Research Article

Concentration-dependent differential effects of udenafil on viability, proliferation, and apoptosis in vascular endothelial and smooth muscle cells

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ABSTRACT

Objectives: Local strategies directed against vascular smooth muscle cell (VSMC) proliferation, such as drug-eluting stents (DES), reduce the occurrence of restenosis. However, these approaches may also inhibit vascular endothelial cell (VEC) proliferation and impair reendothelialization, and hence, increase susceptibility to late thrombosis. In this study we examined the differential effects of various concentrations of the type 5 phosphodiesterase (PDE-5) inhibitor, udenafil, on viability, proliferation, and apoptosis of VEC and VSMC, in order to identify the optimal concentration of udenafil that minimizes inhibition of VEC survival and growth, and maximizes inhibition of VSMC survival and growth.

Materials and Methods: VEC from human umbilical veins and VSMC from human aorta were exposed to various concentrations of udenafil (1, 10, and 100 μmol/l and 1 mmol/l) for 24 h, and its effects on cell viability, proliferation, and apoptosis were studied using 5-bromo-2′-deoxyuridine (BrdU), methylthiazoletetrazolium (MTT) assay, trypan blue dye exclusion, and flow cytometry.

Results: Udenafil inhibited the survival and growth of VEC and VSMC in a concentration-dependent manner over a range of concentrations. At 100 μmol/l, udenafil, inhibited VEC proliferation significantly less than VSMC proliferation ($P < 0.05$), and could significantly induce VEC apoptosis less than VSMC apoptosis ($P < 0.05$).

Conclusions: Udenafil has a differential effect on survival and growth in VEC and VSMC. The maximal differential effect, with minimal inhibition of VEC and maximal inhibition of VSMC, occurs at 100 μmol/l. This characteristic suggests that udenafil is a promising agent for use in DES.

KEY WORDS: Drug-eluting stent, proliferation, udenafil

Introduction

Drug-eluting stents (DES), which release antiproliferative drugs into blood vessel walls to inhibit neointimal hyperplasia, dramatically reduce the incidence of in-stent restenosis.[1-3] However, these agents not only inhibit the proliferation and migration of vascular smooth muscle cells (VSMC), they also suppress the multiplication of vascular endothelial cells (VEC), thereby, potentially impeding reendothelialization and increasing susceptibility to late thrombosis.[2,4,5] Thus, an ideal agent for DES should be able to inhibit VSMC proliferation without inhibiting VEC proliferation. To date, no satisfactory agent of this kind has yet been reported.

The concentration-proliferation inhibition curves of agents differ depending on the target cell [Figure 1]. By varying the concentration of an inhibitory agent, one may hope to find a concentration that is relatively selective for the target cell type and might therefore simultaneously prevent in-stent restenosis and thrombosis. The desired concentration in the tissues could then be achieved by controlled release.

Type 5 phosphodiesterase (PDE-5) inhibitors are known to exert an antiproliferative effect on VSMC.[6] In this study, we investigated the differential effects of various concentrations of udenafil, a PDE-5 inhibitor, on viability, proliferation, and

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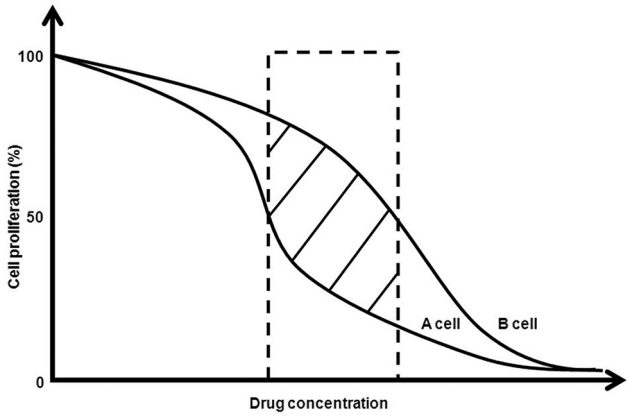
Figure 1: Theoretical concentration-proliferation inhibition curves of a drug on two different cell types



apoptosis in VEC and VSMC with the aim of identifying a concentration of udenafil with a selective effect on VSMC survival and growth.

