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THE ROLE OF mTOR INHIBITORS AND HMG-CoA REDUCTASE INHIBITORS ON YOUNG AND OLD ENDOTHELIAL CELL FUNCTIONS, CRITICAL FOR RE-ENDOTHELIALISATION AFTER PERCUTANEOUS CORONARY INTERVENTION: AN *IN VITRO* STUDY

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Percutaneous coronary intervention (PCI) has become a standard treatment in patients with acute coronary syndrome. However, it is associated with endothelial cell denudation, which may predispose to in-stent thrombosis and restenosis. Pharmacological methods which prevent restenosis can delay post-PCI re-endothelialisation. We have therefore examined how atorvastatin (HMG-CoA reductase inhibitor), sirolimus and everolimus (mTOR inhibitors) affect young and old endothelial cell functions which are responsible for wound healing after PCI. Replicative senescence was induced by serial passages of human umbilical vein endothelial cells (HUVECs). The cells which were examined at their first passages and last passages were designated as 'young' and 'old' respectively. Young and old endothelium were grown to confluence and were wounded by scraping. Scratch healing in the presence or absence of atorvastatin (AT), rapamycin (SR) and everolimus (EV) was monitored by time-lapse microscopy. In addition cells were assessed for viability (MTT assay), migration (chemotaxis chamber), proliferation (^{3}H-thymidine), and cytokine production (immunoassays). Senescent endothelial cells produce more proinflammatory cytokines, angiogenic VEGF and extracellular matrix proteins. They stop proliferating and have diminished migration. When compared to young endothelium, they have similar viability and can regenerate wounds in comparable time. The drugs that have been tested have anti-inflammatory properties but even after pretreatment old cells still produced significantly higher concentration of tested mediators in comparison with young ones. In the concentration obtained in serum after stent implantation, mTOR inhibitors in dose-dependent manner reduced cell proliferation, migration and wound healing. Reduced healing is more pronounced in young endothelium. Atorvastatin, at clinically relevant concentration, is safe for young and old cells. Atorvastatin, sirolimus and everolimus inhibited the secretion of pro-inflammatory mediators in young and old endothelium. In concentrations seen in serum during standard therapy, rapalogs impair endothelial cell regeneration after injuries mimicking those occurring during PCI, while atorvastatin does not affect the healing.

Key words: *endothelial cells, atorvastatin, HMG-CoA reductase inhibitors, sirolimus, everolimus, mTOR inhibitors, percutaneous coronary intervention, cytokines*

INTRODUCTION

In recent decades percutaneous coronary intervention (PCI) with stent implantation has been the predominant surgical procedure in patients with acute coronary syndromes (1, 2). Stent implantation has resulted in significant vessel remodelling at the site of intervention, leading to neointima formation (3). Inoue *et al.* reported that in vascular injury bone marrow-derived cells may differentiate into endothelial cells and also into smooth muscle cells, leading to both re-endothelialisation and neointimal thickening and restenosis (4). Even in successful PCI procedures, more than twenty percent of patients required additional invasive procedures over the following 3 years (2). To stop smooth muscle cell proliferation in intima, drug-eluting stents (DES) were used. The first generation of DES used sirolimus (rapamycin) and paclitaxel as antiproliferative agent. The second generation of DES, use sirolimus derivatives (everolimus, zotarolimus) (5). Despite stent improvement, in-stent re-endothelialisation is still insufficient. The mechanism of the inhibition of cell proliferation by rapamycin and its derivatives consists in the inhibition of mTOR kinase (mammalian target of rapamycin). mTOR kinase is responsible for cell proliferation, migration, regulation of translation and transcription and inhibition of the immune response. The effects of antimitotic drugs used in DES do not always seem to be beneficial to the vascular endothelium. They accelerate the aging process, potentiate stent thrombosis and suppress the proliferation of endothelial cells (6-8).

Statin therapy is also one of the pharmacological methods which prevent restenosis (9). In animal models stents coated with statins reduced the neointimal formation and improved endothelial function (10). The reduction of neointima is based on

the inhibition of smooth muscle cell proliferation (11) but, depending on their concentration statins may also affect the endothelial cell proliferation (12, 13). Besides the prevention of restenosis, statins promote re-endothelialisation by increasing the number of circulating progenitor cells which colonize denuded parts of the vessel (11).

Aging is a relevant risk factor in any cardiovascular disease. Elderly people constitute a major group of patients treated with PCI and are much more prone to complications after angioplasty, owing to the senescence of the cardiovascular system (14-16). Cessation of replication is the hallmark of senescence (17). Although senescent cells stop proliferating, they remain metabolically active and synthesize high amounts of proinflammatory cytokines changing the local tissue homeostasis (18). Progressive accumulation of senescent endothelial cells, deregulation in cytokine production and the reduction in the number of circulating endothelial progenitor cells during the aging process all affect vascular wound healing (19). Senescent endothelial cells were found in arterial vessels in patients with atherosclerosis (20), coronary artery disease (21), and those exposed to various cardiovascular risk factors (22). Senescence of either vascular endothelial cells or endothelial progenitor cells is one of the most important reasons for restenosis in elderly patients who are particularly more prone to complications after the PCI procedure when compared with young patients (15).

