Effects of Various Adhesive Substrates on the Adhesion Forces of Endothelial Progenitor Cells

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Received 11 Oct 2010; Accepted 1 Jan 2011; doi: 10.5405/jmbe.855

Abstract

This study evaluates the effects of various substrates on the adhesion forces of endothelial progenitor cells (EPCs) and identifies the optimal antibody for EPC-capture stents. Single-cell micropipette aspiration (SCMA) is employed to measure the adhesion forces of single EPCs on various substrates, namely gelatin and antibodies CD34, VEGFR-2, and CD133. The adhesion forces of single EPCs on each substrate are compared with those of human umbilical vein endothelial cells (HUVECs). Compared to HUVECs, our results show that EPCs exhibit better adhesion on gelatin, anti-CD34, and anti-CD133. The adhesion forces of EPCs or HUVECs on surfaces coated with these three substances increases with the concentration of these three substances. The adhesion forces are cell-type- and substrate-dependent. EPCs have stronger adhesion than HUVECs on surfaces coated with anti-CD34 or anti-CD133. The adhesion forces of EPCs on an anti-CD133-coated surface are higher than those on anti-CD34-, anti-VEGFR-2-, and gelatin-coated surfaces. The findings suggest that using CD133 antibody to capture EPCs is more efficient than using CD34 antibody.

Keywords: Endothelial progenitor cell, Adhesive substrate, Single micropipette aspiration technique, Adhesion forces, CD133 antibody

1. Introduction

A “pro-healing” approach for the prevention of post-stenting restenosis is currently favored over cytotoxic or cytostatic local pharmacologic therapies [1]. For example, the emergence of drug-eluting stents has dramatically reduced the incidence of in-stent restenosis (ISR) [2,3]. However, drug-eluting stents fail to completely prevent ISR due to their inhibitory effects on endothelial proliferation. Rapid endothelialization can prevent thrombogenicity, thereby inhibiting ISR and accelerating injured-vessel wall re-endothelialization. However, the adhesion, proliferation, and conditions of cells cultured on the surface of a stent in vitro are still unknown. Recent studies showed that circulating endothelial progenitor cells (EPCs) are a key factor in re-endothelialization [4]. The early establishment of a functional endothelial layer after vascular injury has been shown to assist in the prevention of neointimal proliferation and thrombus formation [5,6]. In addition, EPC-capture stents have been developed using immobilized antibodies targeted at the surface antigens of EPCs [7,8]. Antibody-coated stents planted into a blood vessel are able to capture and hold EPCs from circulating blood; therefore, they accelerate stent endothelialization depending on the adhesion of EPCs onto the antibody. Stents coated with antibody against CD34 have been implanted in patients with de novo coronary artery disease, and follow-up examination results demonstrate that the EPC-capture coronary stent is safe and feasible for such treatment [7]. However, the efficiency of adhesion of EPCs on antibodies is unknown. In the present study, the adhesion forces of EPCs on various adhesive substrates, namely gelatin and antibodies CD34, VEGFR-2, and CD133, were evaluated using an in vitro single-cell micropipette aspiration (SCMA) technique.

2. Materials and methods

2.1 EPC isolation and characterization

Male Sprague-Dawley (SD) rats (400-450 g) were purchased from the Chongqing Medical University (Chongqing, China). An experimental protocol in accordance with principles
of the Chinese Laboratory Animal Care and Use Committee was followed.

Bone marrow isolated from SD rats was diluted by Dulbecco’s Modified Eagle’s Medium and Nutrient Mixture F-12 (DMEM/F12) with a ratio of 1:1, and mononuclear cells (MNCs) were separated by density gradient centrifugation with 1.077 g/ml Percoll solution for 20 min at 4000g. Cells were cultured separately for 3 weeks in DMEM/F12 with 20% foetal bovine serum. Fresh medium was added every three days. The non-adherent cells were re-seeded in gelatin-coated six-well plates. After 7-10 days of culture, non-adherent cells were washed and removed by phosphate buffered saline (PBS) solution, and adherent cells were continuously cultured in 25-cm² culture flasks and six-well plates. First-passage cells were used in this experiment.