Materials and Methods

Cell Culture

Human umbilical VEC

Human umbilical VEC (BioBud, Seoul, Korea) were cultured in M199 medium (Gibco, Burlington, Canada) with high glucose and supplemented with heat-inactivated 20% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 1% penicillin-streptomycin (Gibco, Rockville, MD, USA), 10 U/mL Heparin (Han-Lim, Seoul, Korea), and 20 ng/mL basic fibroblast growth factor (BioBud, Seoul, Korea) at 37°C under 5% CO_2, 95% air as described previously.[7] Cells were passaged after reaching confluence every 7-10 days, and passage numbers from 4 to 6 were used for experiments.[8]

Human aortic VSMC

Human aortic VSMC (Cascade, Portland, Oregon, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) with high glucose and supplemented with heat-inactivated 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO_2, 95% air as described previously. Cells were passaged after reaching confluence every 7-10 day, and passage numbers from 3 to 5 were used for experiments.[9]

Reagents

Udenafil (Dong-A Pharmaceutical, Seoul, Korea) was dissolved as a 100 mmol/l stock solution in 100% ethanol (Merck KGaA, Darmstadt, Germany) and filter sterilized. To obtain different test concentrations (1, 10, and 100 μmol/l and 1 mmol/l), serial dilutions were prepared in culture medium. Ethanol (0.1%) was used as a non-drug control throughout the study.

Cell proliferation analysis

The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, Roche Molecular Biochemicals, Mannheim, Germany) was used to evaluate cell proliferation.[10] Cells were seeded in 96-well plates at 5×10^3 cells/well in 100 μl of medium. They were cultured for 24 h to allow adherence. Then, cells were made quiescent by incubation in each medium without FBS for 24 h. After further incubation for 24 h, the medium was replaced with fresh medium containing 10-20% FBS and different concentrations of udenafil for 24 h. During the last 4 h of udenafil treatment, 10 μl/well BrdU labeling reagent (final concentration, 10 μmol/l) was added to the medium and incubated for labeling. After cell fixation and DNA denaturation, a peroxidase-conjugated anti-BrdU monoclonal antibody was added. Color reaction was developed with tetramethylbenzidine and absorbance of the reaction product was measured at 370 nm wavelength in an enzyme-linked immunosorbent assay (ELISA) reader (Spectra Max 190, Molecular Devices, CA, USA). The experiments were repeated three times.

Cell apoptosis analysis

Quantification of apoptotic and viable cells was accomplished with a flow cytometry assay.[11,12] Cells were seeded in 6-well plates at 1.6×10^5 cells/well in 1.5 ml of medium, and cultured at 37°C overnight. After washing with phosphate-buffered saline (PBS), the cells were cultured with medium containing different concentrations of udenafil for 24 h. They were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA), washed twice with cold PBS, and resuspended in 1 × binding buffer containing 5 μl annexin V-fluorescein isothiocyanate (FITC) and 5 μl propidium iodide solution (BD Biosciences, San Diego, CA, USA), and incubated for 15 min at room temperature. They were then analyzed with a FACScan flow cytometer (Becton Dickinson, Mansfield, MA, USA), and the data were evaluated with CellQuest software (Becton Dickinson, Mansfield, MA, USA). Cell viability was quantified as a percentage compared to the control. The experiments were repeated three times.

Cell Viability Analysis

Methylthiazoletetrazolium (MTT)

The MTT assay was performed as previously described.[13] Cells were seeded in 96-well plates at 1×10^4 cells/well in 200 μl of medium.[14] They were cultured for 24 h to allow adherence. The medium was then replaced with fresh medium containing different concentrations of udenafil. After further incubation for 24 h, 100 μl of MTT (5 g/L in PBS, Calbiochem, CA, USA) was added to each well and the plates were incubated at 37°C for 4 h. To each well 150 μl of dimethyl sulfoxide was added, and the plates were agitated on a plate shaker for 10 min. Optical density at 570 nm was read with an ELISA reader (Spectra Max 190, Molecular Devices, CA, USA). The experiments were performed in triplicate.

Trypan blue dye exclusion

Trypan blue dye exclusion assays and cell counting were used to determine viable cell numbers.[15] Cells were seeded at 1.6×10^5 cells/well in 1.5 ml of medium in 6-well plates, and cultured at 37°C overnight.[16] After washing with PBS, they were incubated with different concentrations of udenafil for 24 h, harvested with trypsin-EDTA (Gibco, Burlington, Canada) and stained with 0.4% trypan blue dye (Gibco, Rockville, MD, USA). Trypan blue-positive and -negative cells were counted with a hemocytometer (Hausser Scientific, Horsham, PA, USA) under a phase-contrast microscope (Nikon Diaphot-300, Tokyo, Japan). The experiments were performed in triplicate.

