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(54) Title: VASCULAR REPAIR AND ENDOTHELIAL CELLS

(57) Abstract: This document relates to methods and materials involved in obtaining endothelial cells (ECs) and repairing vascular tissue. For example, methods and materials for obtaining fat derived ECs as well as methods and materials for using ECs for vascular repair are provided.

VASCULAR REPAIR AND ENDOTHELIAL CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U. S. Provisinal Application Serial No. 61/001,975, filed November 5, 2007.

Statement as to Federally Sponsored Research

This invention was made with government support under grant number HL075566 awarded by the National Heart, Lung, and Blood Institute. The government has certain rights in the invention.

BACKGROUND

1. Technical Field

This document relates to endothelial cells (e.g., endothelial cells derived from omental fat) and methods of using endothelial cells for to repair vascular tissue.

2. Background Information

Percutaneous transluminal coronary angioplasty (PTCA) and percutaneous transluminal angioplasty (PTA) revolutionized the treatment of coronary artery disease (CAD) and peripheral artery disease (PAD). Nevertheless, restenosis appears to occur in about 30 to 50 percent of cases within six months following PTCA. The incidence of restenosis after PTA is about 30 to 60 percent. To prevent the high number of restenosis, stenting supplanted PTCA and PTA in many ways. Bare metal stents (BMS) were the first stents used. Although the use of BMS has been demonstrated to be superior to PTA and PTCA alone in reducing restenosis to 20 to 30 percent for coronary arteries, short-term and long-term outcome was not optimal. The implementation of drug eluting stents reduced the risk of restenosis, but was led to other problems like acute early instent thrombosis, which can lead to partial or complete occlusion.

SUMMARY

This document relates to methods and materials involved in obtaining endothelial cells (ECs) and repairing vascular tissue. For example, this document provides methods and materials for obtaining fat derived ECs (FDECs) as well as methods and materials for using ECs for vascular repair.

In general, one aspect of this document features an enriched population of fat (e.g., omental fat) derived endothelial cells. The cells can be positive for vWF, eNOS, VEGFR-2, and *Griffonia* lectin, and can be negative for SM-actin.

In another aspect, this document features a method for isolating an enriched population of fat derived endothelial cells. The method comprises obtaining DiO acetylated LDL positive endothelial cells from a population of cells.

In another aspect, this document features a method for repairing vascular tissue in a mammal. The method comprises administering an amount of an enriched population of fat derived endothelial cells to the mammal under conditions effective to repair vascular tissue.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1. Fat derived cells after one day (A) and 7 days (B) in culture showing cobblestone morphology. The FDECs were highly proliferative and did not exhibit a difference in proliferation in use of FBS or autologous rabbit serum (C). After 14 days, almost 5 x 10^7 cells could be cultured (D).

FIG. 2. Fat derived endothelial cells in culture exhibit positivity for vWF (A), eNOS (B), are negative for SM-actin (C) (SM actin positive cell in side window), are positive for VEGFR-2 (D), and provide a high uptake for DiI acetylated LDL (E) also in co-staining with eNOS (F). To determine the amount of positive cells for DiO, FACS was used (G).

FIG. 3. Fat derived endothelial cells from rabbit exhibited two times longer tube formations than rabbit carotid artery endothelial cells.

FIG. 4. Reendothelialisaton after 48 hours.

FIG. 5. Cells stained for DiI acetylated LDL are covering the whole vessel lumen (A: 100x), (B: 50x), (C: 200x). D reveals the vessel wall of a untreated vessel. Reendothelialization was also proved with *Griffonia* lectin (E) and vWF (F) staining on injured vessels.

FIG. 6. Results on endothelial dependent vasoreactivity in organchamber plotting relaxation in percent.

FIG. 7. Intima media ratio of 28 day survivors. Unselected FDECs did not reveal a significant improvement in morphology, whereas the DiO sorted cells exhibited a significant improvement.

DETAILED DESCRIPTION

This document provides purified populations of ECs (e.g., FDECs) and methods and materials for using ECs (e.g., FDECs) to treat vascular conditions. For example, this document provides enriched populations of FDECs. The term "enriched" as used herein with reference to FDECs means that the population has at least a two fold increase (e.g., at least a 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 75, 100, 500, 750, 1000, 2500, or 5000 fold increase) in ECs from the crude population of cells present in the fat tissue from which the ECs are isolated. The ECs (e.g., FDECs) provided herein can be isolated from mammals, including rodents such as mice and rats, farm animals such as pigs, cattle, sheep, and goats, and humans or other primates. In some cases, ECs can be obtained from fat tissue of a mammal. For example, omental, arterial, kidney, or subcutaneous fat tissue can be used to obtain FDECs. Once obtained, the fat tissue can be treated to that a

population of ECs are obtained. For example, the methods and materials described herein can be used to obtain an enriched population of FDECs.

In some cases, an enriched population of FDECs can be obtained directly from a source of fat. For example, EC isolation techniques can be used to obtain an enriched population of FDECs. In some cases, *in vitro* culture techniques can be used to obtain an enriched population of FDECs. For example, ECs for fat tissue can be cultured as described herein to obtain an enriched population of FDECs. In some cases, EC isolation and in vitro culture techniques can be used to obtain an enriched population of FDECs.

In some cases, endothelial cell markers can be use to increase the purity of a population of ECs. For example, DiO acetylated LDL can be used to obtain an enriched population of ECs.

Once obtained, ECs (e.g., FDECs) can be expanded in culture to increase the number of cells. For example, the culture techniques described herein can be used to obtain a large number of ECs.

