Experimental Assessment of Effects of Antiproliferative Drugs Used in Drug-Eluting Stents on Endothelial Cells

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Abstract

Background: Late and very late stent thrombosis after drug-eluting stent implantation is a major concern. The present study evaluated difference in the effects of sirolimus, paclitaxel and zotarolimus on endothelial cells.

Methods: Mouse endothelial cells were seeded in a 6-well plate. Cells were cultured with an antiproliferative drug at the expected concentrations for each well for 24 hours before making 3 scratch lines with a pipette tip. After a 4.5 hour incubation period, 3 reference scratch lines, vertically across the original scratch lines, were made in the same way. The experiment was repeated at least 6 times (6 plates). Measurements were performed at 9 crossings of each well. Wound healing ratio was calculated as 1 - (distance of the first scratch/distance of the second scratch). % cell migration was calculated as (wound healing ratio at an expected drug concentration/wound healing ratio with no drug) \times 100. Average % cell migration at 54 crossings of 6 plates was calculated.

Results: Paclitaxel inhibited cell migration in a concentration-dependent manner. On the other hand, concentration-dependent inhibition was not observed for sirolimus or zotarolimus. Sirolimus showed a stronger inhibitory effect on migration of endothelial cells compared to zotarolimus.

Conclusions: The difference in the effect of antiproliferative drugs of drug-eluting stents on endothelial cells may be associated with relatively faster re-endothelialization of zotarolimus-eluting stent compared to the 1st generation DES.
1. Introduction

Drug-eluting stent (DES) has dramatically reduced in-stent restenosis. However, late and very late stent thrombosis after DES implantation has emerged as a major concern. The antiproliferative drugs used in DES not only inhibit intimal hyperplasia but also delay re-endothelialization. Recent trials have shown a lower stent thrombosis rate of the 2nd generation DES compared to the 1st generation DES [1]. Thus the effect of the antiproliferative drugs of the 1st and 2nd generation DES on re-endothelialization may be different. The present experimental study evaluated difference in the effect of the antiproliferative drugs of the 1st and 2nd generation DES on endothelial cells.

2. Methods

2.1. Chemicals

Chemicals were purchased from the following commercial sources: paclitaxel (Wako Chemicals, Japan), zotarolimus (Toronto Research Chemicals, Canada), and rapamycin (Sigma, USA). Ethanol was used as solvent for paclitaxel and rapamycin, and DMSO for zotarolimus.

2.2. Cell culture

Mouse vascular endothelial cell line, UV2 (RCB1994) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were cultured at 37 °C in a 5% CO₂, humidified atmosphere. Cells were grown in Dulbecco’s Modified Eagle’s Medium (High glucose, Sigma, USA) with 10% fetal bovine serum (HyClone, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin from Nacalai Tesque (Kyoto, Japan).

2.3. Scratch assay

We assessed the effects of antiproliferative drugs of DES on cultured endothelial cells by scratch wound assay. Cells were seeded at the density of 0.7-1.5×10⁵ cells/well in a 6-well plate. Following the pre-culture period for 24 hour (Figure 1), the volume of culture medium in each well was adjusted to 2 ml to start the incubation of cells with a drug at the
expected concentrations for each well in the plate. The drug concentrations were selected according to those in previous studies [2,3]. Cells were cultured for another 24 hours before making 3 scratch lines with a pipette tip (200-μl size), and the closure of the scratch lines was measured after a 4.5-hour incubation period. Three reference scratch lines, vertically across the original scratch lines, were made in the same way just before fixing cells with 4% paraformaldehyde for 10 min at room temperature prior to take images under the microscope at 40× magnification (Figure 2) [4]. The images acquired for each sample were analyzed quantitatively by using ImageJ 1.48v (NIH, USA). The experiment was repeated at least 6 times (6 plates).

Cell migration was evaluated by comparing the images between the solvent-treated control cells and the drug-treated cells. For each image, measurements were performed at 9 crossings of each well (Figure 1). The distances of the first scratch and the second scratch were measured at 6 points (Figure 2). The mean distances of the first scratch and the second scratch were calculated. Wound healing ratio was calculated as 1 - (mean distance of the first scratch/mean distance of the second scratch). % cell migration was calculated as (wound healing ratio at an expected drug concentration/wound healing ratio with no drug) × 100. Average % cell migration at 54 crossings of 6 plates was calculated.

2.4. Statistical analysis

Statistical analysis was performed with SAS9.4 (SAS Institute Inc. Cary, NC, USA). Values are shown as mean ± SD. Continuous variables were compared using Student’s t test or one-way analysis of variance. Statistical significance was defined as p <0.05

3. Results

Paclitaxel inhibited cell migration in a concentration-dependent manner (Figure 3). On the other hand, concentration-dependent inhibition was not observed for sirolimus or zotarolimus. Pharmacokinetics of sirolimus-eluting stent and zotarolimus-eluting stent were reported [5,6]. However, there is little information about pharmacokinetics of paclitaxel-eluting stent. Thus we compared the effect of sirolimus and zotarolimus at the
concentrations that were closest but higher than the maximum concentration after sirolimus-
(0.86 ± 0.21 ng/ml) and zotarolimus-eluting stent implantation (1.80 ± 0.53 ng/ml) [6]. Sirolimus showed a stronger inhibitory effect on migration of endothelial cells compared to zotarolimus (Figure 4), although concentration of zotarolimus compared to that after stent implantation was much higher than that of sirolimus.