Statistical Analysis

Data are expressed as means ± standard deviations. Comparisons of parameters among the groups were performed

with a one-way analysis of variance (ANOVA) followed by post hoc Tukey's test using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Morphologic Changes of VEC and VSMC

Confluent cultures of adherent VEC had the typical cobblestone morphology under control conditions. After exposure to udenafil, they became rounded and partially detached, and had the abnormal appearance of apoptotic cells. Moreover, the density of adherent cells was reduced. In 100 μmol/l udenafil, approximately 40% of the VEC remained attached to the culture dish; in 1 mmol/l, there were few adherent cells [Figure 2].

Confluent cultures of adherent VSMC had their typical elongated ribbon- or spindle-shaped appearance and formed parallel arrays under control conditions. In 1-100 μmol/l udenafil, the VSMC lost their typical spindle-shaped appearance and some cells appeared swollen and detached from the culture dishes. In 100 μmol/l udenafil, most of the VSMC became detached or had the abnormal appearance of apoptotic cells. In 1 mmol/l udenafil, there were few adherent cells [Figure 3].

Effects of udenafil on cell proliferation

In VEC and VSMC, DNA synthesis was suppressed in a concentration-dependent manner. In 100 μmol/l udenafil, the VSMC proliferation was significantly reduced compared with VEC (45.75 ± 11.38 vs 79.77 ± 14.34, $P < 0.05$). In contrast, in 1 mmol/l udenafil, the VEC proliferation was significantly reduced compared with VSMC (5.08 ± 18.29 vs 42.77 ± 7.79, $P < 0.05$). In 1 or 10 μmol/l udenafil, there were no significant difference between VEC and VSMC proliferation [Figure 4a].

Effects of udenafil on cell apoptosis

Udenafil increased the apoptosis of VEC and VSMC in a concentration-dependent manner [Figure 5a-j]. In 100 μmol/l udenafil, but not in 1 μmol/l, 10 μmol/l, or 1 mmol/l udenafil,

Figure 2: Phase-contrast microscopic appearance of cultured vascular endothelial cell (VEC) exposed to various udenafil concentrations. Confluent cells incubated with (a) 0 μmol/l udenafil, (b) 1 μmol/l udenafil, (c) 10 μmol/l udenafil, and (d) 100 μmol/l udenafil for 24 h displayed dose-dependent cytopathic changes. In 1 mmol/l udenafil (e), there were few adherent cells. Scale bars, 50 μm

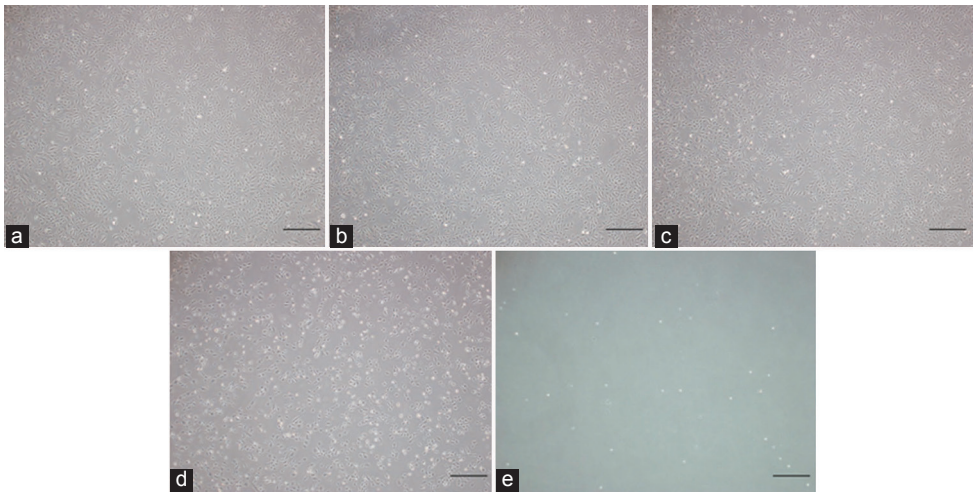


Figure 3: Phase-contrast microscopic appearance of cultured vascular smooth muscle cell (VSMC) exposed to various udenafil concentrations. Confluent cells exposed to (a) 0 μmol/l udenafil, (b) 1 μmol/l udenafil, and (c) 10 μmol/l udenafil for 24 h displayed dose-dependent cytopathic changes. In 100 μmol/l udenafil (d), most of the cells were detached. In 1 mmol/l udenafil (e), there were few adherent cells. Scale bars, 50μm

