Phenotypic and Functional Changes in Regenerated Porcine Coronary Endothelial Cells

Increased Uptake of Modified LDL and Reduced Production of NO

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Abstract—Porcine coronary arteries with regenerated endothelium exhibit impaired endothelium-dependent relaxations. Experiments were designed to analyze the structural and functional changes occurring in regenerated endothelial cells. Primary cultures from regenerated endothelium contained giant endothelial cells, with an increased number of cells with diameter >14.5 μm, a reduced ability to proliferate, and signs of apoptosis. The uptake of fluorescent acetylated LDL was increased 2-fold in cultures from regenerated endothelium. The increased uptake of acetylated LDL was confirmed ex vivo in injured coronary arteries. In cultures from regenerated endothelium, cGMP production was decreased under basal conditions and during stimulation with serotonin, bradykinin, and A23187. Thus, during regeneration, there is accelerated senescence of endothelial cells accompanied by increased incorporation of modified LDL and reduction of NO production without decrease in endothelial NO synthase expression. These alterations help to explain the altered endothelium-dependent responses 28 days after balloon injury. (Circ Res. 2000;86:854-861.)

Key Words: endothelial dysfunction ■ modified LDL ■ cGMP ■ endothelial NO synthase ■ senescence

In porcine coronary arteries, removal of the endothelium in vivo leads to proliferation of vascular smooth muscle. The adjacent endothelial cells migrate and proliferate to reline the vascular wall. Twenty-eight days after injury, coronary arteries with regenerated endothelium exhibit impaired endothelium-dependent relaxations to serotonin or α₂-adrenergic agonists, whereas those to ADP or bradykinin are maintained. 1,2 This selective dysfunction of the pertussis toxin–sensitive signaling pathway is accompanied by a morphological heterogeneity of the regenerated endothelial cells, 3 as is also observed with atherosclerosis and senescence. 4–6 The present study was designed to further compare cells derived from control and regenerated endothelium.

Materials and Methods

Denudation

These experiments were carried out in accordance with the guidelines of the French Ministry of Agriculture for the use and care of animals. Large White pigs (18 to 25 kg) were anesthetized by intramuscular injection of tiletamine and zolazepan (15 mg/kg) containing atropine sulfate (50 μg/kg). The animals were intubated and ventilated with a respirator. Part of the left anterior coronary artery was denuded by inflating a balloon catheter. 3 Twenty-eight days later, the animals were sedated (intramuscular zolletil) and euthanized by exsanguination.

Histology

Control and previously denuded coronary arteries were immersed in cold physiological salt solution containing (in mmol/L) CaCl₂ 2.5, EDTA 0.016, NaCl 118, NaHCO₃ 24.8, KH₂PO₄ 1.18, KCl 4.7, MgSO₄ 1.2, and glucose 11. A ring of each artery was fixed (4% formaldehyde). Different nonserial cross sections (5 mm in length, 200 mm apart) were prepared from paraffin blocks and stained with hematoxylin-eosin-safran for light microscopy.

Primary Cultures

Endothelial cells from native and injured coronary arteries were cultured as described. 3

Cell Size

Light Microscopy

Cells on 24-well plates were fixed (ethanol), stained (Hemacolor reagents; Merck) 7 and examined with a computerized image-analysis system (Histo Software, Biocom).

Flow Cytometry

Cells were washed with EBSS and treated with trypsin-EDTA to obtain a single-cell suspension. Diameter was analyzed with a flow cytometer (EPICS XL/MCL (Beckman Coulter, Villepinte, France).

DNA Content

DNA content was determined in permeabilized fixed cells with propidium iodide. 8 Subconfluent cultures were trypsinized, washed, fixed/permeabilized (ice-cold 70% ethanol overnight at −20°C), incubated with 50 μg/mL propidium iodide (Sigma Chemical Co)
Mitochondrial Alterations

Transmembrane mitochondrial potential and oxidative capacity were measured with 3,3′-dihexylocarbocyanine iodide (40 nmol/L) and dihydroethidium (20 μmol/L), respectively (Molecular Probes).

