.90%), although not effected by ICI alone (39.8±7.66%). Furtheremore, in a cell death detection assay, the value in the E2 treated group (1.22±0.06) was significantly smaller vs control (1.56±0.05), E2 with ICI (1.58±0.05), or ICI alone (1.53±0.08)(SF1D, E).

These data show that the differentiation, proliferative, migratory, and antiapoptotic activities of EPCs is regulated by E2 under the pathway via functional ERs.

SF-II;

Enhanced Cornea Neovascularization following E2 Administration

(A) Cornea neovascularization in P- and E2- treated OVX mice (*; 200ng of rh-VEGF per cornea pellet). (B) Cornea micropocket assay data on the vessel length and circumferential angle of cornea neovascularization, n= 8 /each group, *p< 0.05. The vascular length and circumferential extent of neovascularity (maximum 360°) in mice treated with E2 or P were 0.56±0.03 mm vs 0.42±0.05 mm, and 101±9° and 62±11°. Cornea implants of pellets containing only E2 yielded no evidence of neovascularization, confirming that the effect of E2 on neovascularization in this model relies upon systemic rather than local E2 effects (data not shown).

SF-III;

Incorporation of BM-derived EPC into endothelial layer of vessel wall in uterine tissue of a Tie-2/EGFP/BMT/OVX mouse at day 7 after E2 pellet implantation

(a) Tie-2 positive cell indicated by EGFP positivity. (b) Endothelial layer of vessel wall

disclosed by CD31 positivity. (c) DAPI staining of nuclei. (d) The merged picture of (a), (b), and (c). (e) Magnified immunohistochemical image of Figure 3A-l.

SF -IV;

Recruitment of BM-derived EPCs into cornea neovascularization of Tie-2/LacZ/BMT/OVX mice following E2 administration

(A) Representative macroscopic findings of corneas stained with LacZ in E2 implanted mice as shown upper two pictures (*; 200 ng of rh-VEGF per cornea pellet). As shown lower immunohistological pictures, BM-derived EPCs were more incorporated into neovasculature or adjacent area in cornea in E2 implanted mice (scale bar; 50 μm). (B) The numbers of LacZ stained cells incorporated into foci of cornea neovascularization for each tissue section were counted manually, n= 2 eyes /mouse x 4 mice/each group, 5 tissue sections /eye. EPCs incorporated into the vasculature significantly increased in E2 group, *p< 0.05. Immunohistochemistry of CD31 demonstrated more incorporated BM-derived EPCs into cornea neovasculature of E2 implanted mice (SF A). The number of EPCs was found significantly in the cornea neovasculature of E2 implanted animals than the P group (P; 7.0±1.02 /section vs E2; 14.9±1.75 /section) (SF B).

SF-V;

EPC colony forming activity during human menstrual cycle

(a,b) The features of CFU-EPC at lower magnification (x 40) and higher magnification (x 100), (c) The merged image of CFU-EPC with acLDL-DiI uptake and UEA-1 lectin-FITC binding, scale bar = 200 μm, (d) The time course of CFU-EPC number in PB-MNCs ($2x10^5$ cells) during the menstrual cycle, *p< 0.05 vs T2, **p< 0.05 vs T1, ***p< 0.05 vs T3.

Online Table I; Cell Number of EPC Culture Assay and Gender Hormones in Human Menstrual Cycle

	T1	T2	T3	T4	T5
EPCs /mm^2	362.68± 53.52	236.69± 65.32	347.90± 39.74	397.58± 60.78*	425.48±78.17*
E2 (pg/ml)	61.0±12.01	181.2±29.04*	114.5±32.19	133.4±29.98*	98.5±26.24
PG (ng/ml)	1.22±0.20	1.33±0.31	3.63±1.49	9.47±2.34*	7.03±3.13*
LH (mIU/ml)	6.78±0.95	14.97±2.91*	7.05±1.58	6.53±2.29	5.40±0.71
VEGF (pg/ml)	90.98±24.16	212.43±35.63*	138.21±31.91	123.03±36.97	104.63±26.32

T1; Early proliferative phase, T2; Preovulatory phase, T3; Postovulatory phase, T4; Mid luteal phase, T5; Late luteal phase. E2; 17β-estradiol, PG; Progesterone, LH; Lutenizing hormone. Cultured EPC number and values of gender hormones and VEGF at each phase are presented as mean±SE. *; Statistical significance vs T2 in EPC number and vs T1 in gender hormones and VEGF ($p < 0.05$, n= 6).

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Original Research Report

Nicotine Enlivenment of Blood Flow Recovery Following Endothelial Progenitor Cell Transplantation into Ischemic Hindlimb

ATSUHIKO SUGIMOTO,[1,2] HARUCHIKA MASUDA,[1] MASAMICHI EGUCHI,[1] HIDEKI IWAGURO,[1] TERUHISA TANABE,[2] and TAKAYUKI ASAHARA[1,3]

ABSTRACT

Nicotine has been demonstrated to stimulate postnatal angiogenesis, having an antiapoptotic effect on endothelial cells. Given the extent of this angiogenesis-promoting effect, we hypothesized that nicotine may also stimulate postnatal vasculogenesis on endothelial progenitor cells (EPCs). Our analyses reveal some intriguing results using an in vitro assay with 2×10^{-6} M of nicotine (smoker's average nicotine concentration and the dose of nicotine replacement therapy). The proliferation and migration activities of human EPCs cultured from peripheral blood mononuclear cells of non-smoking healthy volunteers were not affected by nicotine. The effect of nicotine on EPC survival was significantly enhanced under serum starvation on the ratio of Hoechest 33342-stained pyknotic nuclear cells as well as Annexin-V-stained cells to total cells. Furthermore, the antiapoptotic effect of nicotine was blocked completely by nicotinic acetylcholine receptor (nAChR) antagonist hexamethonium. Next, we verified how nicotine acts in vivo. Nicotine (100 ng/ml) was administered orally for 7 days before and 4 weeks after injection of cultured EPCs (1×10^5/mouse) into the tail veins of 8-week-old athymic nude mice with ischemic hindlimbs. Laser doppler imaging analysis indicated that blood perfusion in the ischemic hindlimb was significantly enhanced in EPCs plus nicotine, as compared with EPCs alone. These findings suggest nicotine improves blood flow following EPC transplantation in patients with ischemic diseases.