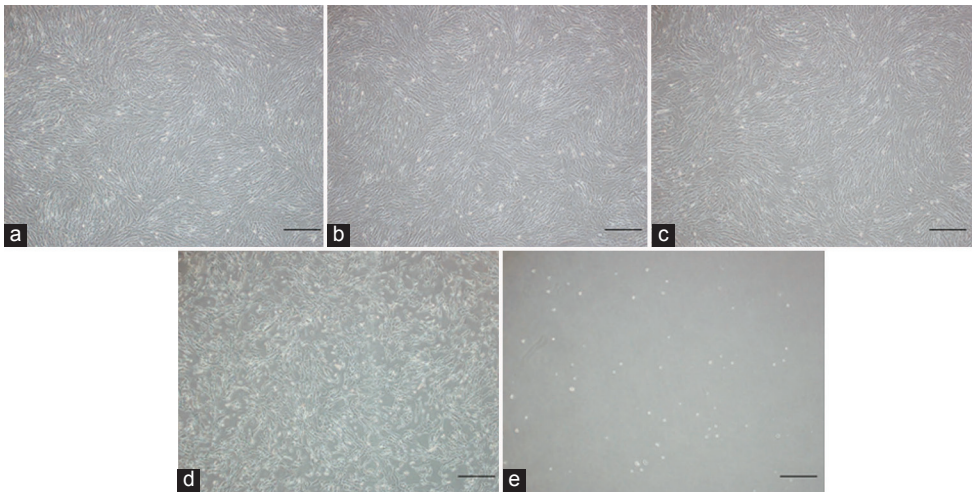


Figure 4: Effects of udenafil on the survival and growth of VEC and VSMC. (a) VEC proliferation was significantly less inhibited by 100 μmol/l udenafil than VSMC. (b) VEC apoptosis was significantly less induced by 100 μmol/l udenafil than VSMC. (C-E) VEC viability was significantly less inhibited by 100 μmol/l udenafil than VSMC. (c) Cell viability measured by MTT assays. (d) Cell viability measured by manual cell counting and trypan blue staining. (e) Cell viability determined by flow cytometry. Data are means ± standard deviation (SD). *$P < 0.05$ vs corresponding VSMC group. BrdU = 5-bromo-2'-deoxyuridine, MTT = methylthiazoletetrazolium

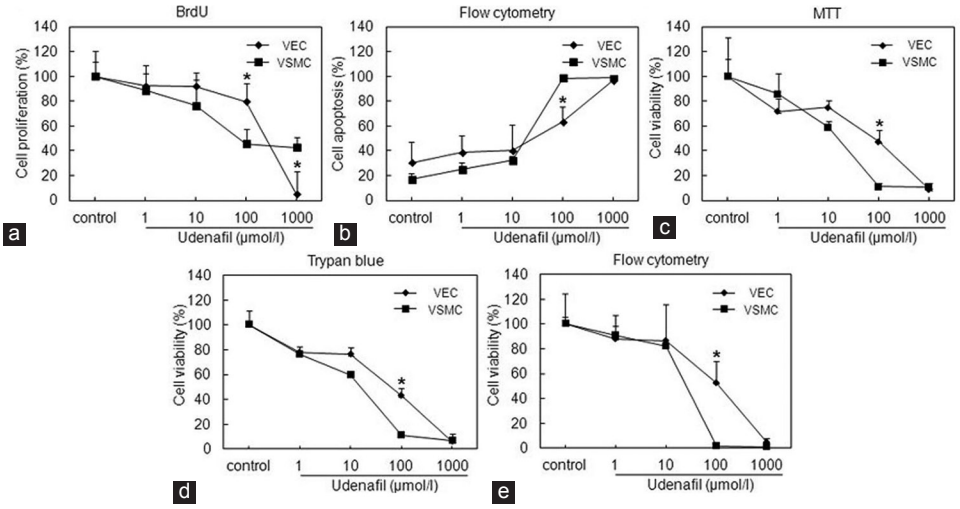
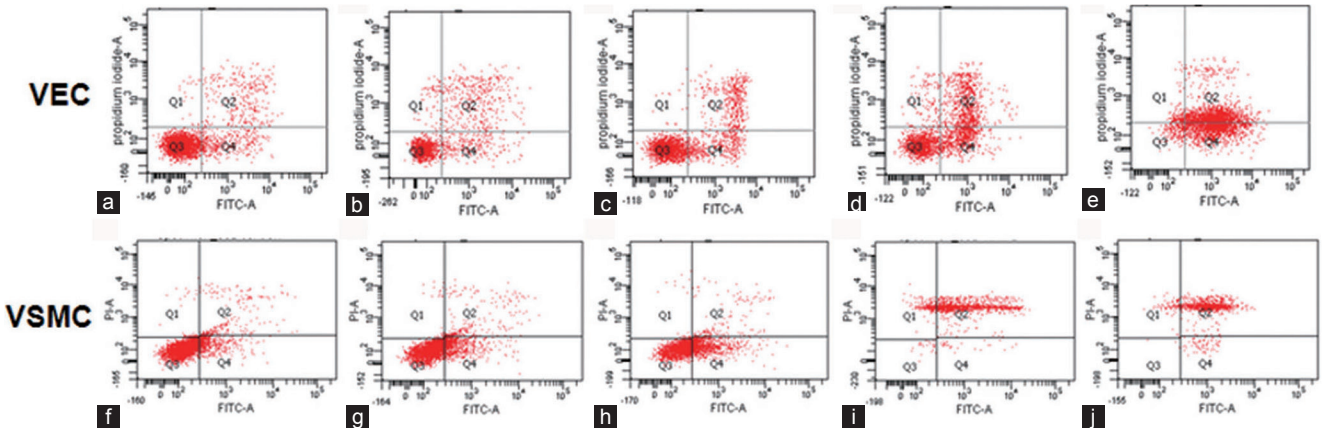


