Recovery of Senescent Endothelial Cells From Injury

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Percutaneous coronary intervention (PCI) is an effective and safe means of coronary revascularization. The main long-term complication of the procedure is restenosis. Although the introduction of stents radically reduced its incidence, restenosis of the treated vessel still remains a significant clinical problem (see ref. [1] for a recent review). The pathophysiology of restenosis is not entirely clear, however, the PCI-induced endothelial cell (EC) injury and subsequent inflammation are thought to be key triggering factors. Denudation of the endothelium and subintimal hemorrhage exposes vascular smooth muscle cells (VSMC) to serum-derived growth factors, resulting in VSMC proliferation and neointima formation. It has been observed that minor EC injuries are associated with only minimal intimal hyperplasia, whereas extensive endothelial denudation results in greater intimal thickening (2). Moreover, VSMC seem to appear in the neointima only in those areas that fail to re-endothelialize soon after injury (3). Thus, prompt restoration of the endothelial integrity is of paramount importance for the prevention of restenosis.

Ageing is a well-known risk factor for cardiovascular disease. Indeed, elderly patients are more likely to suffer from PCI-related complications as a result of both age-associated physiological decline and comorbidities (4). On the other hand, PCI may significantly improve health-related quality of life of elderly patients (5,6). Although animal studies indicate that the responsiveness of VSMC to growth-promoting stimuli may increase with age and thus contribute to neointima formation (7), the existence of a direct link between advanced age and post-PCI restenosis is not certain. Its presence has been suggested by some studies (8), but not the others (9). The issue bears major clinical significance as almost 25% of all PCIs are performed in patients aged 75 and older (4).

Re-endothelialization of denuded areas can occur through cell proliferation, cell migration from wound edges, and by repopulation with bone marrow–derived progenitor cells (10). Because loss of proliferative potential is a hallmark of cellular senescence, one may theorize that ageing will lead to delayed endothelialization. In addition to irreversible growth arrest, the phenotype of senescent ECs is characterized by altered secretion of adhesion molecules, cytokines, and the regulators of vascular tone and fibrinolysis (11). It is not clear, however, how senescence affects the release of nitric oxide (NO) by ECs. Although NO bioavailability declines with age (12), the expression of endothelial eNOS has been reported to decrease, increase or remain unchanged with ageing (13). Clarification of this detail is important given that NO was suggested to support EC regeneration after balloon injury (14).

In the present study, we have therefore examined how young and old ECs in culture recover from scratch injuries mimicking those occurring during PCI.

Methods

Unless indicated otherwise, all reagents were from Sigma-Aldrich. Cell culture plastics were from Nunc and Costar.
WOUND HEALING IN SENESCENT ENDOTHELIAL CELLS

Cell Culture

Clonetics human umbilical vein endothelial cells (HUVEC) pooled from several donors were obtained from Lonza. Cells were propagated in M199 culture medium, supplemented with amphotericin (2.5 \( \mu \)g/mL), gentamycin (50 \( \mu \)g/mL), L-glutamine (2 mM), HEPES (25 mM), hydrocortisone (1 \( \mu \)g/mL), heparin (10 U/mL), EGF (10 ng/mL), and 15% v/v fetal calf serum (Invitrogen).

Induction of Cell Senescence

Replicative senescence of HUVEC was induced by serial passages performed at weekly intervals with a seeding density of 5 \( \times \) 10^3 cells/cm^2. Cells were regarded cells as “young” when the percentage of cells expressing senescence-associated \( \beta \)-galactosidase (SA-\( \beta \)-Gal; 15) was less than 10% of the population, which corresponded to less than 28 days in culture (Figure 1C). In contrast, cultures were considered senescent when cells did not increase in number during 4 weeks, exhibited altered morphology, and when more than 70% of cells stained positively for SA-\( \beta \)-Gal. This corresponded to more than 150 days in culture.

Cell Proliferation Rate

HUVEC proliferation was assessed by \[^{3}H\]-thymidine incorporation. Briefly, cells were plated at a density of 2 \( \times \) 10^4 cells/cm^2, allowed to attach for 4 hours and then pulsed with \[^{3}H\]-thymidine (1 \( \mu \)Ci/mL; Institute of Radioisotopes, Prague, Czech Republic) for 24 hours. The cells were harvested, precipitated with 10% (w/v) trichloroacetic acid, and dissolved in 0.1 M NaOH. The radioactivity released was measured in a beta liquid scintillation counter (Wallac Perkin Elmer).