INTRODUCTION

CIRCULATING ENDOTHELIAL PROGENITOR CELLS (EPCs), which originate from the common hematopoietic stem cell, are mobilized from bone marrow and incorporated into foci of neovascularization. These cells may proliferate, migrate, and differentiate into mature endothelial cells (ECs), thus contributing to postnatal vasculogenesis and assisting in postnatal neovascularization (1,2). As previously described (3), postnatal neovascularization was originally recognized to occur by "angiogenesis," which is neovessel formation by in situ proliferation and migration of preexisting endothelial cells. However, the isolation of EPCs resulted in the addition of "vasculogenesis," which is de novo vessel formation by in situ incorporation, differentiation, mi-

[1]Department of Regenerative Medicine Science, and [2]Department of Internal Medicine, Division of Cardiology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan.

[3]Stem Cell Translational Research, Institution of Biomedical Research and Innovation/RIKEN Center of Developmental Biology, Chuo, Kobe, Hyogo 650-0047, Japan.

gration, and/ or proliferation of bone marrow-derived EPCs (1,2).

Neovascularization is a complex process that is regulated by a balance between proangiogenic and antiangiogenic molecules (4). This balance is disturbed under pathological angiogenic conditions, which are a feature of tobacco-related diseases. Cigarette-smoke, which contains around 4,000 compounds including nicotine, has been shown to reduce EC activities and thus impair neovascularization (5–7). Furthermore, circulating EPCs of healthy smokers exhibit impaired functional activities particularly involving blood vessel repair and growth (8).

However, nicotine has been demonstrated to promote angiogenesis and arteriogenesis (9,10). Nicotine has presented as a proangiogenic agent via a nonneuronal nicotine acetylcholine receptor (nAChR) expressed in differentiating ECs, promoting the production of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), EC prostacyclin, and apoptosis inhibition (11–13).

Recent research reported that nicotine also has the potential to up-regulate circulating EPC activities (14). Wang et al. demonstrated that nicotine dose-dependently increased EPC number, proliferation, migration, and adhesion, as well as in vitro vasculogenesis. However, the therapeutic impact of nicotine on regenerative property of EPCs in vivo has not been elucidated.

A number of preclinical studies have started to use transplanted autologous EPCs isolated from the peripheral blood of patients with ischemic diseases to initiate vasculogenesis (15–18). Nicotine may enhance "therapeutic vasculogenesis" via transplanted EPCs co-operating with in situ ECs.

Transdermal nicotine administration, as an aid to smoking cessation in patients with cardiac disease, has been performed in the clinical setting (19). Such nicotine replacement therapy did not increase cardiovascular events in high-risk patients with cardiac disease.

Given these reports, we investigated whether administration of the dose of nicotine replacement therapy (approximately 2×10^{-6} M), improves blood flow recovery in ischemic hindlimbs following EPC transplantation therapy.

MATERIALS AND METHODS

Plasma nicotine concentration

The plasma nicotine concentration for this study was 2×10^{-6} M (a smoker's average nicotine concentration and the dose of nicotine replacement therapy), as previously described (20).

Human EPC culture

EPCs were isolated and cultured as described by Asahara et al. (1). In brief, human peripheral blood mononuclear cells (PB-MNCs) were isolated from healthy nonsmoking human volunteers by density-gradient centrifugation with Histopaque-1077 (Sigma) and plated on culture dishes coated with human fibronectin (Sigma). The cells were cultured in endothelial cell basal medium-2 (EBM-2; Clonetics) and supplemented with 5% fetal bovine serum (FBS), human VEGF-A, human FGF-2, human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, and antibiotics. Nonadherent cells were removed with phosphate-buffered saline (PBS) at day 4, and adherent EPCs were obtained after 7 days.

Proliferative activity assay

To evaluate EPC proliferative activity at each nicotine concentration (2×10^{-4}, 2×10^{-6}, 2×10^{-8} M), cultured EPCs (1×10^4 cells) were reseeded on a fibronectin-coated 96-well plate filled with 100 ml of EBM-2 containing each nicotine concentration, according to previously described methods (21). After 48 h in culture, 3-(4,5-dimethylthiazol-2-*yl*)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium phenazine methosulfate (MTS/PMS; Promega) solution was added to each well for 3 h, and light absorbance at OD490 nm was detected by an enzyme-linked immunosorbent assay (ELISA) plate reader (Bionetics Laboratory), according to the previously published protocol. The experiment was performed in triplicate.

Migration assay

EPC migration activity in response to nicotine was evaluated by a modified Boyden chamber method using a Transwell with 5-μm pore size filters (Corning Costar), as previously described. In brief, 600 ml of 0.1% bovine serum albumin (BSA)/EBM-2 medium containing nicotine (2×10^{-6} M) was applied to each lower chamber. Harvested cultured human EPCs (1×10^5 cells) in 100 ml of 0.1% BSA/EBM-2 medium, were applied to each upper chamber containing 0.1% gelatin-coated filters. After 6 h incubation at 37°C, the filters were retrieved and fixed with 99% methanol. The nuclei of cells that migrated to the lower side of each filter were mounted in Vectashield mounting media with DAPI (Vector Lab) and counted manually under 13 random high-power fields (200×) using fluorescent microscopy (AX-80, Olympus).

Apoptosis assay

EPC apoptosis, induced by serum starvation, was quantified to determine whether nicotine exerts a survival

effect on EPCs. The proportion of apoptotic EPCs versus the total EPC number after serum starvation was determined by staining with fluorescein isothiocyanate (FITC)-conjugated Annexin-V (Sigma) (22). Pyknotic nuclei (white condensed nuclei) were stained with Hoechest 33342 (Roche Diagnostics) and counted manually, as previously described (23). In brief, cultured EPCs (1×10^5 cells in 500 ml of EPC culture media per well as described above) were reseeded onto four-chamber glass slides (Becton Dickinson). After 24 h of incubation, culture medium was removed and replaced with 500 ml of serum-depleted EBM-2 medium with the following four conditions: (1) nicotine (2×10^{-6} M) and one of the nAChR antagonists, hexamethonium (100 mg/ml, Sigma); (2) nicotine alone; (3) hexamethonium alone; (4) control group. Following culture conditions for 72 h, cells were incubated with Annexin-V or Hoechest 33342 for 3 h. Annexin-V-stained cells and pyknotic changed cells were counted manually and expressed as a percentage of 100 cells in each well in high-power fields (200×), using fluorescent microscopy. Each group was studied in triplicate.