Recently, we have demonstrated a model for the regeneration of young and old vascular endothelial cells after sustaining injuries mimicking vascular damage after PCI (23). Using permanent cell line HUVEC line EA.hy926 (24) we have also demonstrated that atorvastatin (AT) at clinically relevant doses does not affect vascular endothelial cell wound healing *in vitro* (12). Therefore, in the present paper we have focused on pharmacological methods preventing restenosis and their impact on young and old endothelial cell functions, critical for re-endothelialisation after percutaneous coronary intervention (PCI). Using young and old endothelial cells we have tested how acute exposure to atorvastatin, sirolimus and everolimus, at clinically relevant doses, affects wound healing in an *in vitro* model mimicking endothelial injuries occurring during angioplasty.

MATERIALS AND METHODS

Cell culture

In vitro studies were performed with pooled primary human umbilical endothelial cells purchased from Clonetics (Lonza, Switzerland). Cells were propagated in HEPES (25 mM) buffered M199 culture medium, supplemented with L-glutamine (2 mM), amphotericin (2.5 μg/ml), gentamycin (50 μg/ml), hydrocortisone (1 μg/ml), heparin (10 U/ml), EGF (10 ng/ml), and 15% v/v foetal calf serum. All reagents were from Sigma (St. Louis, MO, USA) and cell culture plastics were from Costar (USA).

Experimental design

Young and old endothelial cells were exposed to the cultured medium supplemented in mTOR inhibitors sirolimus (SR), everolimus (EV) and in HMG-CoA reductase inhibitor atorvastatin (AT) in the concentration obtained in serum after stent implantation (SR, EV) and (25, 26) typical medical treatment before PCI (AT) (27). We used the following concentration of the tested drugs: SR and EV 1 – 100 nmol/l and 0.01 – 0.1 μmol/l for AT.

Induction of senescence

Replicative senescence was induced by serial passages of HUVECs (23). Briefly, upon reaching confluency, cells from the culture were harvested using a tripsin-EDTA solution (0.05 – 0.02%) and seeded into a new culture flask at a density of 5000 cells/cm^2. The next passages were performed at 5 – 9 day intervals with the same seeding density. The passages were carried out until cells (i) remained the same in their number for 4 weeks, (ii) exhibited a senescent morphology (irregular shape, hypertrophy, vacuolization), (iii) more than 70% of senescent endothelial cells stained positive for senescence-associated β-galactosidase (SA-β-Gal). Endothelial cells, after serial passages, reached final population doublings (PD) which resulted in cell senescence. The number of PDs was calculated using the equation $PD = \log_2(C_t/C_0)$, where C_0 is the number of cells seeded, and C_t is the number of cells harvested after 5 – 9 days. Cells which were examined at their first passages and last passages were designated as 'young' and 'old' respectively.

Detection of senescence-associated β-galactosidase (SA-β-Gal)

The presence of senescence-associated β-galactosidase was visualized according to Dimri *et al.* (28), using Cell Staining Technology (USA). Cells were considered senescent when more than 70% of cells stained positive for SA-β-Gal.

Cell viability

Cell viability was measured using an MTT assay. Briefly, monolayers of 2×10^4 young and old endothelial cells were exposed to medium M199 with supplements ± SR, EV and AT for 24 hours. After the exposition, cells were incubated in a medium containing 1.25 mg/ml of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 4 h at 37°C. The generated formazan product was dissolved with the addition of acidic solution of 20% w/v sodium dodecyl sulphate and 50% v/v N,N-dimethylformamide. The absorbance of the converted dye was recorded at 595 nm with a reference wavelength of 690 nm. The data were expressed as the percentage of control.

Proliferation assay

Young and old endothelial cells were seeded into a 48-well plate at a density of 2×10^4 cells, and allowed to attach for 4 hours. Then cells were exposed to a culture medium with supplements ± SR, EV and AT for 24 hours in the presence of [^{3}H]-thymidine - 1μ Ci/ml (Institute of Radioisotopes, Prague, Czech Republic). After the incubation, cells were harvested and precipitated with 10% trichloroacetic acid (TCA) and dissolved in 0.1 mmol/l NaOH. The radioactivity was measured in a beta liquid scintillation counter (Wallac, Perkin Elmer). The data was expressed as CPM (Count Per Minute - value comes from the beta liquid scintillation counter).