Phenotypic analysis of the cells was performed on days 7 and 21. Adherent cells were examined for expression of several endothelial cell markers, such as vascular endothelial growth factor receptor 2 (VEGFR-2)/fetal liver kinase-1 (Flk-1), and CD34 and CD133 antibodies, by immunofluorescence (IX81, Olympus, Tokyo, Japan), immunochemistry (IX81, Olympus, Tokyo, Japan) and flow cytometry (FACSCalibur Becton Dickinson, Beckman Coulter, Miami, FL, USA).

As a control, human umbilical vein endothelial cells (HUVECs) were cultured in six-well plates under endothelial cell growth conditions. Two types of cell suspension with cell densities of 1 x 10⁷ were obtained with the conventional method before micropipette aspiration experiments.

2.2 Single-cell micropipette aspiration (SCMA)

SCMA provides a simple method for probing the mechanical properties of single cells. SCMA (Fig. 1) is commonly applied to study the effects of substrates on the adhesion forces of single cells [9-11]. The system consists of a micromanipulator (MR5170, Eppendorf Co., Germany), a microscope (Axiovert35, Zeiss Co., Germany), an image recording system (NV-HD100MC, Panasonic Co., Japan), an image processing system (Vidas 21, Kontron Co., Germany), and pressure transducers (Bioengineering College of Chongqing University, China). The detachment of a cell from the substrate is here defined as a whole single cell leaving the bottom of the chamber (Fig. 2).

Chambers were submerged in 75% alcohol overnight, washed with distilled water 10 times, and were placed in sterilized culture dishes for open-air drying. Chambers were coated with various substrates in various concentrations; uncoated chambers were used for control experiments. The substrates covered the bottoms of chambers. Chambers were dried under a bacteria-free environment for 30 min after they were coated with various substances. Both of steps were repeated 3 times to prepare the coating chambers. Gelatin (Sigma Co., USA) substrates with concentrations of 20, 40, 60, and 80 mg/ml were made. CD34, VEGFR-2, and CD133 antibody (Santa Co., USA) substrates with concentrations of 1:100, 1:200, 1:300, and 1:400 volume ratios were made for this study.

Capillary tubes with an inner diameter of about 0.9 mm were pulled into a micropipette using a Flaming/Brown Micropipette Puller (P87, Sutter Instrument Co., USA). The inner radii (R₀) of the micropipettes ranged from 2.5 to 3.5 μm. The tips of the micropipettes were cut and fire-polished by a microforge.

2.3 Adhesion force measurements by SCMA

The adhesion forces of single EPCs on substrate materials were measured using SCMA and compared with those of HUVECs. Details of the use of SCMA for adhesion forces measurements can be found elsewhere [11-13]. Briefly, glass micropipettes were used and the adhesion forces were measured under direct microscopic observation in conjunction with a video recording system. The forces (product of aspiration pressure and the cross-sectional area of the micropipette tip) required to detach a cell from the substrate-coated surface was measured by stepwise increases in aspiration pressure followed by retraction of the pipette. The cell suspension was plated onto the bottoms of the chambers for 30 min at room temperature. Cells were randomly chosen for measurements and the aspiration pressure (ΔP) was recorded when a single cell had detached from the substrate-coated surface.

\[
F = -\Delta P \tau \cos \theta
\]

where \( R_0, \theta, \) and \( \Delta P \) are the microtube inner radius, the included angle between the sticking surface and microbule axial ray, and the transition under pressure made cell separate
sticking surface, respectively.

In this system, the horizontal orientation of the micropipette is defined as θ = 15°. The relative adhesion forces (F) can be calculated as:

\[ F = \Delta P \pi (R_p)^2 \]  

(2)

Figure 2 shows the process of a single EPC being detached from the surface of coated substrates by a micropipette.

2.4 Statistical analysis

The data shown are mean values of at least three independent experiments and expressed as mean ± standard deviation (SD). An unpaired Student’s t-test or ANOVA was used to evaluate the significance of the differences. Statistical significance was set to \( p < 0.05 \), with \( p < 0.01 \) being highly significant.