Murine EPC culture assay

An EPC culture assay was performed to ascertain the effect of nicotine on EPC activity in vivo. Briefly, in 8- to 10-week-old C57BL/6J male mice ($n = 8$; Clea, Japan), nicotine (100 ng/ml diluted with 2% sucrose water) was administered orally. After 3 weeks, mononuclear cells (MNCs) were isolated from whole blood of mice under anesthesia with sodium pentobarbital (160 mg/kg) by intraperitoneal administration. Isolated MNCs per 500 ml of whole blood were seeded into each well of 0.5% gelatin-coated four-chamber glass slides and cultured for 7 days. The adherent cells were stained by DiI-acetylated low-density lipoprotein (LDL) (Biomedical Technology) and *Bandeiraea simplicifolia* lectin I (BS-I lectin) (Vector Laboratories). The doubly stained cells were enumerated manually for EPCs under a fluorescent microscopy, as previously described (21).

Animal model of ischemic hindlimb

Eight-week-old BALB/cAJcl-nu nude male mice ($n = 6$; Clea, Japan) were anesthetized with sodium pentobarbital, as described above. One femoral artery was resected according to the previously published severe hindlimb ischemia model (15). Mice were then euthanized with an overdose injection of pentobarbital.

Transplantation of ex vivo-expanded EPCs

The impact of systemic nicotine administration after EPC transplantation on therapeutic neovascularization was investigated using a murine model of hindlimb ischemia. Following EPC transplantation, nicotine (100 ng/ml diluted with 2% sucrose water) was orally administered for 7 days before and 4 weeks after cultured EPC transplantation (1×10^5 in 100 ml of PBS/mouse) into the tail veins of 8-week-old BALB/cAJcl-nu nude male mice with induced hindlimb ischemia, the same day as EPC transplantation. The nicotine administration to mice with the ischemic hindlimb was performed according to the same protocol as previously described by Heeschen et al. (11).

Physiological assessment of transplanted animals

Laser doppler perfusion imaging (LDPI) (Moor LDI2, Moor Instruments) was used to record serial blood flow measurements on days 0, 3, 7, 14, 21, and 28 postoperatively, as previously described (15). In these digital color-coded images, a red hue indicated the region of maximum perfusion, medium perfusion values were shown in yellow, and the lowest perfusion values were represented by blue. The evaluation of blood flow perfusion was represented as the ratio of ischemic compared to the healthy limb.

Animal experiments and human subjects

The human protocol was approved by the Tokai University School of Medicine ethical committee, whereas all animal experiments were performed in the animal facility under the approval of animal experimentation committee of the Isehara Campus, Tokai University School of Medicine.

Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was evaluated using the unpaired Student's *t*-test. Multiple comparisons between three groups were done by analysis of variance (ANOVA). A probability value of $p < 0.05$ indicated statistical significance.

RESULTS

No effect of nicotine on cultured EPC proliferation

To investigate whether nicotine promotes EPC proliferative activity, we performed a MTS assay (Fig. 1). The proliferative activity of cultured EPCs was unaffected by the smoker's average nicotine concentration (2×10^{-6} M).

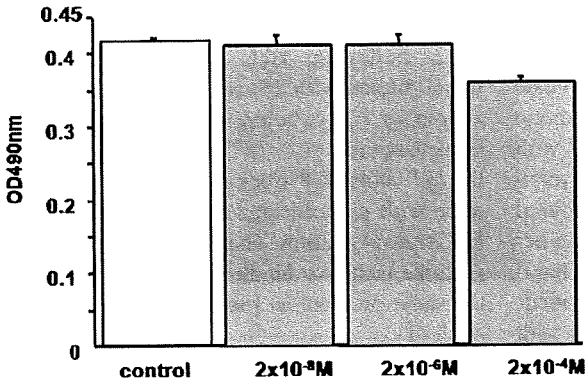

FIG. 1. No effect of nicotine on EPC proliferation. The proliferative activity of cultured EPCs was unaffected by nicotine at various smoker serum concentrations (2×10^{-4} to 2×10^{-8} M). The gray bars indicate nicotine-treated cultured EPCs.

No effect of nicotine on cultured EPC migration

The effect of nicotine on EPC migration was assayed using a modified Boyden chamber assay. The migratory activity of cultured EPCs was not affected by a smoker's serum nicotine concentration of 2×10^{-6} M (migrated cell no./200× high power fields (HPF) = 28.5 for controls, 33.6 at 2×10^{-6} M of nicotine, and 44.8 at 100 ng/ml of VEGF; $p < 0.05$) (Fig. 2).

Antiapoptotic effect of nicotine on cultured EPCs

To elucidate the effect of nicotine on cultured EPC survival, we quantified apoptosis induced by serum starvation. To evaluate early- or late-stage apoptosis by Annexin-V or Hochest 33342 staining, nicotine significantly attenuated the percentage of apoptotic EPCs, 58.5 ± 2.04% to 35.8 ± 1.98% (Fig. 3A, B) or 21.5 ± 1.57% to 13.6 ± 0.89% (Fig. 3C,D) versus controls ($p < 0.0001$).

We also investigated whether nicotine gave rise to an antiapoptotic effect of EPCs through the nAChR using the Annexin-V apoptosis assay with hexamethonium, a nAChR antagonist, as previously reported (Fig. 4). Although the percentage of apoptotic cells did not change with hexamethonium alone, as compared to control, the antiapoptotic effect of nicotine on serum-depleted EPCs was completely blocked upon combination with nicotine, resulting in an antiapoptotic effect via the nAChR (% of Annexin-V-stained cells to total cells = control, 58.5%; nicotine, 35.8%; hexamethonium, 56.2%; nicotine + hexamethonium, 58.5%; $p < 0.0001$).

Up-regulation of circulating EPC activity

To assess the effect of nicotine on EPCs in vivo, we performed an EPC culture assay using MNCs isolated from murine peripheral blood (PB). After 3 weeks of nicotine administration, PB-MNCs were isolated and cultured for 7 days. In the nicotine-administered group, the number of cultured EPCs was significantly increased ($p < 0.0001$) (Fig. 5).