Cell Migration

HUVEC migration was assessed with the use of QCM Chemotaxis 96-well Cell Migration Assay with 8 \( \mu \)m pore size membranes (Chemicon/Milipore). Cells at ~80% confluence were rendered quiescent by incubation in culture medium with reduced FCS concentration (2%) for 24 hours. After that cells were harvested, washed, resuspended in serum-free medium, and placed in a fibronectin-coated (1 \( \mu \)g/mL) migration chamber (5 \( \times \) 10^4 cells/100 \( \mu \)L). Cells were then stimulated with the standard 15% FCS-containing medium for
Table 1. Characteristics of Senescent Human Umbilical Vein Endothelial Cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young</th>
<th>Old</th>
<th>Significance (p Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability, %</td>
<td>89 ± 6</td>
<td>87 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Cell size, μm²</td>
<td>758 ± 253</td>
<td>3,885 ± 1,756</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Nucleus size, μm²</td>
<td>220 ± 85</td>
<td>434 ± 202</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Cytoplasm/nucleus surface area</td>
<td>2.8 ± 1.6</td>
<td>9.0 ± 5.3</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Protein contents, pg/10⁵ cells</td>
<td>92.8 ± 4.6</td>
<td>130.2 ± 6.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IL-6, pg/10⁵ cells</td>
<td>38 ± 18</td>
<td>216 ± 101</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>IL-8/CXCL8, pg/10⁵ cells</td>
<td>1,613 ± 172</td>
<td>6,884 ± 736</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>MCP-1/CCL2, pg/10⁵ cells</td>
<td>618 ± 288</td>
<td>1,770 ± 612</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>TGF-β1, pg/10⁵ cells</td>
<td>60 ± 11</td>
<td>115 ± 32</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>VEGF, pg/10⁵ cells</td>
<td>93 ± 24</td>
<td>221 ± 101</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>sICAM-1, pg/10⁵ cells</td>
<td>104 ± 29</td>
<td>5,247 ± 1,910</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Fibronectin, ng/10⁵ cells</td>
<td>17.6 ± 6.9</td>
<td>45.4 ± 8.0</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

Notes. ns = not significant. The results were obtained from 5–7 independent cultures of each cell population. In morphometric analysis at least 100 cells from each group were examined.

24 hours at 37°C. Migrated cells were detached, lysed, and labeled with a CyQuan GR dye according to the manufacturer’s instructions. Sample fluorescence was measured with a fluorescence microplate reader (Perkin Elmer) using 480 and 520 nm wavelengths for excitation and emission, respectively.

Cell Viability

Cell viability was determined using Trypan blue exclusion test. HUVEC were suspended in Hank’s Balanced Salt Solution and mixed 1:1 (v/v) with 0.4% Trypan blue solution. After 10 minutes the number of blue-stained nonviable cells was counted in a hemocytometer.

Wounding Experiments and Morphometric Evaluation

The monolayers of young and old HUVEC were injured by scratching with a cell scraper (Nunc). The resulting debris was removed by gentle washing with medium. After that the standard culture medium was applied and the cells were placed in an incubator coupled to an Axio Observer D1 inverted microscope (Zeiss). Cells were maintained in a 5% CO₂ humidified atmosphere at 37°C for up to 12 hours, during which the images of the closing wound were acquired by time-lapse microscopy at 30-minute intervals. The images captured were analyzed using the AxioVision Rel. 4.6.3 image analysis software (Zeiss).

Cytochemical Staining

SA-β-Gal and bromodeoxyuridine were visualized using staining kits from Cell Signaling Technology and In VitroGen, respectively. All procedures were carried out in Lab Tek Chamber Slides (Nunc) as per manufacturers’ instructions. Staining with hematoxylin and eosin was performed according to standard histological protocols.

Cellular Protein Measurement

Equal numbers of young and old HUVEC (2.5 x 10⁵) were solubilized with 0.1M NaOH, and analyzed for protein with the Bradford method. The calculated ratio of total cell protein to the corresponding cell number was used as an indicator of cellular hypertrophy.

Cytokine Measurements

Concentrations of cytokines, growth factors, and adhesion molecules secreted by HUVEC were measured in post-culture supernatants with appropriate DuoSet Immunoassay Development Kits (R&D Systems). The assays were designed and performed according to the manufacturer’s instructions. The immunoassay used to measure fibronectin was described in detail elsewhere (16).

Immunoblotting

Antibodies against pAktSer473, Akt, and eNOS were from Cell Signaling Technology; an antibody against β-actin was from Sigma, horseradish peroxidase-labeled secondary antibodies were from Dianova.