Migration assay

QCM™ Chemotaxis 96-well cell migration assays (Chemicon, USA) with 8 μm pores were used to assess the migration of young and old HUVECs. Endothelial cells were grown to 80% confluency in a culture medium ± SR, EV and AT for 24 hours. Eighteen to twenty-four hours before the assay, cells were starved by incubating in the medium M199 with supplements and 2% v/v FCS and then washed twice with PBS, harvested using tripsin/EDTA solution, resuspended in a serum-free medium and placed in a migration chamber (5×10^4 cell/100 μl) covered with fibronectin (1 μg/ml overnight at 4°C). Cells

were then stimulated for 24 hours with a standard 10% serum-containing medium with or without SR, EV and AT. Migrated cells were detached and treated for 15 minutes with the CyQuant GR dye in the lysis buffer, as per manufacturer's instructions. Fluorescence of cell lysates was measured with a fluorescence microplate reader (Victor 2, Perkin Elmer, USA) using 480 nm and 520 nm wavelengths for excitation and emission respectively.

Wound healing

Young and old endothelial cells were grown to confluence in 6-well dishes. Before the experiment, the cells were incubated in M199 with supplements ± SR, EV and AT for 24 hours. Under these conditions the cells remained viable in a nonproliferating state. The quiescent monolayer was injured by scraping with a disposable cell scraper (Nunc, Denmark). To compare the data from all experiments it was very important to make a comparable injury in all monolayers exposed to medications. The monolayer was washed twice with the culture medium and then incubated with M199 with supplements ± SR, EV and AT. The closure of the denuded area was monitored using a Zeiss Axio Observer D1 inverted microscope (Carl Zeiss, Germany) equipped with a CO_2 module and an incubator. During the experiments, cells were maintained in a 5% CO_2 humidified atmosphere at 37°C for the duration of the experiments. A video camera was attached to the microscope, and images of the wounded area were captured as a digitalized sequence every 30 minutes, using Axio-Vision Rel. 4.6.3. image analysis software. The results were calculated as a percentage of the healed area at six separate time intervals (2 h, 4 h, 6 h, 8 h, 10 h, 12 h) along the original margin of the wound. The assay is a well-established method to study wound healing *in vitro* and is thought to be particularly suited for measuring cell migration (29). In our study migration was additionally verified by using a Boyden chamber.

Cytokine measurements

Young and old endothelial cells were cultured with M199 with supplements ± SR, EV and AT for 24 hours. Media were collected and analyzed for the constitutive concentrations of cytokines: IL-6, IL-8, MCP-1, sICAM-1, TGF-β, fibronectin, VEGF. Mediators were measured by using DuoSet Immunoassay Development Kits (R&D Systems) according to the manufacturer's instructions. The sensitivity of the assay was: 2.6 pg/ml for the IL-6, 4.4 pg/ml for the IL-8, 5.8 pg/ml for the MCP-1, 17.5 pg/ml for the sICAM, 17.6 pg/ml for the TGF-β, 0.96 ng/ml for the fibronectin and 12.2 pg/ml for the VEGF. The results were normalized per number of young and old cells in the culture wells.

Statistical analysis

The data were analyzed with repeated measures analysis of variance using GraphPad Prism 6.00 software (GraphPad Software Inc.). A post hoc test was used to make multiple comparisons. A P-value less than 0.05 was considered significant. Results are presented as means ± S.D.

RESULTS

Senescence of endothelial cells

Endothelial cells, after approximately 28-31 passages, reached about 35 population doublings (PD), which resulted in cell senescence. Senescent cells increased in cell size (*Fig. 1A*) when compared with young cells (*Fig. 1B*). Young cells lost their proliferative capacity during serial passages (*Fig. 2B*). The senescence of endothelial cells was associated with the appearance of the senescence marker SA-β-Gal.

Effect of sirolimus, everolimus and atorvastatin on young and old endothelial cell viability and proliferation

Both young and old endothelial cells had the same viability entering the experimental protocol (OD at baseline: control young: 0.435 ± 0.016, control old: 0.444 ± 0.023) (*Fig. 2A*). Exposure of HUVECs to SR and EV resulted in a dose-dependent inhibition of young and old cells viability and proliferation. Figures show only results at the highest dose. The detrimental effect was more pronounced in young cells (*Fig. 2A*).

Using [^{3}H] labelled thymidine we estimated young endothelial cell proliferation and DNA turnover in senescent cells. This assay confirmed that old cells lost their ability to proliferate and only a small amount of them was still in the cell-division cycle (control young: 5838 ± 310 CPM; control old: 1122 ± 146 CPM) (*Fig. 2B*). We observed a dose dependent decrease in DNA turnover in old cells exposed to the medium supplemented with SR and EV. The proliferation of young endothelial cells was also suppressed by the increasing concentration of mTOR inhibitors. In contrast, AT at clinically