LDL

LDL (d=1.019 to 1.063) from plasma of healthy, normolipidic volunteers was oxidized by exposure to 5 μmol/L CuSO4 at 37°C for 24 hours. Oxidation was arrested with 200 μmol/L EDTA. Oxidized LDL was dialyzed against PBS-200 μmol/L EDTA at 4°C. The protein concentration was determined. The amount of thiobarbituric acid–reactive lipid peroxides was checked using a colorimetric assay and malondialdehyde as a standard. Thiobarbituric acid reactivity of 24-hour–oxidized LDL after dialysis was 5.1±1.1 nmol of malondialdehyde/mg of protein (starting LDL, 0.8±0.6 nmol/mg).

Uptake of Fluorescent LDL

Confluent monolayers (passage 1) were incubated with 5 μg/mL fluorescence-conjugated acetylated LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil; 37°C in a CO2 incubator). Then, cells were trypsinized to obtain a single-cell suspension. The incorporation of Dil-LDL was quantified by flow cytometry. Cells were incubated with Dil-acetylated LDL for 4 hours or with Dil-native LDL for 5 hours. The concentration of acetylated LDL needed for half-maximal incorporation (EC50) was determined using Micropharm software (LOGINSERM).

For competition studies, cells were incubated with Dil-acetylated LDL (1 μg/mL) and 50 μg/mL of native LDL, modified LDL, or polyinosinic acid (2 hours at 37°C). Coronary arteries with control or regenerated endothelium were incubated ex vivo with Dil-acetylated LDL (5 μg/mL, for 4 hours, at 37°C in a CO2 incubator). For microscopy, the coronary arteries were fixed with 4% formaldehyde and frozen. To perform flow cytometry, cells were harvested and trypsinized.

cGMP

Endothelial cells seeded in 96-well plates were placed in HEPES-Tris buffer (20 mmol/L) containing (in mmol/L) NaCl 140, KCl 5.4, CaCl2 2.4, and MgSO4 0.7, including 10 μmol/L indomethacin and 100 μmol/L isobutyl methylxanthine. cGMP production was determined under basal conditions and after 1 minute of stimulation with agonists under agitation (37°C). Agonists were the following (in μmol/L): bradykinin 0.1, serotonin 1, and A23187 1. The level of cGMP was measured by radioimmunoassay (Amersham, Amerlex method). Cellular density in each well was defined (Hemacolor method). Results are expressed as fmol per million cells. Cells from control and regenerated endothelium were studied in parallel.

NO Synthase (NOS) Activity

After trypsinization, cells were collected in buffer containing 50 mmol/L Tris base, EGTA (100 μmol/L), DTT (100 μmol/L), and a mixture of protease inhibitors, pH 7.4 at 4°C. For control cell membranes, cells were sonicated (20 strokes) on ice and centrifuged (200,000g for 60 minutes). The protein content of the pellet was measured.

The assay was conducted at 37°C for 1 hour under agitation in a reaction mixture of (in μmol/L) [14C]arginine (11 GBq/mmol) 1.7, calmodulin 1, flavin adenine dinucleotide and flavin mononucleotide 1, and tetrahydrobiopterin 50, as well as (in mmol/L) DTT 1, NADPH 1, and CaCl2 2, with 100 μg of cell membrane protein. The assay was terminated with HClO4 (11.6 mol/L; 4°C). The mixture was centrifuged (1000g, 10 minutes). The supernatant was analyzed by HPLC. [14C]Arginine, [14C]ornithine, and [14C]citrulline were separated using a 150×4.6-mm Hypersil BDS C18 column isocratically eluted at 1 mL/min with 4 mL heptafluorobutyric acid, 125 mL acetonitrile, and 900 mL water. The radioactivity of the effluent was recorded. NOS activity was expressed as citrulline formed per minute and per mg of protein.

