Knockdown of P120 catenin aggravates endothelial injury under an impinging flow by inducing breakdown of adherens junctions

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Abstract. At present, the mechanisms underlying intracranial aneurysm (IA) development remain unclear; however, hemodynamics is considered a crucial factor in the induction of IA. To elucidate the association between hemodynamics and endothelial cell (EC) functions, a modified T chamber system was designed to simulate the adjustable hemodynamic conditions of an artery bifurcation. Normal human umbilical vein ECs (HUVECs) and HUVECs with P120 catenin (P120ctn) knockdown were cultured on coverslips and placed in the chamber. A flow rate of 250 or 500 ml/min impinged on the cell layer. Subsequently, the expression levels of P120ctn and other proteins, and EC morphological alterations, were examined. In normal HUVECs, after 3 h under a flow rate of 500 ml/min, the expression levels of P120ctn, vascular endothelial (VE)-Cadherin, Kaiso and α-catenin were decreased, whereas matrix metalloproteinase-2 (MMP-2) was increased. In HUVECs with P120ctn knockdown, the period during which ECs adhered to the coverslip was reduced to 1 h under a flow rate of 500 ml/min. In addition, the expression levels of VE-Cadherin, Kaiso and α-catenin in ECs were decreased, whereas those of MMP-2 were increased after 1 h; more prominent alterations were detected under a 500 ml/min flow rate compared with a 250 ml/min flow rate. Adherens junctions (AJs) are critical to the maintenance of normal morphology and EC functioning in the vascular wall, and P120ctn is an important regulator of AJs. Loss of P120ctn may be induced by hemodynamic alterations. In response to changes in hemodynamic conditions, a loss of P120ctn may aggravate AJs between ECs, thus inducing inflammation in the vascular wall. Clinically, hemodynamic alterations may result in a loss of P120ctn and endothelial injury; therefore, P120ctn may have a critical role in inducing intracranial aneurysms.

Introduction

The mechanisms underlying intracranial aneurysm (IA) remain unclear; however, hemodynamics is considered a crucial factor in the induction of IA. Li et al (1) demonstrated that alterations in hemodynamic conditions may lead to vascular endothelial injury and a decrease in P120 catenin (P120ctn) expression in endothelial cells (ECs). P120ctn has emerged as a master regulator of cadherin retention and stability at the cell surface. The stabilizing effects of P120ctn are associated with its direct interaction with the cytoplasmic region of cadherin; however, how this interaction is modulated to control cell adhesion remains unclear. Sakamoto et al (2) described the changes in morphology, shape and distribution of ECs after being subjected to an impinging flow for 24 h. Endothelial injury and vascular wall inflammation may contribute to the formation of IA, and the bifurcation of an artery is a common location where IA occurs (3). The aneurysm-promoting environment is characterized by a high wall shear stress (WSS) and a high positive wall shear stress gradient (WSSG) (4).

Adherens junctions (AJs) are specialized areas of the plasma membrane where bundles of the actin cytoskeleton attach to the membrane through transmembrane linkers. Vascular endothelial (VE)-Cadherin is the major cell-cell adhesion molecule in vascular ECs, and is regarded as a master organizer of the endothelial phenotype (5) and an active guardian of vascular integrity (6). VE-Cadherin attaches through its extracellular domains to cadherin in the neighboring cell membranes. P120ctn has been identified as one of several cofactors that interacts with the cadherin tail and modulates cadherin function (7). P120ctn and VE-Cadherin form a cadherin-catenin complex (CCC) to stabilize the AJs between ECs, as well as the EC phenotype.

Inflammation in the vascular endothelium is considered to be associated with the induction of endothelial injury and remodeling (8). The expression of P120ctn has been reported to
exert a positive effect on preventing inflammation in ECs (9). Loss of P120ctn may upregulate the expression levels of some proinflammatory factors, including Kaiso and matrix metalloproteinase-2 (MMP-2), thus inducing inflammation in ECs, and affecting the morphology and functions of ECs (10). Kaiso is a ubiquitously expressed BTB/POZ domain transcription factor, which was initially identified as an interaction partner of P120ctn in the cytoplasm (11), and is able to induce inflammation in vitro and in vivo (12). Further studies are required to clarify whether inflammation, induced by various hemodynamic conditions, leads to endothelial injury, and to investigate the roles of P120ctn, Kaiso and MMP-2 during this process.