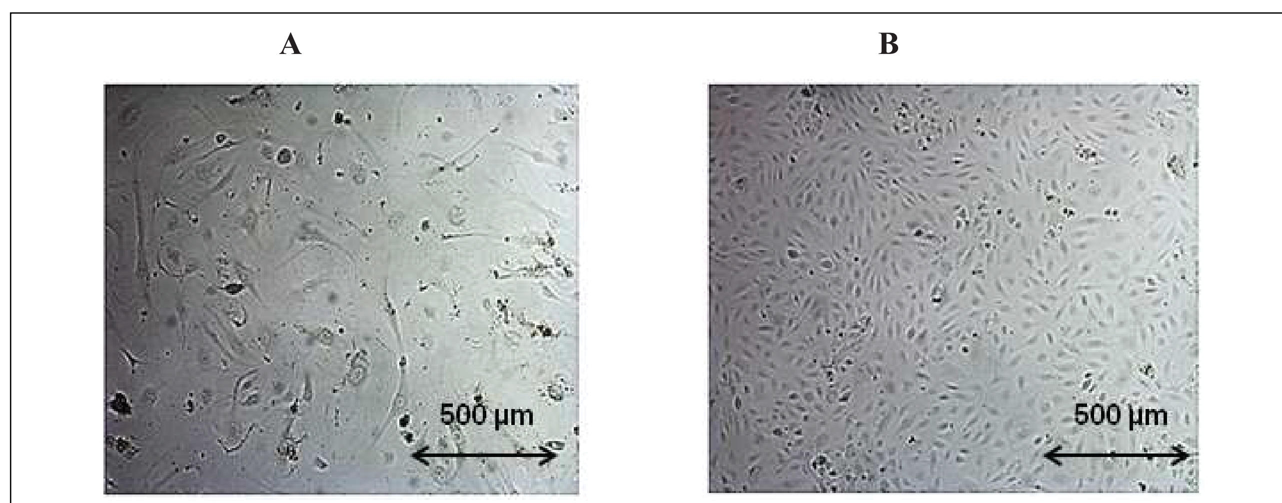

Fig. 1. Exemplary microphotographs of old (*A*) and young (*B*) human umbilical vein endothelial cells (HUVECs). Magnification × 100.

relevant doses did not impair young and old endothelial cell proliferation and viability (*Fig. 2A* and *2B*).

Effect of sirolimus, everolimus and atorvastain on young and old endothelial cell migration

mTOR inhibitors in a dose-dependent manner inhibited young and old endothelial cell migration (*Fig. 2C*, shows only the results at the highest dose). Young endothelial cells exhibited stronger response to the chemoattractant than old cells by approximately 30% (*Fig. 2C*). The response to the increasing concentration of SR and EV was similar in both groups (*Fig. 2C*). Migration of young and old endothelial cells exposed to AT did not differ significantly from the controls (*Fig. 2C*).

Effect of sirolimus, everolimus and atorvastatin on young and old endothelial cell wound closure

The closure of denuded area was monitored by the time lapse microscopy. Although control senescent HUVECs displayed

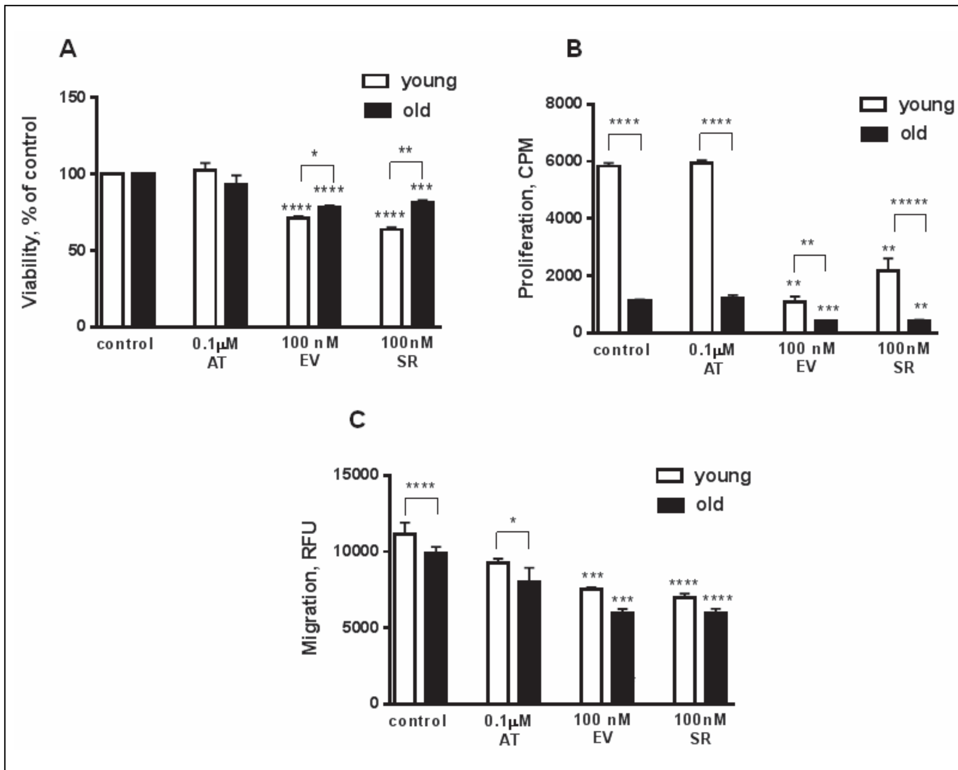

Fig. 2. The effect of the highest dose of atorvastatin (AT), everolimus (EV), and sirolimus (SR) on endothelial cell viability (*A*), proliferation (*B*) and migration (*C*). Young and old cells were treated with medications or vehicle control for 24 hours. After the exposure cell viability (MTT test), proliferation (^{3}H-thymidine incorporation) and migration (Byden chambers) were tested. Results are expressed as mean ± S.D. The data were derived from 4 independent experiments. Asterisks represent a significant difference compared with representative control cells (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001).