Nicotine improved blood perfusion in hindlimb ischemia following EPC transplantation

After nicotine administration and systemic EPC transplantation (EPC-TX), LDPI was used to evaluate proportion of improvement in ischemia to healthy limbs on days 0, 3, 7, 14, 21, and 28, where LDPI indicated significant improvement of blood perfusion (Fig. 6). The ratio of ischemic/nonischemic blood flow in the nicotine-treated group with EPC-TX improved to 74.5 ± 5.2% as compared to 58.0 ± 5.7% in the EPC-TX group, 39.6 ± 5.3% in the nicotine alone group, and 26.9 ± 0.3% in the PBC control group at day 28 ($p < 0.05$). This finding suggests that nicotine enhances EPCs activity through ameliorated EPC survival.

DISCUSSION

Since the discovery of EPCs in human subjects, recent studies proved that EPC transplantation improved blood flow in patients with ischemic diseases, i.e., myocardial or limb ischemia, through therapeutic enhancement of vasculogenesis (15–17).

Proangio/vasculogenic agents such as cytokines, hormones or growth factors, e.g., granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, SDF-1, or VEGF, reportedly promote the

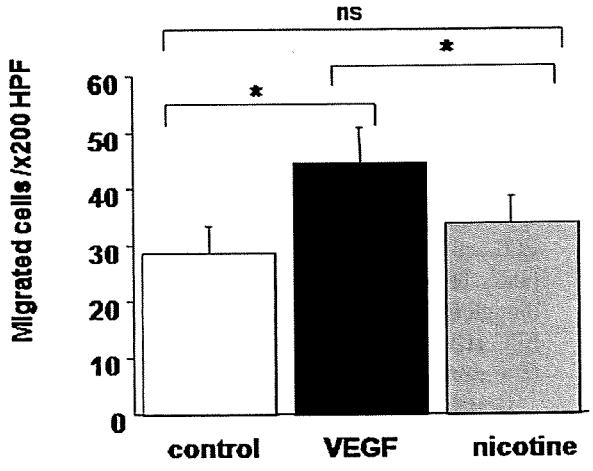

FIG. 2. No effect of nicotine on EPC migration activity. Migratory activity of cultured EPCs was not affected by nicotine compared to control and was significantly up-regulated by the positive control VEGF (*$p < 0.05$).

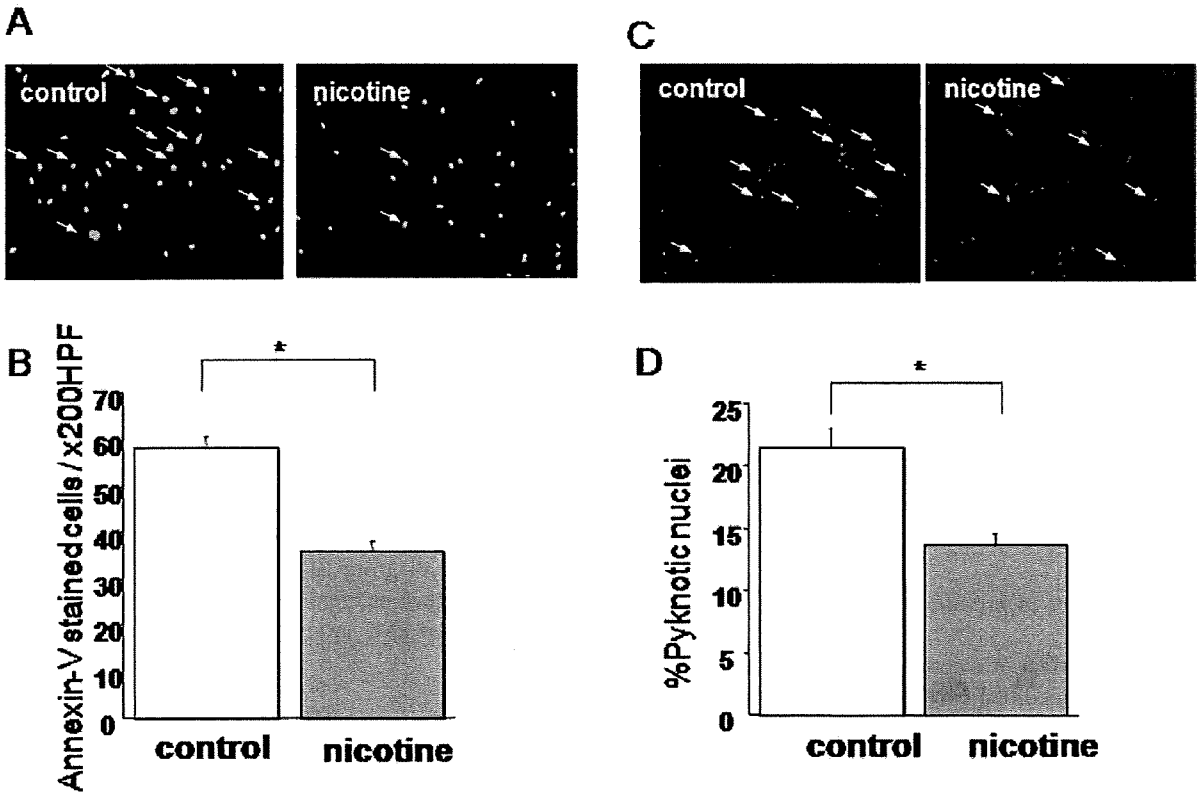

FIG. 3. Anti-apoptotic effect of nicotine on cultured EPCs. (**A**) Annexin-V staining was performed to determine the proportion of apoptotic cells. (**B**) Percentage of Annexin-V-stained cells. (**C**) Enumeration of pyknotic nuclei following Hochest 33342 staining (white condensed nuclei). (**D**) Percentage of pyknotic nuclei ($*p < 0.0001$).

circulating activity of EPCs (21,23–26). Recently, nicotine has also been found to promote angiogenesis through antiapoptotic or proliferative effects on differentiated ECs in vitro or in vivo (11,13), as well as through vasculogenesis via proliferative, migratory or adhesive actions on EPCs in vitro (14).