At present, to the best of our knowledge, in vitro studies regarding the effects of impinging flow with various hemodynamic conditions on AJs between ECs are few. In the present study, the role of P120ctn in endothelial injury was investigated in vitro.

Materials and methods

T chamber system and experimental protocols. A modified T chamber system, which is able to mimic the bifurcation of an artery with various hemodynamic conditions, was designed (13). The patent for this system was registered at the state intellectual property office of the People's Republic of China (14). The T chamber system consists of a ‘T’ shape chamber with two pressure gauges set at branches, a pump, a reservoir containing flow media and connecting pipes. Forces generated by impinging flow were demonstrated as WSS and WSSG. The formula to calculate average WSS is force per unit area, as follows: \( \tau = \frac{F}{A} \). Where \( \tau \) is shear stress; \( F \) is the force applied and \( A \) is the cross-sectional area of material with area parallel to the applied force vector (15). The flow media used, methods of morphological examination and specific protocols of the modified system were elucidated in our previous study (13). In the present study, ImageJ 1.48 software (National Institutes of Health, Bethesda, MA, USA) was used to count the number of ECs on the coverslip. The flow rates selected were 250 and 500 ml min⁻¹. ECs that were not subjected to any impinging flow were identified as the static control group. All experiments were performed in a humidified incubator at 37°C. Characterization of the morphology, including shape, size and orientation, of ECs on the coverslip was achieved by observing cells under an inverted phase contrast microscope (IX71; Olympus Corporation, Tokyo, Japan), at x10 magnification. Digital images were captured using an inverted microscope (Olympus Corporation) and IP Lab software (7.1; Olympus Corporation).

Cell culture. Cell cultures were prepared and maintained according to standard cell culture procedures. Briefly, human umbilical vein endothelial cells (HUVECs; Lonza Group, Ltd., Basel, Switzerland) were cultured in endothelial basal medium (RPMI-1640; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, 10 ng/ml epidermal growth factor and 10% fetal calf serum, which were all provided by Gibco (Thermo Fisher Scientific, Inc.). HUVECs were incubated at 37°C and were cultured in an incubator containing 5% CO₂. Cells were passaged at regular intervals depending on their growth characteristics using 25% trypsin (Biochrom GmbH, Berlin, Germany) as described by Walter et al (16).

Targeted silencing of endogenous P120ctn with small interfering (si)RNA. Knockdown of P120ctn expression in ECs was performed using a siRNA oligonucleotide duplex synthesized by GE Healthcare Dharmacon, Inc. (Lafayette, CO, USA). The siRNA was generated based on the human P120ctn gene sequence, accession number NM_001331, and its sequence was as follows: 5'-GAAUGUGAUGUUAGUU UU-3'. The siCONTROL Non-Targeting siRNA (NT-siRNA; GE Healthcare Dharmacon, Inc.) acted as negative control. Transfection was conducted according to the protocol described by Zhang et al (17). HUVECs, at 50-60% confluence, were seeded in 60-mm dishes. Cells were transfected with 200 pM siRNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 24, 48 and 72 h post-transfection, cells were trypsinized and plated onto 35-mm dishes to assess knockdown efficiency. The confirmation of P120ctn knockdown was determined by western blot analysis.