The ECs (e.g., FDECs) provided herein can be used to treat vascular conditions such as acute injury, chronic endothelial injury, hypertension, pulmonary hypertension, response to angioplasty or stenting, atherosclerosis, and endothelial dysfunction. For example, the ECs (e.g., FDECs) provided herein can be injected into the vascular system upstream of acute or chronic injury or endothelial dysfunction under conditions that result in vascular repair.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 - Vascular Repair using Endothelial Cells derived from Omental Fat

Cell Culture

All animal procedures were approved by an institutional animal care and use committee. Omental-Fat was harvested from New Zealand White rabbits (3.5-4.5 kg) under deep anesthesia with Isoflurane. Through a one-inch incision in the epigastric region right below the sternum along the linea alba, about 3-5 g of omentum was exposed with a hook, and the visible vessel were tight before the fat was removed. Following

mechanical disaggregation, the omentum was washed with Ca^{2+}- and Mg^{2+}-free PBS (Cellgro). A 1% solution of HBSS with Ca^{2+} and Mg^{2} (Cellgro) and Collagenes Type I (Worthington, 142u/mg) was used to digest the tissue. After one hour in room temperature, the supernatant was removed, and the solution gently centrifuged at 1600 rpm. The cells were washed once with HBSS and once with EGM-2 and afterwards plated on fibronectin-coated 6-well plates (1 μg/cm^2; Becton Dickson). EGM-2 is a product of Lonza and EBM-2 with 2% serum, hydrocortisone, recombinant Fibroblastic Growth Factor (hFGF), Vascular Endothelial Growth Factor (VEGF), recombinant Insuline-Like Growth Factor (R-IGF), ascorbic acid, recombinant Epidermal Growth Factor (hEGF), heparin, antimicrobial (penicillin and streptomycin), and antimicotic (amphotericin B) agents. Cells were passaged before reaching confluence, and the medium was changed daily until harvest. EGM-2 medium was slightly modified using 2% autologous serum (AS) instead of 2% Fetal Bovine Serum (FBS).

Characterization of Cultured Cells

Laser confocal microscopy was used to image day 7, 10, and 14 cells. Cells were fixed and blocked with goat serum and incubated with primary antibodies to von Willebrand Factor (vWF), endothelial nitric oxide synthase (eNOS), smooth muscle actin (SM-actin), Griffonia lectin, and vascular endothelial growth factor receptor-2 (VEGFR-2). Also, a staining with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyliodocarbocyanine iodade) acetylated LDL and DiO (3,3' dioctadecyloxacarbocyanine perchlorate) acetylated LDL was performed. Cells were fixed, permeabolized, and blocked with either 10% normal goat serum or 10% normal donkey serum followed by incubation with 5 μg/mL mouse anti-eNOS (BD Transduction Labs, Lexington, KY), 0.7 μg/mL mouse anti-human SM-actin (Dako, Carpinteria, CA), or 19 μg/mL sheep anti-human vWF (The Binding Site, Birmingham, UK). Concentrated matched mouse and sheep IgG served as negative controls. eNOS and actin were visualized using goat anti-mouse fluorescein (Molecular Probes, Eugene,OR). vWF was visualized using donkey anti-sheep biotin followed by Streptavidin fluorescein (Amersham Biosciences Corp, Piscataway, NJ). Hoescht staining was used to identify the nuclei. DiI acetylated LDL was also used to co-stain with vWF, eNOS, and SM-actin as described elsewhere (Fraser et al., Nat. Clin.

Pract. Cardiovasc. Med., 3 Suppl. 1:S33-37 (2006)) for coexpression studies. Fluorescence-activated cell sorting (FACS) detection of DiO acetylated LDL was performed to detect the purity of the cultured cells. For a long-term group sub-study of 28 days, FACS selection was used to select only strongly positive cells who had a high uptake for DiO acetylated LDL. This was used to raise the purity of delivered cells.

Endothelial cell proliferation assay

FDEC's from 12 different rabbits were cultured. Six cultures were grown with EGM-2 with 2% FBS, and six cultures with EGM-2 and 2% autologous serum. The cultures were detached using 0.05% Trypsin and 0.53 mmol/L EDTA in HBSS (Cellgro) every other day, and the cell-numbers were determined with a hemocytometer. Afterwards, the cells were plated again on a freshly fibronectin coated well.

Tube forming Assays

To determine the angiogenetic potential of the fat derived ECs, tube forming assays were performed using Matrigel (Invitrogen, Geltrex Reduced Growth Factor Basement Membrane Matrix). 96 well plates were coated with 150 μL of Matrigel. After detaching the ECs with 0.05% Trypsin and 0.53 mmol/L EDTA in HBSS (Cellgro), cells were plated in a density of about 1000 cells per 96 well plate. As a control rabbit carotid artery endothelial cells were used. After 24 hours, pictures of each well were taken, saved, and imported into Image Pro Plus software program. The total length of each tube or the long axis of single cells or groups of adjacent cells was measured. The assay was tested in triplicate and repeated three times.

Model of acute balloon injury

New Zealand White rabbits weighting 3.5 to 4.5 kg were anesthetized with Isoflorine. The left and the right common carotid artery were exposed from the suprasternal notch to just below the internal/external bifurcation. After looping with a 4-0 suture, vessel clamps were used to isolate the vessels. 8-0 purse-string suture was placed through which a small arteriotomy was created. A 2F Fogarty balloon catheter (Baxter) was introduced antegrade into the vessel lumen. The balloon was inflated to cause a

visible distension and withdrawn three times to denude a 3-cm length of artery as described elsewhere (Miranvill *et al.*, *Circulation*, 110(3):349-55 (2004)). Through the arteriotomy, a 24-gauge catheter was placed in the vessel lumen. In the right carotid artery, 250 μL of Ca^{2+} and Mg^{2+} PBS was injected, and in the left carotid artery, 250 μL of Ca^{2+} and Mg^{2+} PBS with FDECs (2.0 to 3.0 x 10^6 cells). The instillation time was 20 minutes for both sides. Afterwards, the arteriotomy was closed with a purse string suture, and the vessel clamps were removed to restore blood flow. To prevent clotting after the treatment, 81 mg ASA was administered per os daily to the rabbit.

