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Effect of thrombin on human endothelial progenitor cells

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Abstract

Background: Thrombin is the key serine protease of the coagulation cascade which also exerts cellular non-haemostatic effects that are primarily mediated by the protease-activated receptor-1 (PAR-1).
Aim: In the present study we investigated the effect of thrombin on the membrane expression of adhesion molecule ICAM-1 on $CD34^+$-derived late-outgrowth endothelial cells (OECs) and human umbilical vein endothelial cells (HUVECs).
Methods: $CD34^+$ cells were isolated from cord blood mononuclear cells using human CD34 Microbead Kit and appropriately cultured for the formation of OECs. HUVECs were purchased from Lonza. Confluent OECs (passage 4) and HUVECs (passage 3) were incubated for various time intervals up to 24 h with 1-8 U/mL thrombin and the effect of thrombin-induced ICAM-1 expression (anti-CD54-PE) was evaluated using flow cytometry. PECAM-1 (anti-CD31-FITC) was used as an endothelial marker.
Results: Thrombin (8 U/mL) significantly increased ICAM-1 expression on OECs by 3.3-fold, after 24 h of incubation. In the resting state, ICAM-1 expression was significantly higher by 2.1-fold on OECs, compared with HUVECs. On the contrary, the % increase of ICAM-1 expression following activation with thrombin was significantly lower on OECs, compared with HUVECs.
Conclusions: Thrombin is a mediator of ICAM-1 expression on endothelial progenitor cells and this expression at baseline and after thrombin activation is different compared with HUVECs. The biochemical basis of this difference, as well as its pathophysiological significance remain to be established in future studies.

Key words: thrombin; protease-activated receptor-1; endothelial progenitor cells; ICAM-1

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1. Introduction

Thrombin is the key serine protease of the coagulation cascade as it activates platelets and converts fibrinogen to fibrin, thus leading to thrombus formation and stabilization.[1, 2] Apart from its haemostatic effects, thrombin also exerts cellular effects that are mediated by protease-activated receptors (PARs).[2, 3]

PARs are a family of G protein-coupled receptors that are activated by proteolytic cleavage of the N-terminal domain, exposing a new tethered ligand that binds intramolecularly to activate the receptor and to induce intracellular signal transduction.[4, 5] Four members of the PAR family have been identified, PAR-1 to -4.[6-10] PAR-1[6], -3[8] and -4[9] are activated mainly by thrombin[11], whereas PAR-2 is primarily activated by trypsin and trypsin-like proteases.[12]

Thrombin activates various cell types, including platelets, vascular smooth muscle cells (VSMCs), lymphocytes and endothelial cells (ECs) through the PARs activation, thus linking coagulation with inflammation.[3, 13, 14] Thrombin induces proinflammatory signaling, leading to the expression of adhesion molecules and P-selectin on the membrane of platelets and ECs, as well as expression of various cytokines and chemokines from fibroblasts, VSMCs and ECs, leading to leukocyte recruitment to the vessel wall and contributing to inflammatory and fibrotic processes.[2, 15, 16]

Particularly on ECs, thrombin induces the expression of cytokines such as interleukin -6 and -8[17, 18], chemokines such as MCP-1[19-21] and adhesion molecules such as ICAM-1, VCAM-1 and E-selectin[22-25], mainly through PAR-1 activation. In addition, thrombin induces ECs apoptosis through the activation of nuclear factor κB (NF-κB) and caspases,[26, 27] regulates prosctacyclin and nitric oxide production leading to ECs shape change and to the enhancement of barrier permeability.[28-30] Finally, thrombin stimulates the secretion of von Willebrand factor (vWF) and P-selectin from Weibel-Palade bodies of ECs, linking inflammation with atherosclerosis.[31]

Late-outgrowth endothelial cells (OECs) are a type of non-hematopoietic endothelial progenitor cells that have high proliferative capacity[32] and express endothelial markers on their surface.[33, 34] OECs contribute to the repair of the vessel wall and to neovascularisation by differentiation into ECs.[35, 36] OECs can be formed in vitro by differentiation of cord blood $CD34^+$ mononuclear cells in culture.[37]

The aim of the present study was to investigate the effect of thrombin on the membrane expression of ICAM-1 on OECs in culture, in comparison with its effect on mature human umbilical vein endothelial cells (HUVECs).