Western blot analysis. ECs were collected and routinely prepared for western blot analysis and protein concentration determination. Cells were lysed in radioimmunoprecipitation assay buffer (cat. no. p0013; Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cell lysate was directly obtained for western blot analysis. The samples (50 μg) were loaded at a constant protein concentration and were separated by 8 or 12.5% SDS-PAGE, after which, they were electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) at 4°C overnight, and was then incubated overnight at 4°C with the appropriate

Figure 1. (A) Specific wall shear stress and (B) wall shear stress gradient distribution along the EC layer in the T-chamber. Three regions were set to describe different hemodynamic conditions in the modified T chamber system. Region I, a stagnation point and low to normal WSS; region II, a high positive WSSG and high WSS; region III, a negative to zero WSSG and a normal WSS. WSS, wall shear stress; WSSG, wall shear stress gradient.
primary antibodies [monoclonal: P120ctn (cat. no. 610133), VE-Cadherin (cat. no. 610252), α-catenin (cat. no. 610194) and β-catenin (cat. no. 610154), 1:400 dilutions, BD Transduction Laboratories; BD Biosciences, San Jose, CA, USA; polyclonal: Kaiso (cat. no. sc-23871) and β-actin cat. no. (sc-47778), 1:400 dilutions, Santa Cruz Biotechnology, Inc., Dallas, TX, USA] diluted in TBST with 1% nonfat dry milk overnight at 4˚C with agitation. After five washes with TBST, the nitrocellulose membrane was then incubated at 37˚C for 2 h with the appropriate horseradish peroxidase-conjugated secondary antibodies (cat. no. 58802, 1:2,000, Cell Signaling Technology, Inc., Danvers, MA, USA). Immune complexes were detected using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and were semi-quantified by densitometry (ImageJ version 2.0; National Institutes of Health). β-actin was used as the loading control.

Statistical analysis. All experiments were performed five times. Quantitative data are expressed as the means ± standard deviation, whereas categorical variables are presented as percentages. Independent samples t-test, Satterthwaite t-test or Wilcoxon rank-sum test were used to statistically analyze western blot analyses, and two-way analysis of variance was used to analyze endothelial cell density. Data were analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Flow data. According to previous studies (4,13,18,19), three regions were set to define different conditions of hemodynamics in the modified T chamber. Region I: A stagnation point and a low to normal WSS compared with the baseline level in straight vessels. Region II: A high positive WSSG and high WSS. Region III: A recovery region characterized by a normal WSS and a negative to zero WSSG (Fig. 1A and B).

Since normal HUVECs adhered to the coverslips for 3 h (data not shown), the expression levels of proteins and inflammatory factors in normal HUVECs were evaluated after 3 h. However, ECs with P120ctn knockdown adhered to the coverslips for just 1 h under a flow rate of 500 ml/min; therefore, the protocols were altered and the characteristics of ECs with P120ctn knockdown were detected every 15 and 30 min, and 1 h.

P120ctn knockdown by siRNA. To elucidate the role of P120ctn in AJs between ECs, the expression levels of P120ctn were knocked down by siRNA. P120ctn expression was silenced after 24, 48 and 72 h, as determined by western blot analysis,
whereas the expression levels of other proteins were normal, including VE-Cadherin, Kaiso, α-catenin, β-catenin and MMP-2 (Fig. 2A).

**Expression levels of P120ctn, VE-Cadherin and other proteins in HUVECs.** To investigate the effects of impinging flow with different hemodynamic conditions on the function and stability of AJs, the expression levels of specific proteins that constitute AJs were evaluated by western blot analysis (Fig. 2B-D). In normal HUVECs under a flow rate of 500 ml/min, the expression levels of P120ctn (P<0.05), VE-Cadherin, Kaiso (P<0.05) and α-catenin (P<0.05) were decreased, whereas MMP-2 was increased after 3 h (Fig. 2C and D). In normal HUVECs under a flow rate of 250 ml/min, the expression levels of P120ctn, VE-Cadherin, Kaiso and α-catenin were decreased, whereas MMP-2 was increased after 3 h; however, no significant alterations were detected (P>0.05; Fig. 2C and D). More obvious changes in expression were detected in response to a flow rate of 500 ml/min compared with 250 ml/min (Fig. 2B-D).