Reendothelialization

After balloon injury, local delivery of PBS or PBS and 2-3 x 10^6 ECs where delivered. Euthanization was at 48 hours (group 1: n=20) with an overdose of pentobarbital. 20 minutes before group 1 rabbits were killed, they received an intravenous injection of 20 mL of 0.5% Evans Blue dye (sigma) to visualize the denuded vessel areas. After cleaning from adventitia and cutting of three 2 mm vessel rings for the histological evaluation, the vessel were cut longitudinal to allow planimetric analysis (Image ProPlus) of the reendothelialized area.

Histological evaluation

Rabbits were euthanized after 48 hours for group 1. As mentioned before, three rings of the carotid artery were cut from the proximal vessel side. The first ring was cut from a healthy vessel area, the second one from the border line in between, and the third one from the area were reendothelialization took place. Vessel pieces were frozen and cut with a microtome in approximately 10 μm sections. Staining for endothelial cells in the lumen of the carotid artery sections was performed with mouse anti-rabbit vWF and *Griffonia simplicifolia* lectin I isolectin B4 (1:100, BS-1 Lectin, Vector). The specimens were observed under a Nikon EFD-3 microscope, and pictures were taken with the RTKE Spot Digital Camera (Diagnostic Instruments Inc.)

Arterial Vasoreactivity

Four weeks after balloon injury and local delivery of saline or FDECs, the animals were euthanized. After dissection and cleaning, both carotid arteries were immediately cut into about 4 mm vessel rings (3 per artery). Those arterial rings were connected to isometric force displacement transducers and suspended in organ chambers filled with 25 mL of Krebs solution. The procedure was performed as described elsewhere (Gulati *et al.*, *Circulation*, 108:1520-1526 (2003) and Gulati *et al.*, *Am. J. Physiol. Heart Circ. Physiol.*, 287(2):H512-7 (2004)).

Morphometric Analysis

Carotid Artery segments from vasoreactivity studies were cut into two pieces of equal length, fixed in 10% formaldehyde, and imbedded in Paraffin. After removing a 200 μm section, 5 μm sections were generated and stained with hematoxylin and eosin. The slides were manually traced and analyzed with Image ProPlus. Endoluminal, internal elastic laminar, and external elastic lamina were used to calculate intimal and media areas.

Statistical Analysis and Graphs

Data were analyzed with SPSS software version 11.5 (SPSS Inc.) and PRISM GraphPad version 4.03. Comparison between groups was performed with student's t-test, and morphometric data were compared with unpaired t-tests. Vasoreactivity data was compared using ANOVA. A p-value < 0.05 was considered as statistically significant. Graphs were created with Prism GraphPad version 4.03 and Sigma Plot version 9.0. Data were presented as mean ± SEM.

Results

Cell grow and proliferation

Cells were obtained out of 3-5g of omental fat. After 6 hours in culture, first colonies were visible, and after 7 days in culture, the number of cells was 6.81 (+/- 0.83) million in EGM-2 with 2% autologous rabbit serum and 6.69 (+/- 0.91) million using EGM-2 with FBS. There was no significant difference in cell growth between using the

two different types of serum (Fig. 1C). The FDECs exhibited cobblestone morphology (Fig. 1B). The cells were highly proliferative, and after 14 days, the number reached 52 (+/-2.9) million cells in culture (Fig. 1D).

Characterization of Cultured Cells

Under confocal laser microscopy, cells were immunological tested for endothelial cell makers. Cells were positive for vWF, eNOS, VEGFR-2, and *Griffonia* lectin, and negative for SM-actin. Thus, the cells expressed markers of mature endothelial cells. Almost all cells exhibited uptake of DiI acetylated LDL in co-staining with eNOS (Fig. 2). In tube-forming assay using Matrigel, FRECs (9131 ± 1134) were forming more likely capillary-like tubes after 24 hours than rabbit carotid artery endothelial cells (4593 ± 1526) $p<0.01$ (Fig. 3). These results demonstrate that cells obtained from omental fat generate a homogeneous and highly proliferative population of ECs.

To study the potential of enhanced reendothelialization and vascular repair, a rabbit model of acute balloon injury of both carotid arteries was used. Rabbits were treated with 2-3 x 10^6 DiI acetylated LDL labeled cells, and after 48 hours intraluminal coverage of endothelial cells was measured with the absence of Evans-Blue staining as a macroscopic method and with cross-sections of the vessels and the identification of labeled cells. 48 hours after treatment, the vessels in which cells were delivered exhibited almost complete exclusion of Evans- Blue, while the control vessels were stained entirely blue. A mean coverage of 82.2 % (± 26.9) of the treated vessel against 4.2 % (± 3.0) of the untreated vessel ($p<0.001$) was detected (Fig. 4). Immunofluorescence imaging with DiI acetylated LDL revealed intraluminal coverage of delivered cells (Fig. 5A). In cross-sections of the injured and treated vessels, vWF and *Griffonia* lectin staining was performed and revealed positivity through out the whole vessel wall (Fig. 5B).