2. Materials and Methods

2.1 Materials

Culture medium 199, fetal bovine serum (FBS), DPBS, trypsin/EDTA and penicillin-streptomycin (10,000 U/mL) were purchased from Gibco BRL Life Technologies, whereas endothelial cell growth supplement (ECGS), thrombin from human plasma (1 KU) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Heparin, EGM-2 (EBM-2 bullet kit: EBM-2 supplemented with hydrocortisone, human fibroblast growth factor-B, vascular endothelial growth factor, R3 insulin-like growth factor-1, ascorbic acid, human epidermal growth factor, gentamicin/amphotericin-1,000, heparin and FBS), HUVECs were purchased from Lonza (Walkersville Inc, USA). Biocoll separating solution and trypsin/EDTA were from Biochrom AG (Berlin, Germany). CD34 Microbead Kit (human) and MACS separation columns were purchased from Miltenyi Biotec (Germany). FITC mouse IgG1κ Isotype control (anti-IgG1-FITC), PE mouse IgG1κ Isotype control (anti-IgG1-PE), FITC mouse anti-human CD31 (anti-CD31-FITC), FITC mouse anti-human CD34 (anti-CD34-FITC), PE mouse anti-human CD45 (anti-CD45-PE) and PE mouse anti-human CD54 (anti-CD54-PE) were purchased from BD Biosciences (San Jose, CA, USA).

2.2 Isolation of mononuclear cells

Cord blood (50 mL) was collected in a tube containing 20 mM EDTA/PBS solution, as anticoagulant. Blood was diluted 1:4 with 2 mM EDTA/PBS solution and 35 mL of diluted blood were layered over 15 mL of biocoll separating solution. After centrifugation for 35 min at 400 x g and 20°C, mononuclear cells (MNCs) were collected and washed 3 times using 2 mM EDTA/PBS solution. After the first resuspension, MNCs were centrifuged for 10 min at 300 x g and 20°C