In HUVECs with P120ctn knockdown, the expression levels of VE-Cadherin, Kaiso and α-catenin were decreased, whereas MMP-2 expression was increased after 1 h, under both flow rates (250 and 500 ml/min; P<0.05). Alterations in the expression of these proteins were more prominent under the rate of 500 ml/min (Fig. 3A-C). Finally, in HUVECs infected with the control siRNA, no changes in the expression levels of P120ctn, Kaiso, α-catenin and MMP-2 were detected after 1 h at both rates (Fig. 3D).

**Morphology, alignment and distribution of ECs.** The effects of different hemodynamics on the behavior of ECs were examined by observing the morphology, alignment and distribution of ECs under impinging flow produced by the T chamber system. Alterations in the morphology, alignment, distribution and cell density of ECs were conducted as described in our previous research (13).

In HUVECs with P120ctn knockdown, under a flow rate of 250 ml/min, compared with in the static control group (ECs that were not subjected to any impinging flow), the shape of HUVECs remained polygonal after 1 h. The number of ECs in region II was slightly decreased, whereas the number of ECs in region III was increased. At a flow rate of 500 ml/min, no marked alterations in the morphology, alignment and distribution of ECs were observed within 30 min. After 1 h,
ECs in region III were overcrowded, and the number of ECs in region II was decreased. The shape of the HUVECs was elongated in the direction of the flow. The number of ECs in region III was increased and ECs in this region were overcrowded (Fig. 4). To exclude the possibility that the transfected siRNA itself induced the aforementioned alterations in ECs, the morphological changes of ECs transfected with the control siRNA were investigated. No changes in the morphology, alignment and distribution of such ECs were observed compared with in the control group after 1 h at a flow rate of
500 ml/min (Fig. 4). Furthermore, the density of ECs on the coverslip after 15, 30 and 60 min in response to both flow rates was counted (Table I). To calculate cell density, experiments were performed five times. At both flow rates, no significant alterations were detected in cell density after 0, 15, 30 and 60 min (P>0.05).

Discussion

As previously reported by Szymanski et al (4), bovine aortic ECs around the stagnation point maintain a polygonal shape; however, cell density is reduced. Conversely, cells in adjacent regions exposed to very high WSS and WSSG are elongated, aligned parallel to the flow and cell density is increased. However, this previous study did not mention speed control and whether flow rate was adjustable. Furthermore, pressure clamps were used to set the specific pressure value in the chamber following establishment of the experimental flow, yet the authors provided no detailed methods to adjust the velocity of flow and pressure in the chamber.

Different hemodynamic conditions can be established in an artery bifurcation model using a modified T-chamber system; the results of previous studies have demonstrated that in regions with high WSS and WSSG, more severe EC damage is induced compared with in the other regions (1,13). A possible molecular mechanism associated with AJs was investigated. The expression levels of P120ctn, VE-Cadherin and Kaiso were reduced, whereas the expression levels of MMP-2 were increased in response to impinging flow. When P120ctn was knocked down by siRNA, the period during which ECs adhered to the coverslip was reduced to 1 h under a flow rate of 500 ml/min, and more obvious damage to ECs was observed. The expression levels of VE-Cadherin, Kaiso and α-catenin in HUVECs with P120 knockdown were decreased compared with in normal HUVECs. These results confirmed the effects of hemodynamic conditions on the function and morphology of ECs, and demonstrated a potential molecular mechanism; however, this requires further investigation. Different hemodynamic conditions, including various flow velocities and pressure values, can be achieved in the chamber using the present modified system, and may be used to build a more convincing simulation of hemodynamic conditions in humans.