Endothelial NOS (eNOS) Expression

For immunocytochemistry staining of eNOS, monoclonal antibodies (20 μg/mL) recognizing particulate eNOS were applied (room temperature) for 1 hour. Two types of secondary antibodies were used, Alexa 488 rabbit anti-mouse (0.2 μg/mL) and 125I-labeled sheep anti-mouse (0.02 μg/mL). All washes were performed with PBS–1% BSA.

Statistical Analysis

Data are expressed as mean±SEM; n refers to the number of animals. Statistical evaluation was performed by paired Student t test. Multiple comparisons of kinetic curves are based on the Newman-Keuls test. Differences were considered to be significant at P<0.05. An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Histology

Twenty-eight days after balloon injury, porcine coronary arteries were covered with regenerated endothelium and exhibited myointimal thickening (Figure 1).
Cell Size
In primary culture, control cells were mostly uniform in size and displayed a cobblestone pattern (Figure 1). Regenerated cells were heterogeneous with the presence of sparse giant cells. Such cells incorporated Dil-acetylated LDLs and were not stained by antibodies against anti-smooth muscle α-actin (not shown). Flow cytometry (Figure 2) revealed that in control and regenerated cultures, the majority of endothelial cells had a diameter of ≈11 μm. Cells with a diameter >14.5 μm were significantly more numerous in cultures from regenerated than those from control endothelium. Counting of giant cells confirmed a significant increase in the number of enlarged cells after denudation (4.17 ± 1.69 and 9.88 ± 3.3 per 1000 cells for control and regenerated cells, respectively).

Proliferation
The percentage of cells in S and G2/M phases was significantly lower in cultures from regenerated than control endothelium (11.6 ± 0.9 versus 18.9 ± 2.7%, respectively). The reduction in proliferating cells was associated with a significant increase in the sub-G1 population relative to apoptotic cells in cultures from regenerated than those from control endothelium. Counting of giant cells confirmed a significant increase in the number of enlarged cells after denudation (4.17 ± 1.69 and 9.88 ± 3.3 per 1000 cells for control and regenerated cells, respectively).

Mitochondrial Dysfunction
An increased oxidation of dihydroethidium associated with a decreased mitochondrial transmembrane potential was revealed by the biparametric analysis of cultures from regenerated cells. A greater number of cells with altered mitochondrial function was observed in regenerated endothelial cells (Figure 3).

Acetylated LDL
Cultured endothelial cells from control and regenerated endothelium incorporated fluorescent acetylated LDL (Figure 4). Smooth muscle cells remained unstained under the same conditions (not shown). The incorporation of acetylated LDL was time and concentration dependent (Figure 5). It was significantly lower for control than for regenerated cells (Figure 5). The EC50 was similar for the 2 populations of cells (8 to 10 μg/mL). No significant difference in native LDL incorporation was detected between control and regenerated cells (Figure 5).

Incorporation of Acetylated LDL Ex Vivo
Sections of artery with regenerated (Figure 6) or native (not shown) endothelium demonstrated incorporation of...
acetylated LDL in endothelial cells, whereas the underlying smooth muscle cells remained unstained. Flow cytometry revealed that regenerated endothelium exhibited a significantly greater incorporation of acetylated LDL than of native endothelium (Figure 6).

cGMP

N-Nitro-L-arginine (NLA; 10 μmol/L) inhibited basal and bradykinin-induced cGMP production in endothelial cells (basal, 2273±258, and with NLA, 587±64 fmol/million cells; stimulated, 4360±134, and with NLA, 627±127 fmol/million cells). Oxadiazoloquinoxalin (ODQ, 1 μmol/L), a soluble guanylate cyclase inhibitor, also inhibited basal and bradykinin-stimulated production of cGMP in endothelial cells (basal, 1203±328, and with ODQ, 458±69 fmol/million cells; stimulated, 4095±1560, and with ODQ, 638±128 fmol/million cells; n=6). The basal cGMP production was decreased significantly (by 55%) in cultures from regenerated endothelium. cGMP production induced by bradykinin (0.1 μmol/L), serotonin (1 μmol/L), and calcium ionophore (A23187, 1 μmol/L) was also reduced (Table).
NOS Activity