Cells were rinsed twice with ice-cold 10mM HEPES, 150mM NaCl solution, pH 7.5, and scraped in a lysis buffer containing 40mM Tris/HCl, pH 8.0, 4mM EDTA, 20% glycerol, 276mM NaCl, 2% Triton X-100, 1mM sodium vanadate, 2mM sodium pyrophosphate, 10mM sodium fluoride, 10mM β-glycerophosphate, and Complete protease inhibitor cocktail (Roche). The lysate was centrifuged at 14,000 rpm at 4°C for 15 minutes and its protein concentration was determined with the Bradford method. Equal amounts of protein lysates were loaded onto a 12% polyacrylamide/sodium dodecyl sulfate gel and subjected to separation by electrophoresis. Proteins were electroblotted onto polyvinylidene fluoride membrane (Hybond, GE Healthcare) and visualized with ECL chemiluminescence detection system (Thermo Scientific). Specific bands were quantified with ImageJ 1.43 image processing software. Detected proteins were normalized to β-actin as a loading control.

Statistical Analysis

The data derived from young and corresponding old cells were treated as paired and analyzed with the paired t test using GraphPad Prism 5.00 software (GraphPad Software). Results were expressed as means ± SD. A p value of less than .05 was considered significant.

RESULTS

Replicative Senescence of HUVEC

Serial passages of HUVEC led to a gradual loss of proliferative capacity and ultimately to the cessation of cell growth (Figure 1A). Senescent cells remained in a viable state but displayed distorted morphology with extensive vacuolization and hypertrophy (Figure 1B and Table 1). These effects were associated with an increased number of cells expressing SA-β-gal (Figure 1C and D). Moreover, compared with young HUVEC, senescent cells secreted significantly more cytokines (IL-6, IL-8, MCP-1), growth...
factors (VEGF, TGF-β1), and extracellular matrix proteins (fibronectin; Table 1). The most striking effect was observed in the level of soluble form of the adhesion molecule-1 (sICAM-1), which increased approximately 50-fold.

In addition, senescent HUVEC exhibited increased expression of both eNOS and phosphorylated AKT, as assessed by immunoblotting (Figure 2). Expression of eNOS and pAKT in old cells was 6.3 ± 3.0 and 2.2 ± 0.3 times higher, respectively, compared with young cells.

Response of Young and Old HUVEC to Injury

The closure of scratch wounds was monitored by time-lapse microscopy (Figure 3). Repeated measurements revealed that the mean surface area of the wound inflicted on a monolayer of old HUVEC was slightly greater than that observed in young cells (0.493 ± 0.056 mm² vs 0.434 ± 0.055 mm², respectively, \( n = 28, p < .01 \)). This effect was most likely related to the enlarged and irregular shape of old HUVEC, which resulted in wounds having more jagged edges compared with young HUVEC. To normalize for this difference, the results of the assay were expressed as percentage of the initial wound surface area.

It turned out that both young and old HUVEC repopulated the denuded areas within 12 hours. Interestingly, two-way analysis of variance revealed that kinetics of the process in old HUVEC was more intense at earlier time points so that the curve as a whole differed significantly from that describing young cells (\( n = 10, p < .035 \)).

HUVEC Proliferation

\([\text{H}]-\text{thymidine incorporation by young HUVEC was about 8.5-fold greater than by old cells (Figure 4A). To assess the contribution of cell proliferation to wound repair, the incorporation of BrdU was visualized by immunocytochemistry. After injury the BrdU uptake was detected in 9 ± 3% of young and in 6 ± 1% of old HUVEC (\( n = 10, p < .05 \)). Cells staining positively for BrdU were usually found at some distance from the scratch and only sporadically were seen at the wound edges (Figure 4B). Collectively, these data documented the loss of proliferative potential in old HUVEC and suggested that cell proliferation did not play a leading role in wound closure by senescent cells.}

HUVEC Migration

Migration of young and old HUVEC was compared using the QCM assay that utilizes fluorescent dye binding to nucleic acids. Therefore we have first examined if CyQuant GR dye binds to young and old HUVEC with similar efficiency. To
this end, equal numbers of cells were labeled with the dye and then lysed. The signal detected did not differ between young and old HUVEC (119,975 ± 23,095 vs 124,458 ± 23,260 RLU/10⁵ cells, respectively, n = 6). During the assay itself, young and old HUVEC displayed similar baseline migration in the absence of growth factors (Figure 4C). However, they responded differently to stimulation with complete growth medium so that the migration of young, but not of old HUVEC, increased significantly.