With adjustable velocity and pressure, the modified T-chamber system used in this study is able to mimic different hemodynamic conditions of the bifurcation of an artery. In our previous study (13), it was demonstrated that the number of normal ECs in region II is decreased and gaps between cells are enlarged in response to impinging flow, at a rate of 500 ml/min, for 12 h. In addition, the number of cells in region III is increased and cells are accumulated in other regions, whereas the alignment of cells follows the direction of the impinging flow; these findings are in accordance with those of Szymanski et al (4). Since no significant differences were detected in the total density of ECs on the coverslip at different time points, it may be hypothesized that the ECs lost from region II moved to region III and accumulated there. The present study indicated that normal ECs in the region characterized by high WSS and WSSG may be damaged at a higher flow rate, and some ECs will move downstream and crowd there. Therefore, in response to hemodynamic alterations, vessel walls in the bifurcation of an artery may be damaged in this manner; such remodeling of the vascular wall may be an essential and potential factor in the induction of IA, however this needs to be validated.

It has been suggested that damage to AJs between ECs may be the possible mechanism underlying endothelial injury, of which, P120ctn may be a key regulator. In response to hemodynamic alterations, the AJs between ECs may be injured and the vessel wall may be remodeled. In the present study, after 3 h under a flow rate of 250 and 500 ml/min, the expression levels of P120ctn, VE-Cadherin and Kaiso were decreased, whereas MMP-2 expression was increased. The changes in the expression levels of these proteins were more prominent in response to a flow rate of 500 ml/min, compared with a flow rate of 250 ml/min. These results suggested that it is hemodynamic alterations that affect the expression of these proteins and damage the AJs between ECs. The results of the present study were in concordance with those of Noren et al (20), which indicated that the expression levels of P120ctn are positively associated with those of Kaiso.

In the present study, alterations in the expression levels of P120ctn, Kaiso and α-catenin were significant under a flow rate of 500 ml/min for 3 h. The findings suggested that when hemodynamic alterations occur, P120ctn expression may be lost, followed by the destruction of AJs, and a decrease in the expression of VE-Cadherin and α-catenin. Conversely, as the levels of P120ctn are decreased, the combination of P120ctn and Kaiso may break down and the inflammatory ability of Kaiso might be recovered, thus resulting in EC inflammation. The increased expression of MMP-2 detected in this study might verify this conclusion; however, its expression was not significantly altered within 3 h. Further studies are required to clarify the expression of these proteins (VE-Cadherin) and inflammatory factors (MMP-2, Kaiso) after 12 h in normal ECs in response to impinging flow, and to determine whether these changes will be significant.

It is crucial for ECs to maintain the normal function and morphology of the vascular wall. However, the stability of ECs

Table I. Endothelial cell density on coverslips following P120ctn knockdown and impinging flow at 250 and 500 ml/min.

<table>
<thead>
<tr>
<th>Cell density (×10³/ml)</th>
<th>Flow rate (ml/min)</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>500</td>
<td>23.9±0.5</td>
<td>23.3±1.3</td>
<td>22.8±1.4</td>
<td>22.6±0.5</td>
<td>0.6432</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.1±0.7</td>
<td>22.5±1.4</td>
<td>22.3±0.5</td>
<td>21.8±1.3</td>
<td>0.5856</td>
</tr>
</tbody>
</table>

All experiments were performed five times. EC, epithelial cells.
can be damaged by various factors, including, inflammation within the vascular wall and hemodynamic alterations to the blood flow in vessels. P120ctn is considered an important component in maintaining the AJs between ECs, alongside α-catenin and β-catenin (21). P120ctn is able to form the CCC, which has the ability to maintain normal functions and signaling pathways between ECs (20).

P120ctn also has the ability to regulate gene transcription in ECs; for example, P120ctn is able to combine with Kaiso, an inflammatory factor, and inhibit the combination of Kaiso and DNA in nuclei (22). In addition, the inhibitory effects of Kaiso on EC growth, as well as Kaiso-induced inflammatory damage to the vascular wall, are reduced by the combination of P120ctn and Kaiso (10). The expression levels of MMP-2 are increased in vessel walls derived from human IA, and MMP-2 is able to combine with Kaiso (22). These findings may suggest that there is an association between P120ctn and vascular inflammation; however, this requires further investigation.