Cigarette smoking is a well-known major risk factor of cardiovascular diseases. Also, cigarette smoking reportedly abolishes EPC activities, specifically proliferation, differentiation, migration, and adhesion (8,14) and correlates inversely with coronary risk factors, e.g., hypertension and serum hypercholesterolemia (27). Circulating EPC activity in chronic cigarette smokers was recently reported to be impaired, but improved by nicotine patch, although not statistically significantly (28). The abrogated EPC activities may be caused by the overall effect cigarette smoking has over nicotine's EPC bioactivity.

Given these findings, we hypothesized that nicotine might augment the effect of EPC transplantation. In the present study, nicotine promoted blood flow in murine ischemic hindlimbs following ex vivo-expanded EPC transplantation.

As described by Wang et al., nicotine dose-dependently increased EPC number as well as proliferative, mi-

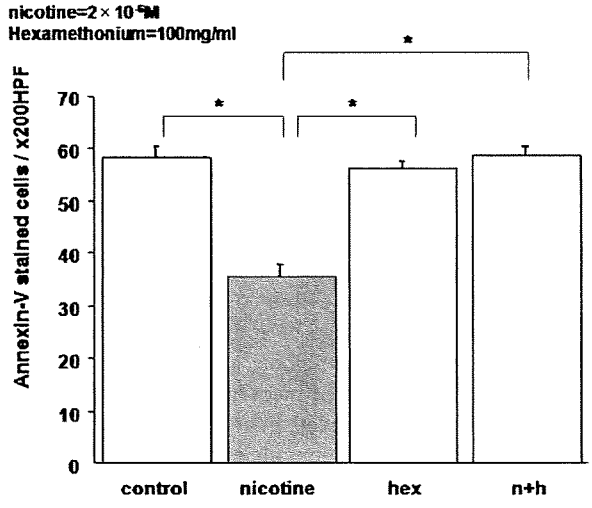

FIG. 4. nAChR antagonist blocked nicotine's antiapoptotic effect on EPCs. The nAChR antagonist Hexamethonium (hex) blocked the antiapoptotic effect of nicotine on cultured EPCs, as was seen using the Annexin-V apoptosis assay described in the Materials and Methods section. The antiapoptotic effect was not observed in the nicotine with hex (n+h) group as compared with nicotine alone ($*p < 0.0001$).

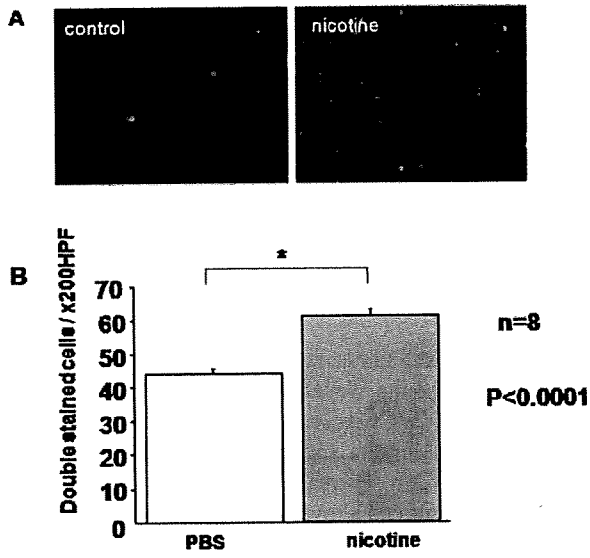

FIG. 5. Nicotine up-regulated circulating EPC activity. Significant increase in cultured nicotine-administered EPCs, stained with both acetylated LDL-DiI and BS-1 lectin-FITC, as compared to control (PBS) ($p < 0.0001$, $n = 8$) is seen using the murine EPC culture assay.

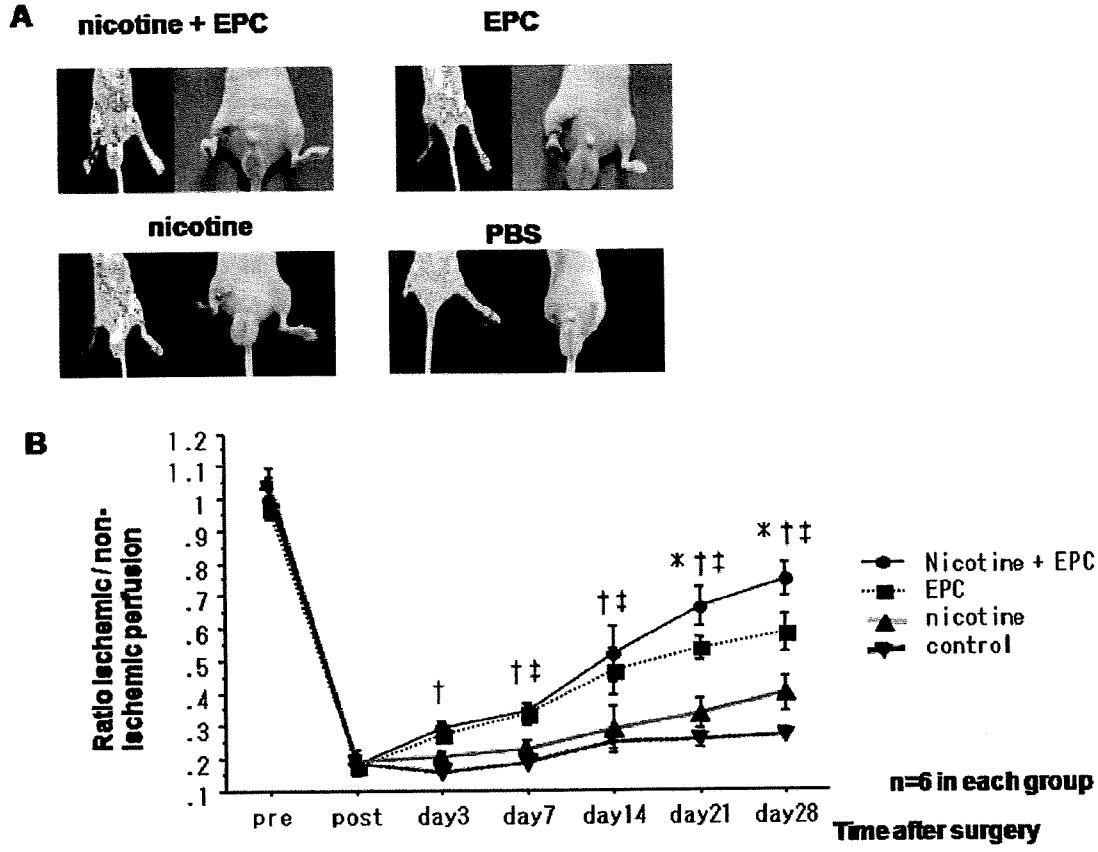

FIG. 6. Nicotine enhanced blood perfusion of ischemic limb transplanted EPCs. (A) Representative blood perfusion imaging analysis in each group at day 28. (B) Ratio of ischemic/nonischemic limbs in each group in time series. *, nicotine + EPC vs. EPC; †, nicotine + EPC vs. control; ‡, EPC vs. control; $p < 0.05$, $n = 6$ in each group.