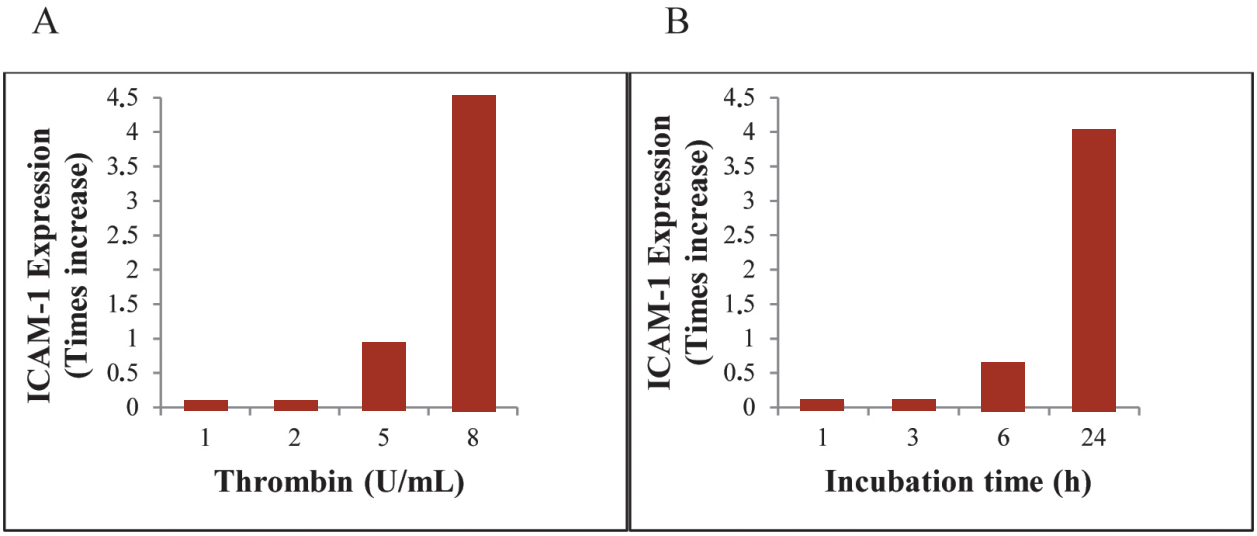

Figure 1. Representative bar graphs of the A) dose- and B) time-dependent effect of thrombin on the membrane expression of ICAM-1 on HUVECs. Cells were incubated for 24 h with various thrombin concentrations ranging from 1 to 8 U/mL (A) or with 8 U/mL thrombin for various time intervals ranging from 1 to 24 h (B)

and for 10 min at 200 x g and 20°C for the other washing steps. Finally, 0.5 x 10^8 MNCs were resuspended in 300 μL of PBS containing 2 mM EDTA and 0.5% BSA (EDTA/BSA/PBS buffer).

2.3 Magnetic labeling and CD34+ separation

For the magnetic labeling of $CD34^+$, MNCs (0.5 x 10^8) were resuspended in 300 μL of PBS containing 2 mM EDTA and 0.5% BSA, were incubated with 100 μL of FcR blocking reagent and 100 μL of CD34-conjugated magnetic microbeads for 30 min at 4°C. After incubation, 5 mL of EDTA/BSA/PBS buffer were added to the MNCs and a centrifugation for 10 min at 300 x g was followed. MNCs (0.5 x 10^8) were then resuspended in 500 μL of EDTA/BSA/PBS buffer. During the above process, all the solutions used were kept cold in order to avoid the binding of the antibodies on the cell surface and non-specific cell labeling. If the number of MNCs was higher than 0.5 x 108, the volumes of solutions and reagents were scaled up accordingly.

For the magnetic separation, the column was washed with 500 μL EDTA/BSA/PBS buffer and placed in a magnetic field suitable for MACS separator. Cell suspension was allowed to pass through the column and the unlabeled cells were collected. The column was washed three times with 500 μL EDTA/BSA/PBS buffer to collect the remaining cells. Subsequently, 1 mL of the above buffer was added, the column was removed from the magnetic field, placed in a tube and the magnetically labeled $CD34^+$ cells were flushed out using a plunger. To increase their purity,the magnetically labeled $CD34^+$ cells were allowed to pass through a second column.

2.4 Evaluation of $CD34^+$ purity using flow cytometry

To evaluate the purity of the $CD34^+$ cells, 20 μL of each unlabeled and labeled fraction as well as the total MNCs before the separation, were incubated with 2 μL of anti-IgG1-FITC, anti-CD34-FITC, anti-IgG1-PE and anti-CD45-PE for 2 min at 4°C, after the addition of 80 μL of PBS. Cells were then washed with 1 mL of PBS, centrifuged for 5 min at 340 x g and resuspended in 500 μL of cold PBS. Cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

2.5 Cell Culture

$CD34^+$ cells were cultured in EGM-2 for 20-30 days on collagen-coated tissue culture dishes at 37°C and 5% CO_2 for the formation of OECs. The medium was changed every day for the first 7-14 days and every 2 days until the coverage of 70-90% of the culture dish (confluent). Cells were detached with trypsin/EDTA solution (0.05%/0.02% in PBS), subcultured and stored in 10% DMSO/FBS at -80°C for 2-3 days and then to liquid nitrogen. All experiments of the present work were performed using OECs of passage 4.