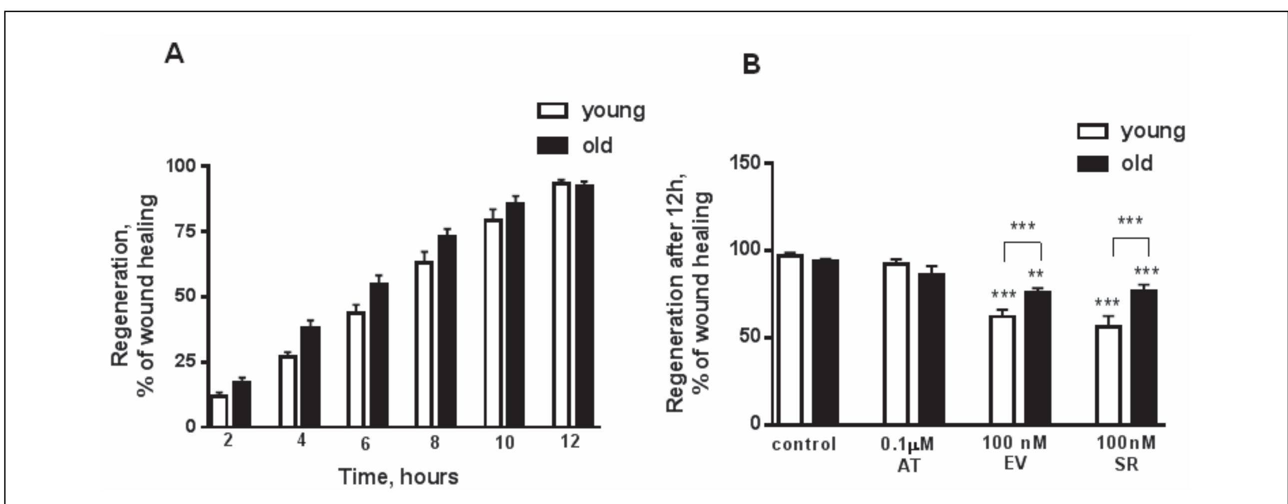

Fig. 3. Wound closure by young and old endothelial cells in a model which mimics vascular damage after PCI. Panel *A* represents kinetics of wound healing in young and old endothelial cells at two-hourly intervals. Panel *B* represents the effect of the highest concentration of AT, EV and SR on endothelial cell regeneration after 12-hour of wound healing. Cells were treated with medications or vehicle control for 24-hour before and during regeneration. Data were expressed as a percent of wound closure after 12-hour. Results are expressed as mean ± S.D. The data were derived from 8 independent experiments. Asterisks represent a significant difference compared with representative control cells (*P < 0.05, **P < 0.01; ***P < 0.001).

markedly reduced potential to proliferate and migrate, they were still able to repopulate the denuded areas as fast as the young cells (*Fig. 3A* and *3B*). The surface area of the wounds generated at the beginning of experiments did not differ between the groups and was confirmed by statistical analysis (*Table 1*). Images of the wounded area were taken at the beginning of the experiment (0 h), and after 2 h, 4 h, 6 h, 8 h, 10 h and 12 h of the progressive closure of the wounded area in both young and old endothelial cells. The results were calculated as a percentage of the healed area at six separate time intervals. The comparison of the kinetics of the regeneration processes in young and old cells revealed that both groups had similar patterns in the repopulation of wounded areas (*Fig. 3A*). They repopulated the denuded areas within comparable time of 12 hours (*Fig. 3A*). We observed that AT, at clinically relevant concentration did not disrupt young and old endothelial cell regeneration (*Fig. 3B*), while mTOR inhibitors attenuated wound healing in a dose dependent manner (data not shown). The percentage of healed wounds after the exposition to SR and EV was higher in old cells compared with young cells (*Fig. 3B*). This may be due to a better condition of old cells (viability) after exposition to mTOR inhibitors (*Fig. 2A*).