Arterial Vasoreactivity

To determine the long term effects on vascular function and structure, the same procedure and the same animal model of acute balloon injury of both carotid arteries was used. Rabbits were treated with 2-3 x 10^6 DiI acetylated LDL labeled cells for the group of rabbits which were treated with unsorted cells (n=8). The same amount of cells, but

with DiO acetylated LDL staining was used for the group of rabbits where FACS sorted cells were used (n=12). To investigate the effects of cells delivered to the injured vessel, endothelial dependent vasoreactivity of carotid artery rings was evaluated four weeks after surgery. After precontraction in the organ chamber, ring relaxation in response to acetylcholine concentration was measured (Fig. 6). Maximal relaxation was in both groups significantly enhanced compared to the saline treated vessel. Carotid artery rings from the saline treated side exhibited a relaxation of 16.2 % (± 3.4). Unselected and selected FDECs improved the maximal relaxation significantly (unselected: 62.1 % (± 8.6), selected: 66.3 % (± 4.1)). Both groups of cell treated vessels exhibited a significant increase of endothelial dependent vasorelaxation ($p < 0.01$) compared to the saline (control) side. Selection did not bring any improvement. There was almost no difference in response to acetylcholine between the selected and the unselected cells. These results demonstrate that the treatment of balloon injured vessels with FDECs remarkably improves the vasoreactivity although the response to acetylcholine did not achieve that of a normal uninjured vessel.

Morphometric Analysis

The effect of FDEC delivery on neointimal formation was determined. The comparison between control (0.49 ± 0.03) and unselected FDECs (0.40 ± 0.09) did not reveal any difference. FDECs selected with DiO acetylated LDL (0.31 ± 0.03) exhibited a significant improvement and lower amount of intimal thickening as compared to the saline treated control (Fig. 7, $p<0.001$). These results suggest that the local delivery of FDECs in balloon injured vessels does not only improve the endothelial dependent vasoreactivity but also decreases neointimal formation after mechanical injury.

The results provided herein demonstrate that autologous microvascular endothelial cells derived from omental fat can be used to enhance re-endothelialization in 48 hours almost completely. Moreover, FDEC delivery was associated with an increase of endothelium dependent vasoreactivity and a decrease in neointimal formation compared to the controls, which were treated with saline. The results provided herein also demonstrate that only a very low amount of fat (3-5 g) is necessary to obtain a very high number of phenotypical and functional microvascular endothelial cells. In addition, the

results provided herein demonstrate that autologous serum can be used to culture those highly proliferative cells.

The astonishingly fast reendothelialization that could be proved after 48 hours with an almost complete lack of Evans Blue at the vessel lumen and the proof of DiI acetylated LDL positive cells throughout the whole vessel lumen help demonstrate that FDECs can be used effectively to vascular repair. In addition, a four times increase of endothelium-dependent vasoreactivity was observed. It seems like that the purity of the FDECs can be increased because the unselected FDECs did not exhibit a beneficial effect on neointimal formation.

After 7 or 10 days in culture, the cells were stained for endothelial and smooth muscle markers, and the purity of endothelial cells was very high. We could not find any cells that did not express endothelial markers like eNOS and vWF, and we could hardly find cells positive for smooth muscle actin in the slides. DiO acetylated LDL was used as a functional marker to select positive cells with FACS to enhance the purity of FDECs. Even though most of the cultures were highly positive for DiO acetylated LDL, we did gate only for 75% of the highest positive cells to improve the purity of the cells. Using this method, we could highlight next to the 4 times increase of endothelium dependent vasoreactivity also a 60% decrease in neointimal formation.

In conclusion, the results provided herein indicate that FDECs provide a extremely fast reendothelialization and an improvement in vascular function and morphology after vessel injury. In addition, the results provided herein demonstrate that FDECs are highly proliferative, have a high angiogenic potential, and can be cultured and used with autologous serum. Thus, FDECs can be used to treat vessel injuries and arterial occlusive disease.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

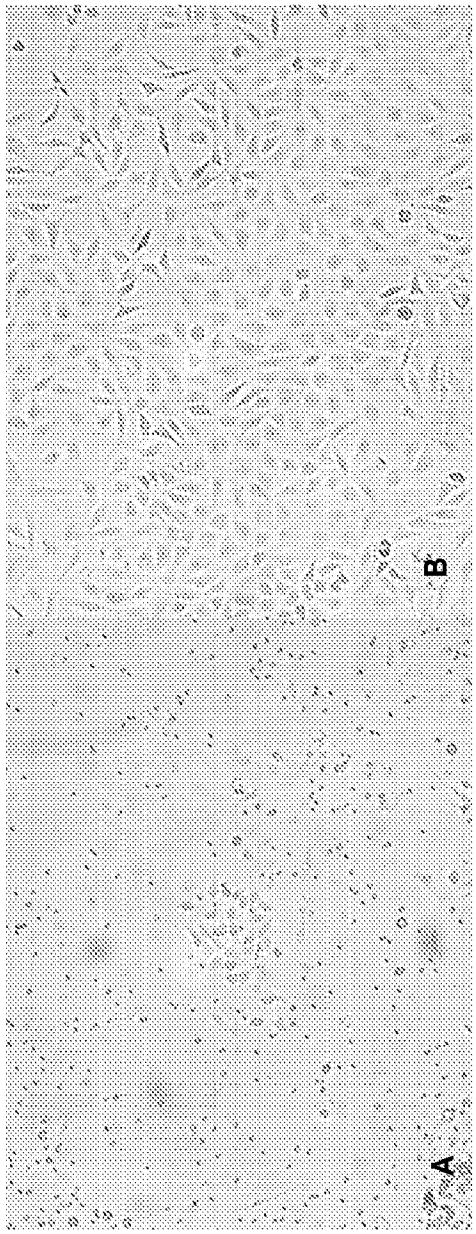
WHAT IS CLAIMED IS:

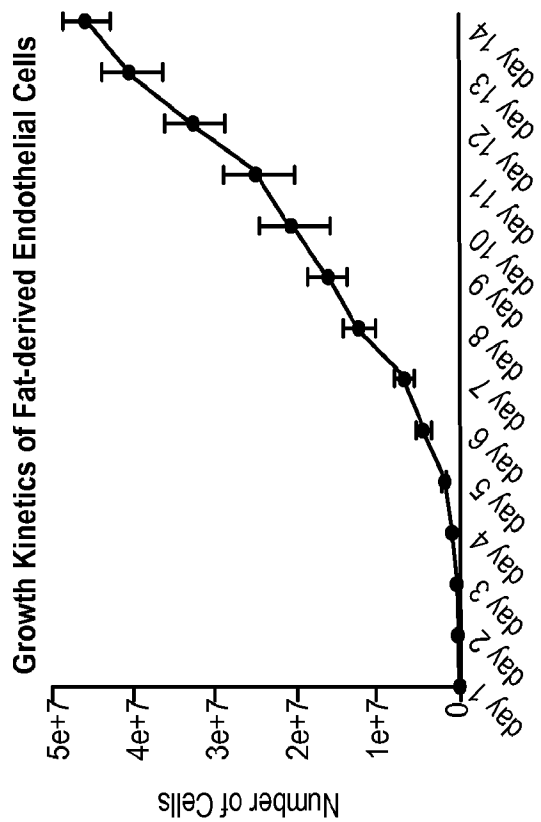
1. An enriched population of fat derived endothelial cells.

2. The enriched population of fat derived endothelial cells of claim 1, wherein said cells are positive for vWF, eNOS, VEGFR-2, and *Griffonia* lectin, and negative for SM-actin.

3. A method for isolating an enriched population of fat derived endothelial cells, said method comprising obtaining DiO acetylated LDL positive endothelial cells from a population of cells.

4. A method for repairing vascular tissue in a mammal, wherein said method comprises administering an amount of an enriched population of fat derived endothelial cells to said mammal under conditions effective to repair vascular tissue.


A
B


Growth Kinetics of Fat-derived Endothelial Cells
Number of Cells
5e+7
4e+7
3e+7
2e+7
1e+7
0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
day 8
day 9
day 10
day 11
day 12
day 13
day 14

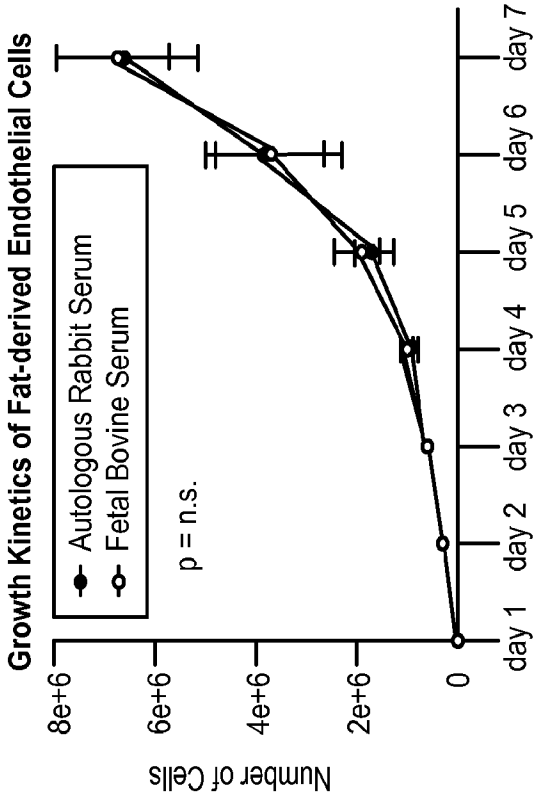
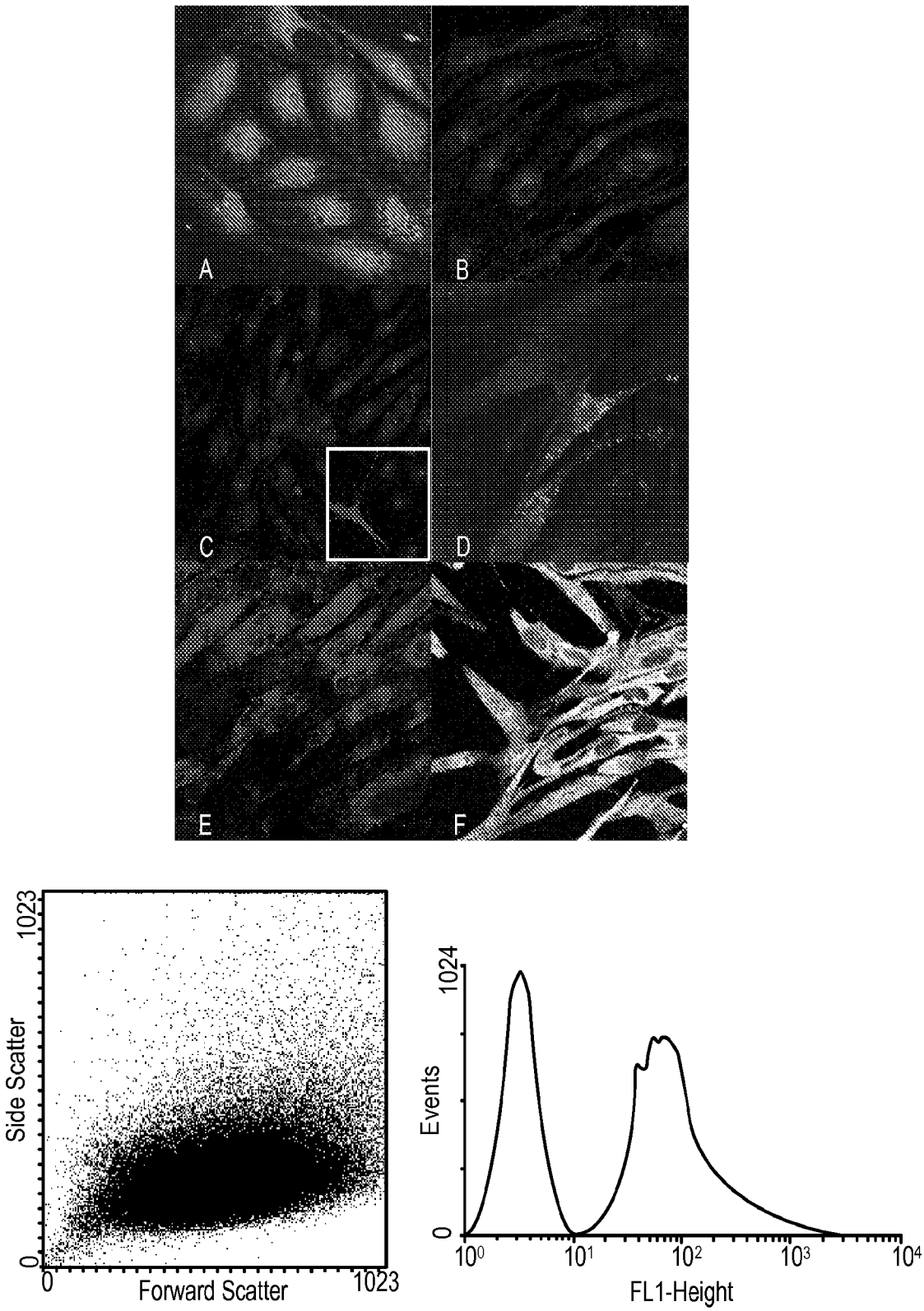