gratory, adhesive, and in vitro vasculogenesis capacity at concentrations of 10^{-12} to 10^{-8} M (14). Nicotine's peak effects on EPCs were observed at concentrations of 10^{-8} M. Cytotoxicity was seen, however, at higher nicotine concentrations (over 10^{-6} M).

We evaluated the effect of nicotine at the dose of nicotine replacement therapy or a smoker's average serum concentration of 2×10^{-6} M on EPC biology. The migratory activity of EPCs was not influenced by this nicotine dose, whereas antiapoptotic effect of nicotine was significant.

EPC proliferative activity was not affected by 2×10^{-6} M nicotine. Nevertheless, in EPC culture assay, the number of EPCs increased following nicotine administration. These data suggest that nicotine contributes to EPC long-term survival activity via its antiapoptotic effect, as described above.

Improved blood flow in ischemic hindlimbs through EPC transplantation was enhanced by nicotine co-administration. Therapeutic vasculogenesis is considered to be brought on by up-regulating transplanted EPC survival and circulating intrinsic EPCs (29). Nicotine's survival effect on EPCs is thought to be caused through the nAChR (9). In addition to promoting exogenously transplanted and endogenous EPC bioactivity, nicotine augments therapeutic angiogenesis through differentiated ECs in ischemic foci in situ via an antiapoptotic effect and VEGF induction of ECs (12,13,30).

Long-term administration of nicotine may raise the growth of aortic atheroma and plaque neovascularization in patients, probably leading to the risk of plaque rupture (9). However, the effect of nicotine in short-term administration promotes arteriogenesis involving collateral vessel development (10).

Clinically, transdermal nicotine administration, i.e., nicotine replacement therapy via a nicotine patch, has been devised to reduce cigarette smoking, the largest coronary risk factor of smokers with ischemic cardiovascular disease. The duration of smoking cessation is reportedly limited by nicotine replacement therapy, resulting in no significant increase in cardiovascular events based on the adverse effect of nicotine in high-risk outpatients with cardiovascular disease (19). Neovascularization in ischemic sites might be promoted, to some extent, in patients by transdermal nicotine administration, probably contributing to the lack of an increase in cardiovascular events.

Nicotine replacement therapy may be useful for neovessel formation, including collateral development in ischemic foci and augmentation of the EPC transplantation effect. However, further studies are needed to determine the appropriate nicotine dose and duration.

Nicotine replacement therapy is practical in clinical medicine to use for risk reduction in patients who suffer from cardiovascular diseases. Therefore, we believe that the concept of enhancing EPC survival activity by nicotine opens the new field for clinical strategy of EPC transplantation therapy. In conclusion, our data provides an advantageous approach using nicotine replacement therapy for therapeutic vasculogenesis via EPC transplantation in conjunction with nicotine administration.

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Address reprint requests to:
Dr. Takayuki Asahara
Department of Regenerative Medicine
Tokai University School of Medicine
Bohseidai, Isehara, Kanagawa 259-1193, Japan

E-mail: asa777@aol.com

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臓器移植・組織移植から再生医療へ
—臓器・組織・細胞の procurement の観点から—

（財）先端医療振興財団　先端医療センター研究所　松山晃文

はじめに

臓器移植、組織移植、細胞移植そして再生医療。これらは医療として特殊である。多くの医療は患者自己完結型といえるのに対し、臓器移植・組織移植そして再生医療の多くは、生体死体問わずヒトから獲得した臓器・組織あるいは細胞を治療目的で摘出あるいは採取（獲得：procurement）する医療であり患者以外の第三者の存在が必須である。

医師は、医行為（医業）の名の下であれば何をしても良いかのような“幻想”がある。例えば、外科的手術のためにメスを入れるという行為は、医師が医療として施術するのであれば傷害罪は成立しない。外形的に構成要件を満たすが、違法性は阻却されるからである。あくまでも患者のためという法の精神と患者の法益を守っているからこそ違法性が阻却され罪に問われないのである。医師が医療行為において罪に問われないのは、ただこの1点によってのみ支えられており、医師法を紐解けば、第17条において医業を医師の独占的業務とし、医師以外の者に対しては罰則によって医業を行うことを禁止しているのみであり、“all mighty”ではないことが理解できよう。医師が、侵襲を伴う医療行為を行っても罪に問われないのは、「患者」のためという法の精神と患者の法益を守っているからである。そう考えると、他者等ヒトからこれら医療のために原材料としての臓器・組織・細胞を procurement する過程においては侵害している法益があるはずで、これを医療行為の視点から是として了とできるのだろうか。

本稿では臓器移植、組織移植、細胞移植そして再生医療について、procurement の観点から議論を行う。すなわち、他者を含めたヒトからの臓器・組織あるいは細胞の procurement が必須であるという臓器移植、組織移植ならびに再生医療の共通した特殊性を鑑み、procurement により侵害される他者等の守られるべき法益との患者の利益とのトレードオフのみならず、医師が患者の治療行為のために他者等の守られるべき法益を侵害することを許容する法的な手当てについて議論を行いたい。薬事法との関係については、第3章9「再生医療と薬事法」において検討する。

各論

臓器移植

患者から治療目的で組織・細胞を採取するのは、本人にとって治療効果という利益が期待されるため、これは違法性が阻却される。ここで、患者の治療のために他者から臓器・組織・細胞を獲得するという行為は明確に違法性が阻却されているのかという疑問を提示