Effect of sirolimus, everolimus and atorvastatin on young and old endothelial cell cytokine production

Young and old endothelial cells produce various amounts of mediators. Senescent cells produce far more proinflammatory cytokines, such as IL-6, MCP-1, IL-8 and sICAM-1 when compared with young cells (*Table 2*). The production of angiogenic VEGF, and extracellular matrix proteins (fibronectin, TGF-β) is also more pronounced in senescent endothelial cells than in young cells (*Table 2*). The modification of cytokine production by the tested drugs was dose dependent. *Fig. 4* presents the percentage change of cytokine production by the highest drug concentration. The modifying effect of rapalogs and AT on young and old endothelium was mostly comparable (*Fig. 4A*, *4B* and *4C*). Even after the exposition to the tested drugs, the detected concentrations of tested mediators was still significantly higher in the old endothelial cells in comparison with the young cells (*Fig. 4A*, *4B* and *4C*). AT, as an anti-inflammatory agent, reduces pro-inflammatory mediators proportionally to its concentration both in young and old cells. It also attenuates VEGF, fibronectin and TGF-β production (*Fig. 4A*).

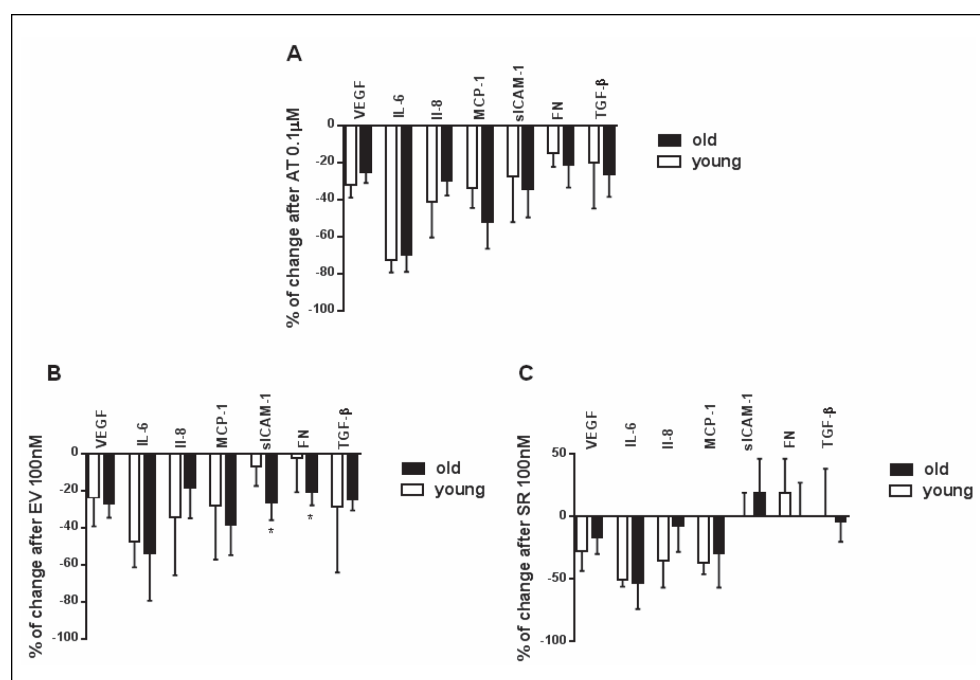

Fig. 4. The percentage change of mediators production by the highest drug concentration in cultured medium, obtained from young and old endothelial cells. Secreted mediators were measured in post-culture supernatants following a 24-hour exposure to AT, EV, SR. The data were derived from 8 independent experiments.
(VEGF, vascular endothelial growth factor; Il-8 (CXCL8), interleukin 8; IL-6, interleukin 6; MCP-1 (CCL2), monocyte chemoattractant protein; sICAM-1, the soluble form of intercellular adhesion molecule; TGF-β, transforming growth factor β. *P < 0.05; young verus old.

Table 1. The surface area of the wounds generated at the beginning of experiments. Results are expressed as mean ± S.D. The data were derived from 8 independent experiments.

Surface area for regeneration, mm^2	**Young**	**Old**	**P**
Control	498 ± 48	516 ± 66	0.1563
0.01 µM AT	555 ± 52	529 ± 41	0.7907
0.1 µM AT	504 ± 80	568 ± 107	0.4375
Control	486 ± 44	483 ± 63	0.8125
1 nM EV	476 ± 49	492 ± 61	0.5781
10 nM EV	499 ± 63	526 ± 35	0.3750
100 nM EV	479 ± 25	503 ± 55	0.4688
Control	506 ± 47	524 ± 84	0.6875
1 nM SR	545 ± 51	482 ± 66	0.1563
10 nM SR	554 ± 75	520 ± 83	0,4375
100 nM SR	590 ± 44	545 ± 38	0.2050

Table 2. The constitutive production of various mediators by young and old endothelial cells. Secreted mediators were measured in post-culture supernatants following a 24-hour exposure to standard culture medium without medications. Results are expressed as mean ± S.D. The data were derived from 8 independent experiments.
VEGF, vascular endothelial growth factor; IL-8 (CXCL8), interleukin 8; IL-6, interleukin 6; MCP-1 (CCL2), monocyte chemoattractant protein; sICAM-1, the soluble form of intercellular adhesion molecule; TGF-β, transforming growth factor β.