In cell membranes from both control and regenerated endothelium, the citrulline production was abolished by LNA (10 μmol/L) without formation of ornithine (Figure 7). The NOS activity (V_max) and the Michaelis constant (K_m) of membranes from native endothelial cells were 1.12±0.10 pmol/min×mg protein⁻¹ and 0.61±0.06 μmol/L (n=4), respectively. The basal NOS activity of membranes from regenerated endothelial cells was significantly lower than that measured in membranes of native endothelial cells (Figure 7).

eNOS Expression

The nonspecific fluorescence represented <14% whatever the origin of the cells, indicating the specificity of the staining. The fluorescence values for control and regenerated endothelial cells were not different (16.5±1.1 and 16.8±1.2 arbitrary units; n=4). The flow cytometric biparametric analysis, comparing the intensity of the fluorescence as a function of cell size, did not show differences in eNOS expression between normal and large endothelial cells.

Likewise, no significant difference in eNOS expression was found between control and regenerated endothelial cells using 125I-labeled secondary antibodies (94 090±19 541 and 79 073±14 555 cpm/million cells, respectively; n=9).

Discussion

In primary culture, cells from regenerated endothelium were morphologically more heterogenous than those from

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**Table 1. Effect of Bradykinin, Serotonin, and Calcium Ionophore on cGMP Production in Cultures From Control and Regenerated Endothelium**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Regenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>4319±438</td>
<td>1831±323*</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>9260±1213</td>
<td>2639±671*</td>
</tr>
<tr>
<td>Basal</td>
<td>3587±443</td>
<td>1672±368*</td>
</tr>
<tr>
<td>Serotonin</td>
<td>4639±796</td>
<td>2773±813*</td>
</tr>
<tr>
<td>Calcium ionophore A23187</td>
<td>11 868±2011</td>
<td>8393±1993*</td>
</tr>
</tbody>
</table>

Experiments performed in the presence of indomethacin (10 μmol/L) and isobutyl-L-methyl xanthine (100 μmol/L). Results are shown in fmol/million cells (mean±SEM; n=9).

*Significant differences between the cGMP production in cells from control and regenerated endothelium (P<0.05).
control endothelium. Quantitative analysis of endothelial cell size variability revealed a significant increase in the number of large and giant cells. These atypical cells were characterized as endothelial cells, because they stained positively with von Willebrand factor antibody, incorporated Dil-acetylated LDLs, and did not stain with monoclonal antibodies specific for smooth muscle cells. Morphological similarities exist between cells from porcine regenerated endothelium and the giant cells contained in the endothelium taken from elderly patients, or regions submitted to increased hemodynamic stress. Hence, even if the factor or factors contributing to their formation are not necessarily the same, the accumulation of enlarged endothelial cells represents a marker of injured endothelium.

The present findings suggest that cells from regenerated endothelium have a reduced capacity to proliferate. In vitro, increase in cell volume and decline in proliferative capacity characterize endothelium that becomes senescent after a large number of subcultures. Hence, the numerous endothelial cell doublings that the cells undergo in vivo to repopulate the luminal surface of the artery after balloon injury could be sufficient to induce an altered phenotype of cells. In mammals, cells damaged by age are eliminated by apoptosis. The presence of apoptotic events in control and cells. In mammals, cells damaged by age are eliminated by apoptosis. The presence of apoptotic events in control and regenerated endothelium was evaluated by measuring par-apoptosis. The presence of apoptotic events in control and cells. In mammals, cells damaged by age are eliminated by apoptosis. The presence of apoptotic events in control and regenerated endothelium was evaluated by measuring par-

A major finding of the present study is that all cells from regenerated endothelium in porcine coronary arteries exhibit an increased uptake of modified LDL. The global shift in fluorescence intensity of cells from regenerated endothelium demonstrates that both normal and enlarged cells present an increased ability to incorporate the modified LDL. In parallel, ex vivo arteries with regenerated endothelium exhibited a greater ability to incorporate modified LDL than those with native endothelium. Thus, the increased uptake of modified LDL was not due to the culture conditions but to an alteration of the phenotype of cells during the regeneration process.