したい。

精神的にも生計的にも一体化した親族であれば、これは患者が得られる健康という法益を共有することが可能である。腎不全患者への生体親族間腎移植であれば、慢性透析という不利益から脱することができ、ひいては臓器を提供した親族にとっても患者の健康という利益を享受しうるとの解釈がなりたつ。解釈だけであれば、限りなく白ではあっても、完全な白ではないのではない、との議論がなされるかもしれない。親族が相互扶助を行うための 1 つの方策として臓器移植を位置づけるのであれば、親族のその想いを止めないためにも、法的な手当てがあってもよい。親が子を思う気持ちから臓器あるいはその一部を提供する、苦しんでいる配偶者のために臓器提供を行う、これらが本当に自由意思の発露として行われるのであれば、法的な手当てがなされないほうが理不尽である。このような観点からの議論は、奥田によってもなされ、彼は生体間臓器移植こそ臓器移植のあるべき姿であると定立している。上記議論でもなされるように、あくまでも一体化していると合理的に推定できる親族関係でのみ生体間臓器移植は進められるべきであり、たとえば精神障害がある者と養子縁組等して臓器を搾取、あるいは自由意思の発露を阻害される環境下での関係で臓器を提供させられることは厳に禁じられるべきであり、それらに加担する医師がいるのであれば、医師として生きることを「プロフェッション（宣言）」した者として、出所進退を明らかにすべきである。

一方で、第三者（他者：third party）から臓器組織等を獲得する場合はどうなのか。患者にとっては健康という利益が得られると想定され求められる医療であるが、他者にとって利益はない。むしろ、生体であれば健康体にメスを入れるわけであるし、健康を損ねる危険性がある。たとえ同意があったとしても、傷害していることに変わりはなく、同意撤回後の原状回復は望むべくもない。違法性が阻却されるのか、法的に不安定ではなかろうか。また、死体由来臓器の移植であれば、死体にメスを入れるわけであるから死体損壊罪が成立するかもしれないし、墓地および埋葬に関する法律に触れるかもしれない。社会全体から見て、患者の健康が得られ社会的遺失利益が回復されるとの法益はまぎれもなく大きいが、ミクロの視点では触法行為となる可能性もある。それゆえ、医師が医行為として臓器移植を実施するにあたり、患者の健康のために診療に専念できる制度の構築は、立法措置として講じられるべきものである。具体的には、生体あるいは死体から臓器組織細胞等を摘出するという行為の違法性が阻却されるという立法が求められるのである。臓器移植にあっては、現行では臓器の移植に関する法律がそれである。生体から臓器（腎臓）を摘出することを可能とする立法であり、法益の親族内での一体化という解釈的運用から解き放たれ、明文化された違法性の阻却が図られている。死体から臓器を獲得すること関しては、患者の健康という法益が死体の尊厳との法益のトレードオフにて勝ることから、それを可能とすることで死体損壊罪には当たらないことを示し、脳死をヒトの死の一形態として明記することで、殺人罪（あるいは傷害罪）には該当しないことを宣言したと認識している。

組織移植

組織移植に関して明確に規定した法はない。臓器の移植に関する法律において、組織移植は同法の適用範囲外である旨触れられているのみである。医学的な定義もあいまいとしたものであり、現状では学会などが自主的に基準を設けているのが実情である。平成 11 年から 12 年にかけて、一定の基準が必要ではないかとの認識の下、研究班が設置され、その研究成果をもとに、日本組織移植学会が学会倫理指針を策定、公表している。皮膚移植では、特に死体より獲得された皮膚を脱細胞処理し、それをバンク化して救急救命の現場で熱傷などの患者の求めに応じて提供されている。組織移植といっても、ヴァリエーションに富んでおり、たとえば膵島移植は組織を viable な状態で単離し 1 型糖尿病患者に経門脈的に投与するものであり、皮膚移植は心停止ドナーより皮膚を獲得し、一時的に貯蔵して必要な時に用いる。

現行では医師が学会などの自主基準に則して行っており、医薬品医療機器としての承認は得ていない。臓器移植においては、レシピエントの主治医が procurement から移植まで一貫して医療行為として行っており頒布性も低いため、薬事法の規制はうけない。一方で、組織移植のなかでも、例えば皮膚バンクあるいは骨バンク等のようにバンク化されうるものは、医療行為としての一貫性の問題、およびその頒布性の高さから議論の余地がある。前者に関しては、組織摘出を行う医師と組織移植レシピエントの主治医が異なるため、一貫した医療行為とみなすには難点があるのではないかとの議論であり、後者に関しては、バンク化して頒布するものであるから、それが無償であっても反復継続して「物」の頒布を行うこととなり、外形的には薬事法規制の対象となる「業」となりうるのではないか、との議論である。現状では、皮膚などの処理を主治医団の医師とみなされる医師が手技として行い、「脱細胞皮膚組織」という「物」ではなく、皮膚を脱衣細胞化させ貯蔵する「手技」であるとし、実務的に運用している。

細胞治療（広義）

細胞を用いた治療（広義）には細胞移植と再生医療（狭義の細胞治療）が内包される。

1）輸血

もっとも古くから実践されている細胞治療（移植）は輸血である。20 世紀初頭、Landsteiner による血液型の発見を epoch making とし、爾来数多くの患者を救命してきた。輸血が医療行為として行われ始めた時点では、薬事法下での規制は行われていなかったが、頒布性の高さによる感染症伝播の発生など公衆衛生上の反省・問題と製品としての有効性・有用性・均一性の確保の観点から、世界的にみても薬事規制を受けることとなった。我が国でも、現行法制度下では、献血により収集された血液は、赤血球、血小板あるいは血漿成分ならびに血漿成分由来製剤として医薬品として薬事法の適用をうける（安全な血液製剤の安定供給の確保等に関する法律第 2 条同施行規則第 1 条別表 1）。健常人から血液を獲得するという行為は、本来であれば傷害罪を外形的にはみたすが、医薬品としての血液製剤を製造するための血液の獲得、すなわち献血について、安全な血液製剤の安定供給