**Wound Repopulation**

Although senescent HUVEC displayed markedly reduced potential to proliferate and migrate, they were still able to repopulate the denuded areas as fast as younger cells. This observation was puzzling and prompted us to examine the morphometric features of healed wounds. It turned out that, compared with young HUVEC, significantly fewer senescent cells sufficed to effectively cover the wound (Figure 5). One might have hypothesized that impaired capacity of old HUVEC to migrate would become apparent during healing of larger wounds. We have therefore compared the rates of recovery from smaller (<0.5 mm²) and larger injuries (>0.5 mm²). Although within the same observation period the percentage of wound closure was less in larger wounds, there was no difference between young and old HUVEC (Table 2).

**DISCUSSION**

Vascular ageing is associated with EC dysfunction, which—in turn—predisposes to increased risk of cardiovascular events in the elderly patients (17). As a result, a significant number of older individuals may require PCI and suffer from the complications of the procedure. It has been demonstrated that endothelial dysfunction that is still present 4 weeks after PCI predicts future clinical restenosis (18). We have therefore examined how senescence of ECs in vitro affects their regeneration after injuries mimicking those occurring during PCI. The experiments were performed using ECs from the umbilical cord, as they entered the culture as very young cells and could be expected to show a clearly different phenotype when senescent. One may argue that given the clinical context of our study, the use of ECs from coronary arteries would be more appropriate. However, global gene expression profiling revealed a great deal of overlapping between these populations (19), which is predominantly attributed to a similar vessel caliber. Because human coronary artery ECs are usually derived from adult donors, we used HUVEC as a convenient tool for initial screening.

Following repeated passages in culture, ECs entered the state of senescence characterized by altered morphology, growth inhibition, and increased expression of pAKT.
These changes were consistent with earlier observations that senescence-associated growth arrest of ECs is mediated by an increase in AKT activity that leads to induction of the p53/p21 pathway (20). Given that growth inhibition is the hallmark of cell senescence, we found it surprising that senescent cells repopulated denuded areas as fast as young cells. This prompt re-endothelialization suggested that old cells retained their ability to migrate swiftly into the wound. However, such a conclusion would be in contrast to previous reports demonstrating reduced migration of senescent ECs (21). To verify our data, we assessed cell migration using a chemotactic chamber. We found that in this setting senescent cells displayed a reduced migratory potential compared with young cells. To reconcile the data from the scratch test and the chemotactic assay, we compared the morphology of cells migrating into the wound and found that far fewer senescent cells repopulated the area. Thus, although the senescent cell migration was decreased, the old cells effectively covered the wound by virtue of their increased size.

Increasing evidence points to a role of NO in EC senescence and regeneration after injury. It has been demonstrated that L-arginine, a substrate for eNOS-mediated NO production, enhanced epithelial cell migration (22) and stimulated wound healing in elderly patients (23). We found that expression of eNOS by senescent HUVEC was markedly increased compared with young cells. This observation supports the view that senescence of EC is associated with increased rather than decreased eNOS expression (24). Augmented eNOS expression in senescent cells is usually interpreted as an attempt to compensate for reduced NO bioavailability, which can result from ineffective NO production or increased NO removal (13). Interestingly, it has been demonstrated that NO donors can delay senescence of HUVEC in culture (25).

The impact of EC senescence in the context of PCI may go beyond the population of elderly patients. It is now recognized that cells may enter the senescence program prematurely in response to harmful environmental stimuli. In this respect,
senescent ECs were detected in patients with atherosclerosis (26) and coronary artery disease (27), as well as in experimental animals, following vascular catheterization (28).

Earlier observations suggested that endothelial regeneration after balloon injury might be delayed in old animals (14). Our findings suggest that if the outcome of PCI-induced EC injury is different in the elderly patients, it is not related to prolonged re-endothelialization, but may rather be linked to different properties of cells filling the gap. Senescent cells secrete increased amounts of proinflammatory cytokines and growth factors and thus may contribute to exaggerated inflammatory and fibrotic reaction in the vascular wall. In this respect, it has been demonstrated that enhanced expression of ICAM-1 by senescent HUVEC leads to increased adhesion and accumulation of monocytes (29).

In addition to providing some clues on how the aged endothelium recovers from PCI-related injuries, our observations may also have some implications for the interpretation of the scratch test. The assay is a well-established method to study wound healing in vitro and is thought to be particularly suited for measuring cell migration (30). Our data indicate, however, that although the assay clearly monitors wound closure, the calculated rate of migration needs to be verified by other methods. This is especially the case if the cell populations analyzed differ significantly in size.

**Conflict of Interest**

The authors have no conflict of interest to declare.

**References**