The incorporation of acetylated LDL was saturable, with an identical apparent EC₅₀ in both types of cells. The maximal uptake was 2-fold higher in cultures from regenerated than those from native endothelium. The remaining nonspecific staining was comparable, demonstrating that the greater fluorescence intensity of regenerated cells is not due to a higher nonspecific staining. These findings suggest an increase in acetylated LDL (scavenger) receptor density in cells from denuded blood vessels. These receptors are present constitutively on endothelial cells and exhibit characteristic ligand specificity different from receptors for native LDL. The modifications of LDL that convert it into a high-affinity ligand for the scavenger receptors include acetylation and oxidation. These binding sites share the property of being completely inhibited by polyinosinic acid but not by native LDL. This is confirmed by the competition experiments performed in the present study in cells from both control and regenerated endothelium. Only a partial competition of acetylated LDL uptake by oxidized LDL was observed. Nonreciprocal cross-competition between modified lipoproteins does not necessarily imply the presence of receptors specific only for acetylated LDL rather than receptors that recognize acetylated and oxidized LDL. Different binding sites on the same receptor or the extent of oxidation of the modified LDL could be involved. The increased degree of competition of acetylated LDL uptake with oxidized LDL in cells from regenerated endothelium demonstrates an increased capacity of such cells to take up oxidized LDL through a scavenger receptor that recognizes both oxidized and acetylated LDL.

As in macrophages, the uptake of acetylated LDL is increased in endothelial cells from older rats. In bovine aortic endothelial cells, growth cessation acts as a signal for upregulation of scavenger receptor activity. Certain inflammatory cytokines upregulate the expression of receptors for modified LDL in macrophages. In the present study, the persisting increased uptake of acetylated LDL in cells from regenerated endothelium separated from components of regenerated lesions implies an intrinsic (genetic) modification of these cells. The numerous cell doublings could be implicated, given that an increased uptake of acetylated LDL has been observed after several passages in vitro of endothelial cells from control porcine coronary arteries (data not shown).

Arteries exposed to modified LDL exhibit impaired endothelium-dependent responses, a phenomenon closely resembling that observed in atherosclerotic arteries. Oxidized LDL or its lipid constituents induce a selective alteration of endothelium-dependent relaxations similar to that observed 28 days after denudation. Transendothelial cholesterol transport takes place at caveolae, where endothelial signal transductions, such as G proteins and NOS, are situated. This colocalization in the plasma membrane may facilitate the interaction of these transduction signals. A second major finding was that the production of NO is reduced in cultures from regenerated endo-
theilium. Indeed, the formation of NO measured as increases in intracellular cGMP under basal conditions or that induced by various agonists was decreased. Likewise, the activity of eNOS, estimated by measuring the citrulline formed during the oxidation of arginine, demonstrated that indeed the enzyme activity is decreased in cultures from regenerated endothelium, which then explains a reduced production of cGMP. However, this abnormal enzyme activity cannot be explained by a decrease of eNOS expression in cultures from previously injured coronary arteries.

In arteries with regenerated endothelium, the relaxation in response to serotonin is impaired severely, whereas that due to bradykinin is mildly to moderately reduced and that due to the calcium ionophore remains unchanged; this appears to be related to the ability of these agonists to stimulate the production of cGMP in the regenerated endothelium. Contrary to the relaxations induced by both bradykinin and the calcium ionophore for which the decreased production of NO can be compensated by the release of endothelium-derived hyperpolarizing factor, serotonin exclusively stimulated NO production and so fails to induce relaxation of coronary arteries with regenerated endothelium. The present findings permit us to propose an explanation for the endothelial dysfunction in arteries with regenerated endothelium. The present findings permit us to propose an explanation for the endothelial dysfunction in which the pertussis toxin–sensitive G protein pathway is impaired severely, whereas that induced by various agonists was decreased. Likewise, this phenomenon modifies the redox homeostasis of the endothelial cells and alters the function of G proteins and of the NOS involved in the endothelium-dependent relaxations.

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References


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