4. Discussion

The present study evaluated effects of antiproliferative drugs of the 1st and 2nd generation DES on endothelial cells. Inhibition of endothelial cell migration with paclitaxel was concentration-dependent. The concentration-dependent inhibition was not observed for sirolimus or zotarolimus. Sirolimus inhibited cell migration more than zotarolimus.

The reported incidence of late and very late stent thrombosis after the 1st generation DES implantation ranged between 0.2% and 0.7% [7-12]. A cohort study reported that definite stent thrombosis continued to occur at the constant rate of 0.6% per year from 30 days to 3 years after the 1st generation DES implantation [11]. Stent thrombosis often results in myocardial infarction or death. Iakovou et al.[12] reported a case fatality rate of stent thrombosis at 45%. Recent studies have shown that stent thrombosis is less frequent after implantation of the 2nd generation DES compared to the 1st generation DES [13]. It may be owing to better stent material, implantation techniques, and antiplatelet therapy. A human autopsy study has shown greater strut coverage with less inflammation and fibrin deposition after implantation of the 2nd generation DES compared to the 1st generation DES [14].

DES usually consists of 3 components: (1) metallic stent, (2) drug as an antiproliferative agent to inhibit neointimal formation, and (3) polymer as a drug-carrier vehicle. The cause of stent thrombosis is multifactorial. Patient-related, lesion-related, and procedure-related factors may be intertwined. A study demonstrated less thrombogenicity of stent with thinner struts [15]. It has been shown that hypersensitivity reaction to the polymer of the 1st generation stents is associated with late and very late stent thrombosis [16]. However, delayed re-endothelialization due to the antiproliferative drugs of DES may be mainly associated with late and very late stent thrombosis [17].
Paclitaxel inhibits endothelial cell migration and intimal hyperplasia by stabilizing microtubules [18,19]. Cytostatic effect of low-dose paclitaxel on human endothelial cells has been demonstrated [20]. Because paclitaxel is an anticancer agent, therapeutic window may be narrow, which may be associated with delay in re-endothelialization in a concentration-dependent manner [21]. Sirolimus [22,23] and zotarolimus [24] inhibit the mammalian target of rapamycin pathway, which results in the antimigration and antiproliferative effect. A previous study evaluated the effects of antiproliferative drugs of DES on endothelial progenitor cells [25]. Sirolimus or paclitaxel inhibited endothelial progenitor cells migration to a significantly greater degree than zotarolimus. Delayed re-endothelialization of DES is a mechanism of late and very late stent thrombosis. Stronger inhibitory effect of antiproliferative drugs on migration of endothelial cells may be associated with delayed re-endothelialization of DES. Cell migration studies may be useful to find a better antiproliferative drug in case a new DES is developed.

5. Limitations

The present study used mouse vascular endothelial cells and compared the effect of drugs on cell migration at each concentration based on known pharmacokinetics. The effects of antiproliferative drugs might be different between mouse and human endothelial cells. Steinfeld et al.[26] used endothelial cell isolated from human coronary arteries and reported paclitaxel inhibited cell migration compared with zotarolimus at 1 μM. The study compared the effect of drugs on same concentration. However, the effect of actual concentrations of each antiproliferative drug at the vascular endothelium adjacent to DES is unknown. In addition, endothelialization can be affected by endothelial progenitor cells and other kinds of cells. Therefore, further analysis based on more information of pharmacokinetics of antiproliferative drugs and using those cells may be needed to assess speed of endothelialization more precisely.

6. Conclusions

Inhibition of endothelial cell migration with paclitaxel is concentration-dependent.
On the other hand, it is concentration-independent for zotarolimus or sirolimus. Zotarolimus has less inhibitory effect compared to sirolimus. It may be associated with relatively faster re-endothelialization of zotarolimus-eluting stent compared to the 1st generation DES.

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Conflict of interest

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References


**Figure legends**

Fig. 1  Experimental flow chart.

Fig. 2  Measurements of cell migration. The distances of the first scratch line (W1-6) and the second scratch line (H1-6) are measured at 6 points. The mean distances of the first scratch and the second scratch are calculated. Wound healing ratio was calculated as 1 - (mean distance of the first scratch/mean distance of the second scratch).

Fig. 3  The effect of antiproliferative drugs on endothelial cell migration. Paclitaxel inhibits cell migration in a concentration-dependent manner. On the other hand, concentration-dependent inhibition is not observed for sirolimus or zotarolimus.

Fig. 4  The effect of sirolimus and zotarolimus on endothelial cell migration at each concentration based on pharmacokinetics after stent implantation. Sirolimus shows a stronger inhibitory effect on migration of endothelial cells compared to zotarolimus.
Endothelial cells are seeded in a 6-well plate.

An antiproliferative drug at the expected concentrations for each well is added.

3 scratch lines with a pipette tip are made.

3 reference scratch lines, vertically across the original scratch lines, are made.
Sirolimus (1.0 ng/ml) vs Zotarolimus (9.7 ng/ml)

Average % cell migration

p=0.002

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