3. Results

3.1 Isolation and Characterization of EPCs

Under the culture conditions used in this study, outgrowth colonies of adherent cells were found between days 5 and 8 after plating. These colonies proliferated rapidly and exhibited a cobblestone morphology (Fig. 3A). Two weeks after plating, most colonies reached near-confluence (Fig. 3B). Cells were then passaged several times; no discernible alteration in cell morphology or growth characteristics was observed during this procedure (Fig. 3C). After 10 days of culture, the majority of cells expressed endothelial cell marker VEGFR-2 (Fig. 3D), and endothelial progenitor cell markers CD34 (Fig. 3E) and CD133 (Fig. 3F). The results from flow cytometry show that there were 73.28 ± 7.43% CD34+/VEGFR-2+ cells and 70.36 ± 10.17% CD34+/CD133+ cells. The isolated EPCs were colony-forming units of HUVECs.

3.2 Effects of Substrate Concentrations on Adherent Forces of EPCs

The chamber surface was coated by gelatin solution (20 mg/ml) and vacuum dried. The chamber was then placed in an antibody solution for 24 h and vacuum dried. The antibody solution was diluted with PBS solution 400, 300, 200, and 100 times (V/V). The lower concentrations of antibodies VEGFR-2 and CD34 did not significantly affect the adhesion forces of EPCs \( (p < 0.05) \) (Fig. 4a, b). The adhesion of EPCs was significantly increased by anti-VEGFR-2 and anti-CD34 when diluted with PBS solution 200 and 100 times \( (p < 0.01) \). Anti-CD133 significantly increased the adhesion of EPCs \( (p < 0.001) \) in a dose-dependent manner (Fig. 4c).

![Image](57x146)

**Figure 3.** Characterization of EPCs from bone marrow of SD rat. (A) Colony of EPCs 5 days after plating; (B, C) cobblestone morphology of EPCs passaged several times 15 days after plating (100X); (D) immunofluorescence detection of VEGFR-2; immunohistochemical detection of (E) CD34 and (F) CD133. Preparations were viewed at 200X unless otherwise indicated.

![Image](212x167)

**Figure 4.** Effects of antibody solution concentration on the adhesion force of EPCs plated on chamber surfaces coated with (a) antibody VEGFR-2, (b) antibody CD34, and (c) antibody CD133. *: significant difference in adhesion force of EPC between chambers with different concentrations of antibody, \( p < 0.05 \); **: highly significant difference \( p < 0.01 \).
These results indicate that antibody concentrations affect the adhesion of EPCs on a modified surface. The increased adhesion forces of EPCs observed is concentration-dependent.

3.3 Effects of substrate type on adhesion forces of EPCs

All three types of antibody substrate, anti-VEGFR-2, anti-CD34, and anti-CD133, significantly increased the adhesion forces of EPCs. Among the three types of antibody substrate, the anti-CD133 substrate had the largest effect on the adhesion forces (Fig. 5). In addition, the adhesion forces increased in an antibody-dose-dependent manner.

3.4 Comparison of HUVEC and EPC adhesion forces

Figure 6 shows the effects of various antibody-coated surfaces on the adhesion of cells. The adhesion forces of HUVECs were not significantly different from those of EPCs when using low concentrations of gelatin. However, a significant difference was observed when using a concentration of 80 mg/ml (Fig. 6a). When the chamber was coated with the other three different antibodies, respectively, the other eleven pair treatments presented highly significant difference \((p < 0.01)\) in the adhesion force between HUVECs and EPCs except one pair low-concentration treatment (400 dilution) of VEGFR-2. The differences in cell adhesion force depend not only on the cell type, but also on the concentration of antibodies used (Figs. 6b, 6c, 6d).

These results show that the adhesion of EPCs was stronger than that of HUVECs when the chamber was coated with anti-CD34 or anti-CD133, but weaker when anti-VEGFR2 was used.