の確保等に関する法律において、健常人からの血液の獲得にかかる項目が規定されていることで、違法性が阻却されていると考える。医薬品としての血液製剤の製造を目的としない健常人からの採血が、古くは売血として行われていたが、血液提供者の健康を害するという公衆衛生上の懸念と、感染症の伝播に関する懸念から禁止規定がある（安全な血液製剤の安定供給の確保等に関する法律16条・32条）。売血でなくても、健常人から血液製剤の製造を目的としない採血に関しては、健康診断など本人に利益があると推定される場合を除外すれば法的に不安定であると言える。

2）骨髄移植

輸血についで実践されてきた細胞治療（移植）は、骨髄移植である。白血病など血液疾患では、骨髄に存在する血液系幹細胞の腫瘍化が起こっているため、抗腫瘍薬剤の投与を行うとともに、根治療法として非自己骨髄細胞の自己骨髄への生着と入れ替えを期待したものである。HLA型が不適合である場合は、移植した非自己骨髄細胞が免疫系の作用で拒絶されてしまうか、あるいは非自己骨髄細胞が自己細胞を認識してGVHDを引き起こして致死的となる危険性がある。HLA型が一致あるいは近似した同胞ないしは親族等から骨髄液を採取し、末梢から点滴で骨髄液を輸注するとの一貫した医療行為である。健常者に全身麻酔をかけて腰部から骨髄を採取するという行為もまた、外形的には傷害を構成するが、精神的にも生計的にも一体化した親族であれば、これは患者が得られる健康という法益を共有することが可能であって、違法性は阻却される。加えて、採取された骨髄は一定程度の時間が経過すれば自然経過として回復するものである。骨髄移植は、上記のように同胞あるいは親族からの提供という形で医療としてその有効性有用性が確立されてきた。しかし、致死性血液疾患の患者のすべてに同胞がいるとは限らず、またHLA型が移植に適さない場合も多く、医療技術として救命できるはずの命が、骨髄提供者がいないために失われるという現実に直面するにいたった。骨髄バンクの登場である。HLA型が一致あるいは移植可能な程度に一致するため、約10万人の骨髄提供candidateをリストアップするというバンクである。バンクといっても実際に骨髄が採取されて貯蔵されているというわけではないため、安全な血液製剤の安定供給の確保等に関する法律には抵触しない。骨髄バンクが事業として行っているのは、ボランティアとしての骨髄提供意思表示者と骨髄移植希望患者のマッチングである。複数回の面談をへて、健常者は自由意思で骨髄の提供を行う。外形的には健常者に全身麻酔をかけて腰部から骨髄を採取するという行為であるため傷害を構成するが、腎移植のように片腎となり腎予備能が低下するわけではなく、採取された骨髄は一定程度の時間が経過すれば自然経過として回復するという点で、違法性の程度は低いと類推される。厚生労働省健康局臓器移植対策室等から各種通知が発出されており、違法性を問われることはない。米国においてもPublic Health Service Act Article 361に基づき実施され(cGTPガイドラインである21 CFR Parts 16, 1270, and 1271. Current Good Tissue Practice for Human Cell, Tissue, and Cellular and Tissue-Based Product Establishments; Inspection and Enforcementを遵守する必要はあろう)、同Article351には規制を受けない組織移植扱い

である。

3）臍帯血移植

臍帯血移植は、骨髄移植におけるドナーへの侵害性を考慮する必要がない治療法として進められてきた。臍帯は、我が国では伝統的に母子のつながりの記念としてその一部が保管されてきたが、残りの臍帯は医療廃棄物として処理されてきた。この臍帯血は、新生児の遺伝情報を有する組織で、そのなかには未分化血液幹細胞が豊富に含まれることが知られている。白血病など血液疾患では、骨髄に存在する血液系幹細胞の腫瘍化が起こっているため、抗腫瘍薬剤の投与を行うとともに、根治療法の１つとして臍帯血の自己骨髄への生着と入れ替えを期待し、HLA 型が一致あるいは近似した臍帯血を末梢から点滴で骨髄液を輸注する。健常者に全身麻酔をかけて腰部から骨髄を採取するという行為を伴わない。臍帯血を廃棄する場合は医療廃棄物としての処理が行われるが、臍帯から臍帯血を採取した残余物は、臍帯血バンクでも規定が定められ適正に廃棄されている。

骨髄バンクと臍帯血バンクの相違点は、前者がドナー情報のバンクであって、患者への治療が必要となった段階で初めてドナーにアクセスして骨髄の提供を受けることとなるのに対し、後者ではあらかじめ臍帯血を貯蔵（バンク）してあり患者への治療が希求されたときに速やかに貯蔵（バンク化）された臍帯血を頒布する、という点にある。臍帯血移植においては、あらかじめ貯蔵した臍帯血を反復継続して（業として）頒布するため、薬事法あるいは安全な血液製剤の安定供給の確保等に関する法律に抵触するかが議論となる。我が国では組織移植の１つとして取り扱われているが、これは臍帯血移植が血液専門医らにより臨床研究として推進され、移植手技として十分に認知され保険点数まで設定されているという歴史的経緯に加え、細胞に分離処理は行っているが加工はしていないという点（minimal manipulate）、1 対 1 という頒布性の低さから公衆衛生上の感染症の伝播蔓延の危険性が著しく低いという点などが背景にあるものと思われ、我が国では厚生労働省健康局臓器移植対策室が所管している。医薬品化するには、臍帯血を得る分娩室等の清潔度の validation が必要となるかもしれず、またこれまで貯蔵してきた臍帯血の取り扱いという課題が生じるかもしれない。

4）再生医療（狭義の細胞治療）

再生医療の実現化にむけ、その出口が薬事法の適用をうける細胞組織利用医薬品医療機器として承認をうけるかあるいは医師の技術・手技として薬事法外で行われる場合がある。いずれにせよ、自己（自家）細胞組織などを採取して加工などを行って移植・投与するか、非自己から細胞組織等を採取して加工などを行って移植・投与し、患者の健康の回復を目的としている。自己（自家）から細胞組織を採取し、それを治療目的に利用するのであれば、医学的にみて採取による侵襲程度が予測される健康回復の程度を凌駕すると科学的合理的に判断できるとの担保の上で、その採取に違法性はないと言える。Procurement において違法性阻却事由にあたるか議論すべきは、非自己よりの細胞組織の獲得の場合である。治療のためとはいえ健常者から細胞組織を得ることは、外形的には傷害を構成する。精神