Growth Kinetics of Fat-derived Endothelial Cells
Autologous Rabbit Serum
Fetal Bovine Serum
p = n.s.
Number of Cells
8e+6
6e+6
4e+6
2e+6
0
day 1
day 2
day 3
day 4
day 5
day 6
day 7

FIG. 1


A
B
C
D
E
F
Side Scatter
1023
0
0
Forward Scatter
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Events
1024
0
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10^1
10^2
10^3
10^4
FL1-Height

FIG. 2

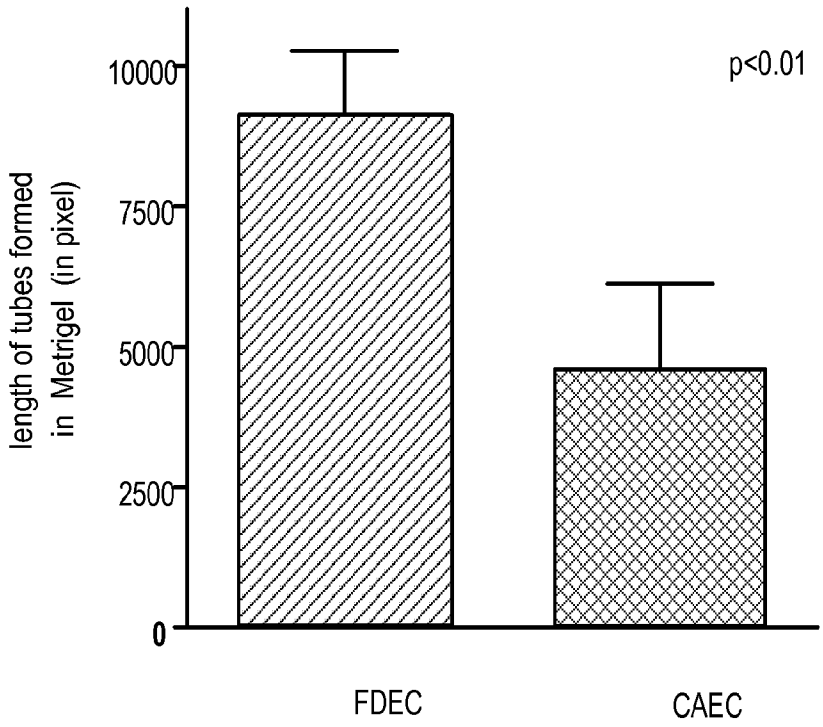
length of tubes formed in Metrigel (in pixel)
10000
7500
5000
2500
0
p<0.01
FDEC
CAEC