Parameters	Control young	Control old	P
VEGF (ng/10^6cells)	0.96 ± 0.09	1.82 ± 0.23	P < 0.0001
IL-8 (CXCL8) (ng/10^6cells)	1.4 ± 0.3	5.9 ± 0.8	P < 0.0001
IL-6 (ng/10^6cells)	0.37 ± 0.04	0.92 ± 0,19	P < 0.001
MCP-1 (CCL2) (ng/10^6cells)	5.1 ± 0.7	9.7 ± 2.3	P < 0.01
sICAM-1 (ng/10^6cells)	2.1 ± 0.8	4.2 ± 0.7	P < 0.01
TGF-β (ng/10^6cells)	23.5 ± 3.7	38.5 ± 4.1	P < 0.001
Fibronetin (ng/10^6cells)	0.84 ± 0.10	1.2 ± 0.13	P < 0.01

Anti-inflammatory effect of mTOR kinase inhibitors is more pronounced after EV exposition, especially in old HUVECs (*Fig. 4B*). The exposure to SR caused a decrease in IL-6, IL-8, MCP-1 and VEGF (*Fig. 4C*). The anti-inflammatory effect of mTOR kinase inhibitors was far less pronounced in young endothelial cells and concerned only statistically significant reduction in IL-6 and MCP-1, proportionally to the tested concentration (data not shown). mTOR kinase inhibitors exerted antiangiogenic effect by lowering VEGF production in both HUVECs groups and additionally had a weak modulatory effect on extracellular matrix proteins synthesis (*Fig. 4B* and *4C*). Due to senescent cell hypertrophy, the effect of cytokine production was more evident when calculated per number of cells than per micrograms of cell protein (*Table 2*).

DISCUSSION

Endothelial denudation is considered to be a primary injury after stent implantation. Prompt regeneration of wounded endothelial cells is a precondition for protecting against vascular restenosis. Our previous study described a model for endothelial cell regeneration in tissue culture using a cell scraper. The repopulation of the wounded area was continuously monitored by time-lapse microscopy, with subsequent morphometric analysis (23). The healing of the endothelial cells after PCI is slow and takes time (30). After stent implantation, re-endothelialisation can be completed by the migration of vessel ECs, proliferation and from bone-marrow-derived progenitor endothelial cells (31, 32). The participation of progenitor endothelial cells in re-endothelialisation is still imperfectly understood (11, 33). However, we must take into consideration that this process could have been slowed down because of progenitor cell's senescence (34). Young cells can regenerate after injuries using both migration and proliferation, whereas in old cells migration seems to be the most plausible mechanism of wound healing since senescent cells have exhausted their replicative potential. In our paper we have proved that senescent endothelial cells produce more proinflammatory cytokines, angiogenic VEGF and extracellular matrix proteins. They also stop proliferating and have diminished migration. When compared to young endothelium, they have similar viability and can regenerate wounds in comparable time because they are hypertrophic. Their size is approximately five times larger than that of young cells, so in spite of a feeble migration and reduced cell proliferation they are still able to heal wounds (23). The tested drugs have anti-inflammatory properties and additionally alter extracellular matrix protein synthesis, but even after pretreatment old cells still produce significantly higher concentration of tested mediators in comparison with young cells. The mTOR inhibitors exhibit antiangiogenic properties by reducing the concentration of VEGF, proliferation and migration. For this reason they are used in tumour therapy (35, 36). Additionally, the modulating anti-inflammatory effect of the tested drugs seems to be more pronounced in senescent endothelium. Atorvastatin, at clinically relevant concentration, is safe for endothelium as we earlier proved using permanent cell line HUVECs EA.hy926 (12). The same effect was observed using primary young and old HUVECs. On the contrary, mTOR inhibitors used in concentration detected in the blood after stent implantation are harmful for both young and old endothelium. They slow down the cell cycle, restrain migration and regeneration, although old cells remain more viable after exposition to rapalogs, when compared to their young counterparts. Old cells are able to repopulate denuded areas faster than young cells after the exposition to SR and its derivative EV due to better viability and hypertrophy. It is worth emphasizing that the main mechanism by which wounds are regenerated is the migration process and only to a small degree it is caused by proliferation (23, 37).

The mTOR kinase is serine/threonine protein kinase belonging to the kinase family that is highly concentrated in all eukaryotes. The dysregulation of mTOR signalling is often associated with cancer and several metabolic disorders (e.g. obesity, type 2 diabetes) (36). Experimental data show that statins and mTOR kinase inhibitors exhibit a biphasic effect depending on their concentration. The mechanism underlying the biphasic effect of statins is attributed to their impact on protein prenylation (38). In high concentrations (>1 μM) they inhibit endothelial cell proliferation, while in very low (nanomolar) concentrations they stimulate cell cycle (12, 13). While in high concentration (μM range) mTOR kinase inhibitors initiate cell death by the induction of autophagy-specific genes (36). The mTOR kinase is inhibited by rapalogs at nanomolar range, and Pallet *et al.* hypothesized that the majority of rapalogs-related adverse effects are not necessarily caused by

mTOR inhibition, but by side effects that remain to be identified (39). Using atorvastatin, sirolimus and everolimus in concentration corresponding to those obtained during clinical therapy makes them close to the *in vivo* conditions (25-27).