Figure 5: Effect of different concentrations of udenafil for 24 h on apoptosis in VEC and VSMC. Distributions of cells treated with different concentrations of udenafil displayed as dot plots: Viable cells (fluorescein isothiocyanate (FITC¯)/propidium iodide (PI¯)), apoptotic cells (FITC+/PI¯), secondary necrotic cells (FITC+/PI+). (a and f) Cells incubated with 0 μmol/l udenafil. (b and g) Cells incubated with 1 μmol/l udenafil. (c and h) Cells incubated with 10 μmol/l udenafil. (d and i) Cells incubated with 100 μmol/l udenafil. (e and j) Cells incubated with 1 mmol/l udenafil. A minimum of 10,000 events was counted per sample



there was a significant difference between VEC and VSMC apoptosis (63.36 ± 12.41 vs 98.63 ± 0.23, $P < 0.05$) [Figure 4b].

Effects of udenafil on cell viability in the MTT assay

Udenafil also decreased the viability of VEC and VSMC in a concentration-dependent manner [Table 1]. Again in 100 μmol/l udenafil, but not in 1 μmol/l, 10 μmol/l, or 1 mmol/l udenafil, there was a significant difference between VEC and VSMC viability (47.26 ± 9.73 vs 10.99 ± 3.14, $P < 0.05$) [Figure 4c].

Effects of udenafil on cell viability as assessed by the trypan blue dye exclusion assay

Udenafil decreased the viability of the VEC and VSMC in a concentration-dependent manner [Table 2]. In 100 μmol/l udenafil, but not in 1 μmol/l, 10 μmol/l, or 1 mmol/l udenafil, there was a significant difference between VEC and VSMC viability (42.85 ± 6.12 vs 11.11 ± 1.48, $P < 0.05$) [Figure 4d].

Effects of udenafil on cell viability as assessed by flow cytometry

The VEC viability of the 1 mmol/l group was significantly reduced compared with the control group (4.65 ± 3.23 vs 100 ± 24.57%, $P < 0.05$). In the 1, 10, and 100 μmol/l groups, there were no significant reductions compared with the control group [Figure 5a-e]. The VSMC viability in the 100 μmol/l and 1 mmol/l groups were significantly reduced compared with the control group (1.64 ± 0.27 vs 100 ± 6.04%, $P < 0.05$; 0.76 ± 0.42 vs 100 ± 6.04%, $P < 0.05$) [Figure 5f-j]. In 100 μmol/l udenafil, but not in 1 μmol/l, 10 μmol/l, or 1 mmol/l udenafil, there was a significant difference between VEC and VSMC viability (52.68 ± 17.85 vs 1.64 ± 0.27, $P < 0.05$) [Figure 4e].