FIG. 3

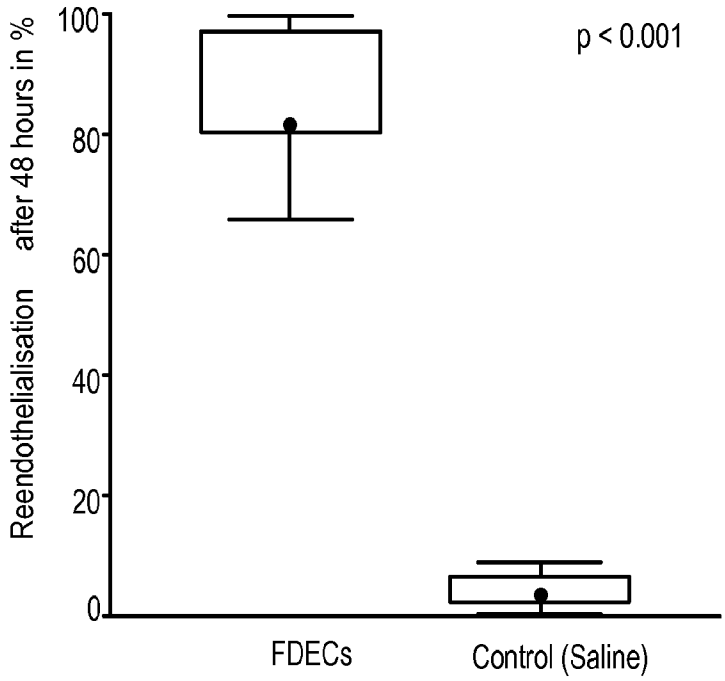
Reendothelialisation after 48 hours in %
100
80
60
40
20
0
p < 0.001
FDECs
Control (Saline)