A large body of evidence indicates that statins and mTOR inhibitors have a pleiotropic anti-atherosclerotic effect and can be used as an addition to therapy to prevent or delay the vascular disease (36, 40-42). By suppressing cell proliferation and promoting cell autophagy the mTOR inhibitors control plaque growth and destabilization but on the other hand they act adversely on endothelium (6-8). Rapamycin and everolimus accelerate the aging process in endothelial cells (8), but it has also been demonstrated that rapamycin extends life span in mice by delaying aging (43). Indeed, mTOR inhibition seems to be an innovative strategy for the treatment of age-associated diseases. The importance of mTOR inhibitors in progeria-related diseases is complex and consists mostly in: (i) activating autophagy, (ii) decreasing age-related inflammation and (iii) mimicking the effects of caloric restriction and its role in extending lifespan (44).

Contrary to rapalogs, statins exert a beneficial effect on endothelial cells improving their hemostatic, vasomotor, anti-inflammatory and angiogenic functions (13). The anti-atherosclerotic effect of rapalogs and statins is attributed to their anti-inflammatory effect. As we have demonstrated atorvastatin not only reduces the pro-inflammatory cytokine synthesis in endothelial cells but also reduces the production of extracellular matrix proteins and VEGF. Anti-inflammatory effect of rapalogs is more apparent in everolimus, especially in senescent endothelial cells. This anti-inflammatory effect of rapalogs has also been seen in macrophages (40). The observed effect is related to the inhibition of protein synthesis by cells exposed to mTOR kinase inhibitors (36). The inhibition of protein synthesis together with autophagy might lead to the gradual decrease of cell viability and cell death. Exposition to increasing concentration of mTOR kinase inhibitors resulted in progressive loss in cell viability, proliferation and migration in young and old endothelial cells. Anti-proliferative, anti-migratory, anti-inflammatory and immunomodulatory effects of rapalogs are well established (7, 8, 36). Hovever, they can also stimulate the production of pro-inflammatory cytokines in vascular smooth muscle cells (45), monocytes and neutrophils (46). This may also account for endothelial dysfunction (39, 45, 47). The adverse effects associated with mTOR inhibitors such as the deterioration of many essential functions of the endothelium and recently identified dyslipidemia can be improved by a combined use of statins (36). The improvement of cardiac contractility and protection against arrhythmia after ACS is one of the most important designations of the cardiac pharmacotherapy. Benova *et al.* using animal model of isolated rat hearts, demonstrated antiarrhythmic effect of atorvastatin. Isolated fibrillating hearts were protected from the lethal arrhythmia and the sinus rhythm was restored after atorvastatin treatment (48).

Vascular aging is associated with EC dysfunction which predisposes to increased risk of cardiovascular events in the elderly (16). A significant number of elderly patients require PCI and suffer from the complications of the procedure. We have previously examined how senescence of ECs *in vitro* affects the regeneration after injuries mimicking those occurring during angioplasty. Our experiments were performed using HUVECs which revealed a great convergence with endothelial cells from coronary arteries (49). It was important to assess the reaction of young and old endothelial cells after exposition to atorvastatin and rapalogs. Because human coronary artery endothelial cells are usually derived from adult donors we used very young endothelial cells derived from umbilical veins. Following serial passages in culture, ECs entered the state of senescence. We found it surprising that senescent cells repopulated denuded areas as fast as young cells in spite of growth inhibition and reduced migratory potential. To reconcile the results we performed a morphometric analysis, which reveals that old cells effectively covered the wound by virtue of their increased size. An additional explanation of this phenomena could be an increased expression of eNOS by senescent HUVECs (23). eNOS-mediated NO production enhanced endothelial cell migration (50) and stimulated wound healing in elderly patients (51). Zemakova *et al.* documented that atorvastatin prevented inflammatory-mediated downregulation of eNOS protein level. This effect was regulated *via* endoglin, a transmembrane glycoprotein, that is part of the TGF-beta receptor complex (52). This endoglin supporting effect on eNOS synthesis could be particularly important for senescent endothelium, since TGF-beta is known to be involved in the aging process and tumorigenesis (53, 54).

Taken together, our results indicate that atorvastatin at clinically relevant concentrations and rapalogs in the concentration obtained in the serum after stent implantation may weaken the inflammatory response associated with angioplasty. Rapalogs delay young and old cell regeneration after injuries mimicking those occurring after PCI, while atorvastatin does not delay endothelial recovery from injuries.

Conflict of interests: None declared.

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