Table 1:

Effect of 0-1 mmol/l udenafil for 24 h on VEC and VSMC viability assessed by MTT assays

	Control		1 μmol/l		10 μmol/l		100 μmol/l		1 mmol/l	
	OD	Viability (%)	OD	Viability (%)	OD	Viability (%)	OD	Viability (%)	OD	Viability (%)
VEC	0.0914±0.0130	100±14.27	0.0656±0.0090	71.77±9.92	0.0684±0.0055	74.83±6.07	0.0432±0.0088	47.26±9.73	0.0084±0.0004	9.23±0.47
VSMC	0.2948±0.0921	100±31.24	0.2530±0.0478	85.82±16.24	0.1746±0.0139	59.22±4.71	0.0324±0.0092	10.99±3.14	0.0324±0.0088	10.85±2.99

The results were expressed as mean±standard deviation. Viability (%) = (experimental OD/control OD)×100. VEC = Vascular endothelial cell, VSMC = Vascular smooth muscle cell, MTT = Methylthiazoletetrazolium, OD = Optical density

Table 2:

Effect of 0-1 mmol/l udenafil for 24 h on VEC and VSMC viability assessed by trypan blue dye exclusion assays

	Control		1 μmol/l		10 μmol/l		100 μmol/l		1 mmol/l	
	Cell number (1×10^4)	Viability (%)	Cell number (1×10^4)	Viability (%)	Cell number (1×10^4)	Viability (%)	Cell number (1×10^4)	Viability (%)	Cell number (1×10^4)	Viability (%)
VEC	16.33±0.57	100±3.53	12.66±0.76	77.55±4.67	12.50±0.86	76.53±5.30	7.00±1.00	42.85±6.12	1.00±1.00	6.12±6.12
VSMC	19.50±2.29	100±11.75	14.83±0.14	76.06±0.74	11.66±0.28	59.82±1.48	2.16±0.28	11.11±1.48	1.33±0.57	6.83±2.96

The results were expressed as Mean±Standard deviation. Viability (%) = (experimental cell number/control cell number)×100. VEC = Vascular endothelial cell, VSMC = Vascular smooth muscle cell

Discussion

In this study we showed that the concentration-proliferation inhibition curves of udenafil for VEC and VSMC differed. In the BrdU, MTT, trypan blue dye exclusion, and flow cytometry assays, udenafil inhibited the survival and growth of VEC and VSMC in a concentration-dependent manner. However, there were no differences between the effects of 1 μmol/l, 10 μmol/l, and 1mmol/l concentrations of VEC and VSMC. VEC survival and growth were significantly less inhibited by 100 μmol/l udenafil than VSMC ($P < 0.05$).

DESs that release either sirolimus or paclitaxel are currently being used clinically to prevent arterial neointimal hyperplasia following revascularization procedures involving stents. Axel *et al.*,[17] indicated that after single-dose application of paclitaxel for 24 h, they observed dose-dependent inhibition of VEC and VSMC proliferation. In 0.01 μmol/l paclitaxel, which had the maximum differential inhibitory effect on VSMC vs VEC proliferation, the difference of cell growth was approximately 15%. Moreover, Matter *et al*.,[18] demonstrated that in 0.1 nmol/l sirolimus, there was a difference of cell growth of approximately 10%. In our study, the difference in cell viability in 100 μmol/l udenafil was approximately 30%, more than with either paclitaxel or sirolimus.

It is likely that the differential effect of udenafil on cell survival and growth in VEC and VSMC is due to an effect on intracellular PDE-5 activity; current research indicates that there is high concentration of PDE-5 in VSMC, but a lower concentration in VEC.[19]

Like other antiproliferative DES agents, udenafil has a property that makes it an attractive candidate for local drug therapy of excessive arterial smooth muscle cell proliferation in restenosis after stent implantation; its highly lipophilic character[20] may promote rapid cellular uptake by enabling it to pass easily through the hydrophobic barrier of the cell membrane.

Although the maximum inhibitory effect of udenafil was comparable to those of paclitaxel and sirolimus (90% for udenafil vs 90% for paclitaxel and 75% for sirolimus), the effective anti-proliferative concentration for VSMC may need to be higher for udenafil than for paclitaxel or sirolimus (0.1-1 mmol/l for udenafil vs 0.01-10 μmol/l for paclitaxel and 2.5-14 nmol/l for sirolimus).[17,18,21] Therefore, it will be necessary to investigate the controlled release of udenafil from DES in an animal model.

In addition, we limited the duration of drug treatment to 24 h, so that we were unable to evaluate the long-term effects of udenafil on survival and growth of VEC and VSMC. Since recovery from stent-induced vascular injury requires a long time, a long-term study of the *in vivo* effects is required.

In summary, our study showed that 100 μmol/l udenafil has a maximum differential effect on VSMC versus VEC survival and growth. This differential effect of udenafil could potentially contribute to preventing late thrombosis due to current DES.

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