FIG. 4

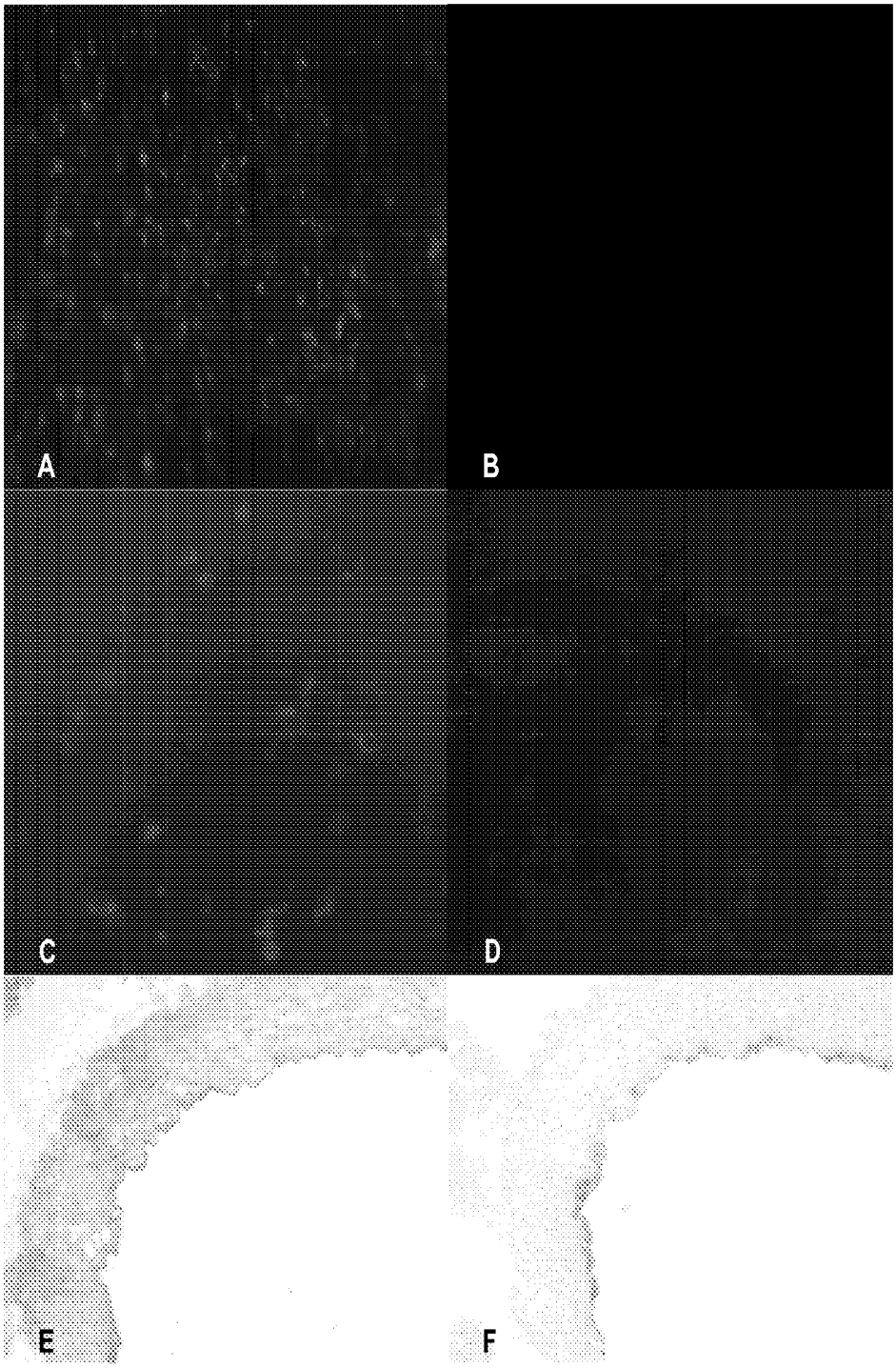

FIG. 5

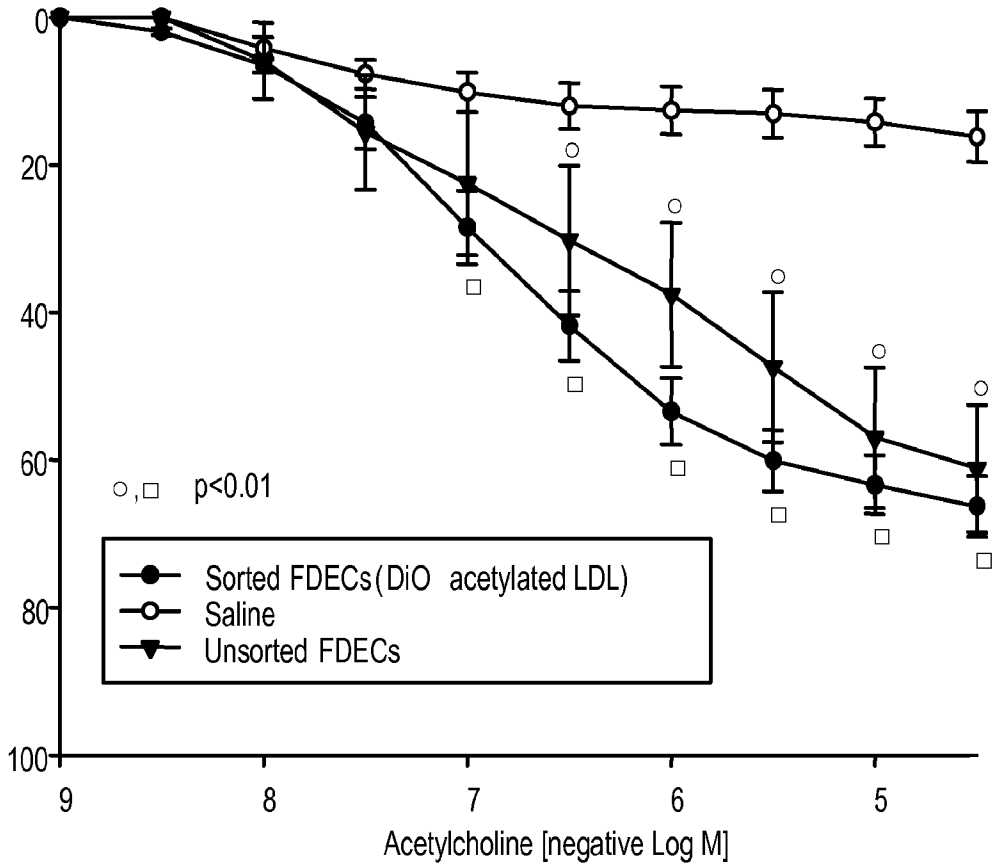
0
20
40
60
80
100
9
8
7
6
5
Acetylcholine [negative Log M]
○,□ p<0.01
Sorted FDECs(DiO acetylated LDL)
Saline
Unsorted FDECs

FIG. 6

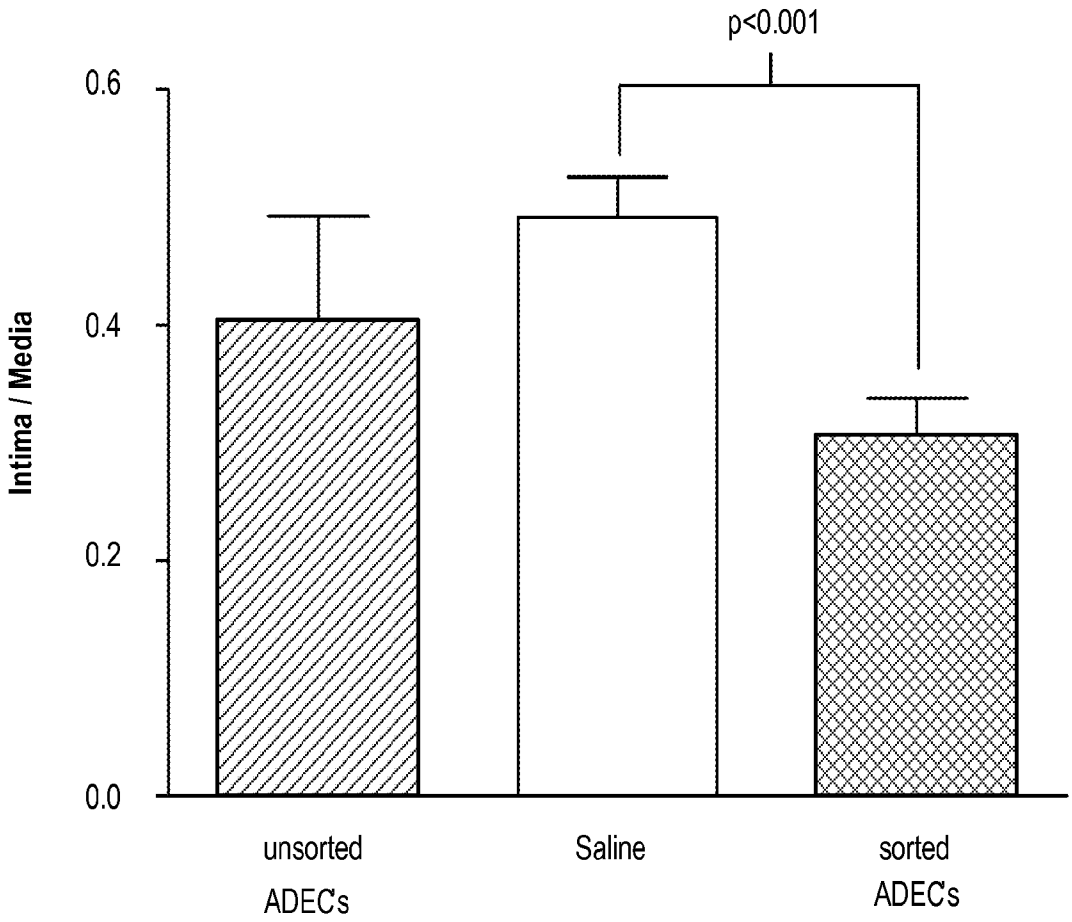
p<0.001
0.6
0.4
0.2
0.0
Intima / Media
unsorted ADEC's
Saline
sorted ADEC's

FIG. 7