When P120ctn expression was knocked down in the present study, the period during which HUVECs adhered to the coverslips was reduced to 1 h; in our previous study, normal HUVECs adhered for 12 h (13). The expression levels of VE-Cadherin and Kaiso were more prominently decreased, whereas the expression levels of MMP-2 were more prominently increased in HUVECs with P120ctn knockdown compared with normal HUVECs. A possible mechanism for this may be that when P120ctn was knocked down, the expression levels of VE-Cadherin and α-catenin were downregulated, leading to decreased CCC complex formation, and damaged AJs and ECs.

In response to hemodynamic alterations, P120ctn may be destroyed first. In the present study, P120ctn was knocked down in ECs by siRNA. In P120ctn knockdown ECs its expression could not be detected by western blotting, whereas the expression levels of other proteins were normal. These findings suggested that alterations in the expression levels of other proteins may not be induced by P120ctn knockdown alone. Hemodynamic alterations may be a key factor in inducing changes in the expression levels of these proteins; therefore, it was hypothesized that P120ctn may be destroyed first.

In response to impinging flow and hemodynamic changes, the stability of the connection between P120ctn knockdown ECs may be decreased, and more VE-Cadherin broke down or was lost; therefore, more prominent morphological alterations in ECs were observed. In addition, the duration that P120ctn knockdown ECs adhered to the coverslip under 500 ml/min impinging flow was decreased to 1 h. Furthermore, in response to P120ctn knockdown, the levels of Kaiso were decreased, whereas those of MMP-2 were significantly increased. It may be hypothesized that the decreased expression of Kaiso may induce an increase in MMP-2 expression. Furthermore, the present study hypothesized that Kaiso may recover its inflammatory ability in response to P120ctn knockdown, which is inhibited when Kaiso is combined with P120ctn in the P120ctn-Kaiso complex. Due to the inflammation caused by free Kaiso (23), the expression levels of MMP-2 may increase, resulting in the induction of a more serious inflammatory injury to ECs; however, further research is required to verify this assumption.

VE-Cadherin is also considered a crucial component of AJs, as is P120ctn. As well as its role in forming the CCC, VE-Cadherin serves an important role in maintaining the normal functions and morphology of ECs. It has been reported that as the levels of VE-Cadherin decrease, the stability of ECs breaks down, which is considered an initial step in inducing remodeling of the vascular wall (6). It has also been revealed that as WSS increases, the levels of VE-Cadherin decrease in this study. However, the specific mechanism underlying the effects of P120ctn, VE-Cadherin and other proteins on ECs remains unclear. In addition, further research is required to clarify whether VE-Cadherin is broken down or is removed by the impinging flow, thus resulting in the decreased expression levels of VE-Cadherin.

The present study was limited by the type of cells selected; since arterial ECs were not available, HUVECs were selected. In addition, it is necessary to subject ECs to a longer period of impinging flow. Other hemodynamic conditions should be selected in future research to elucidate the association between AJs and ECs. Although the present study indicated that the expression levels of P120ctn were dependent on hemodynamics, further research is required to clarify the effects of P120ctn overexpression on AJs in response to impinging flow. Finally, more methods, including confocal microscopy or other histochemical analyses are required to provide stronger evidence to support the present conclusions.

In conclusion, the present study revealed that AJs are crucial for the maintenance of normal EC morphology and functions in the vascular wall, and P120ctn is an important regulator of AJs. In addition, it was suggested that a loss in P120ctn may be induced first by hemodynamic alterations. As hemodynamic conditions change, losing P120ctn may aggravate AJs between ECs and induce the inflammation in the vascular wall. Clinically, in response to hemodynamic alterations, a loss of P120ctn may induce endothelial injury; therefore, P120ctn may have a critical role in inducing IA.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
The study was designed by JLZ and MHL. The experiments were performed and data were collected by JLZ, JWJ and ZPX, and the data were analyzed by JLZ, ZPX and NZY. The manuscript was written by JLZ and ZPX, and was revised and
approved by all authors. The manuscript was finally proofread and approved by MHL.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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