4. Discussion

SCMA has been proven to be an effective method for studying cell mechanical properties \([14,15]\). This technique has also been used to study the movement, transformation, and flexibility of single cells. It can detect the transformation process and yield the critical force values of single cells, which can elucidate cell mechanical characteristics and the interaction between cells and substrates. In this study, the precision of the pressure in SCMA was 0.25 mm-H\(_2\)O. The inner diameter of the micropipette was \(\sim 6 \, \mu m\), and the force precision for measuring cell adhesion was \(10^{-10} - 10^{-11} \, N\). Due to the small \(\theta\), the adhesion force defined here reflects the shearing force between the cell and the coated surface. The results of the adhesion force of cells measured by SCMA are in agreement with those of cell counting after washing under a shearing force.
The rates of cell growth, proliferation, and differentiation depend on a number of factors, including initial cell adhesion, the type of surface material, and the type of extracellular matrix (ECM) protein, such as fibronectin (FN) or laminin. The ECM in a serum can be absorbed non-specifically and integrated with integrin, which may further affect cell behavior. Different material surfaces have different effects on the adhesion properties of the ECM [16]. It has been shown that increasing the amount of FN used for coating the surface can lead to better cell adhesion, spreading, and growth, which are affected by factors including the chemical and physical properties of the material surface [17-19] and the number of ECMs. Inui et al. demonstrated that platelet-derived growth factor in oligosaccharides of chitosans interact with receptors on the surface of vascular smooth muscle cells and promote cell growth [20]. Pierschbacher first reported that the Arg-Gly-Asp (RGD) peptide specifically binds FN and its receptors [21]. Glycoproteins such as FN, fibrinogen, collagen, and osteopontin, that contain RGD peptides have thus been used to coat surfaces. It has been observed that RGD mediates interactions between cells and ECMs [22,23].

Results from this study suggest that an antibody-coated stent employed in the body may capture EPCs but not HUVECs or other cells in peripheral blood. EPCs are precursors of endothelial cells. Growing evidence suggests that bone-marrow-derived EPCs circulating in the blood may play an important role in the formation of new blood vessels, as well as contribute to vascular homeostasis in adults [24].

CD34, VEGFR-2, and CD133 are important antibodies for identifying EPCs. It was found that the adhesion forces of EPCs on surfaces coated with anti-CD133 were significant higher than those of EPCs on surfaces coated with antibody CD34 or VEGFR-2, which is consistent with previously reported results [25,26]. Our previous studies also showed that CD133 antibody had a more obvious influence on EPCs in terms of proliferation, adhesion, and NO secretion than did CD34. Using CD3133 antibody to capture EPCs has been demonstrated to be more efficient than using CD34 antibody [27]. These findings indicate that the specificity of CD133 for EPCs is higher than that of CD34, suggesting that CD133 antibody has more potential for EPC-capture stents [8,28].

This investigation primarily focused on the cellular adhesion forces under static conditions. Stents planted into vessels are subject to flow conditions, with EPCs circulating in the blood. Further research will involve examining EPC adhesion on material surfaces treated with multiple types of antibody, and atomic force microscopy will be used to quantitatively measure the adhesion force between cells and substrates [29]. Additionally, it is also necessary to further investigate the behavior of EPC adhesion, migration, and proliferation under flow conditions.

5. Conclusions

The adhesion force of EPCs on surfaces coated by anti-CD34 and anti-CD133 antibodies increased with the concentration of the antibody solution. The highest adhesion forces were obtained at a concentration of 1:100 for antibodies diluted with PBS solution. The adhesion forces of HUVECs and EPCs had a highly significant difference for chambers coated by three types of antibody. For a given antibody substrate, there was a highly significant difference in the adhesion forces between HUVECs and EPCs. EPCs had stronger adhesion than that of HUVECs on chamber surfaces coated with anti-CD34 or anti-CD133. The adhesion forces of EPCs on the surface coated with anti-CD133 were higher than those on the surface coated with anti-CD34, which were higher than those coated with VEGFR-2 and gelatin. This suggests that anti-CD133 has great potential for applications in EPC-capture stents.

Acknowledgements

This research was supported by grants from the Chinese Ministry of Science and Technology (2004DFA06400), the National Natural Science Foundation of China (30970721), and the Chongqing Municipality, China (DRC2005-1006, CSTC2006AAA5014-3) to Dr. G. Wang.

References