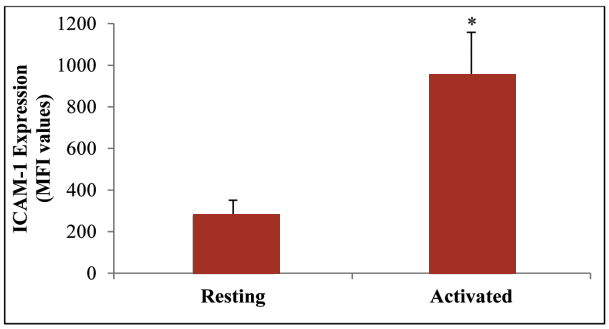

Figure 2. Bar graph that shows the effect of thrombin on the membrane expression of ICAM-1 on OECs. OECs were incubated with 8 U/mL thrombin for 24 h. Results represent the mean ± SD from at least 3 different experiments (*p= 0.004, compared to resting cells)

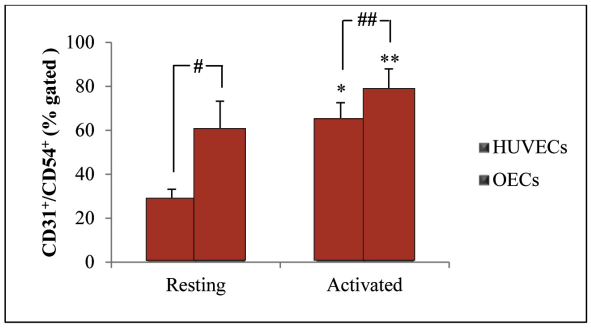

Figure 3. Comparison of ICAM-1 expression on OECs and HUVECs at the resting state and after activation with thrombin. OECs and HUVECs were incubated with 8 U/mL thrombin for 24 h. The results presented represent the mean ± SD from at least 3 different experiments (*p = 0.0001 and **p = 0.0001, compared with activated HUVECs and OECs, respectively. #p= 0.0001 [comparison between the resting states of HUVECs and OECs] and ##p = 0.028 [comparison between the activated states of HUVECs and OECs])

HUVECs were cultured in complete M199 (Medium 199, 20% FBS, 15 mg ECGS, 1% penicillin-streptomycin and 0.05% heparin (2.5 U/mL) on collagen-coated tissue culture dishes at 37°C and 5% CO_2. All experiments were performed using HUVECs of passage 3.

2.6 Cell activation and flow cytometry analysis of ICAM-1 expression

Confluent OECs were cultured on collagen-coated 6-well culture plates in M199 containing 5% FBS and 1% penicillin-strepromycin and were activated with 8 U/mL thrombin for 24 h. After activation the medium was removed, cells were washed two times with PBS 1X and detached by incubation with 200 μL of trypsin/EDTA for 2 min at 37°C. Cells were then diluted 1:10 (v/v) with 10% FBS/M199 and centrifuged for 5 min at 340 x g. Precipitated cells were resuspended in 10% FBS/M199. For flow cytometric analysis, cells (100,000) were incubated with 5 μL of anti-CD31-FITC and 5 μL of anti-CD54-PE or 5 μL anti-IgG1-FITC and 5 μL anti-IgG1-PE (negative isotypic control) for 30 min at 4°C. After incubation, cells were centrifuged for 5 min at 340 x g, washed 3 times with 5% FBS/M199 and resuspended in 500 μL of 5% FBS/ M199. The ICAM-1 membrane expression was expressed as mean fluorescence intensity (MFI) values and $CD31^+$/$CD54^+$ cells (% gated).

In addition, control experiments were conducted

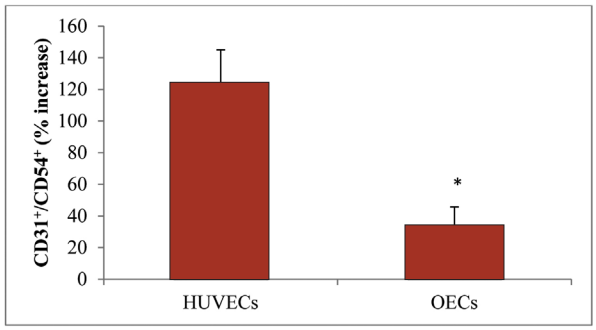

Figure 4. Bar graph that compares the % increase of thrombin-induced ICAM-1 expression on OECs and HUVECs. OECs and HUVECs were incubated with 8 U/mL thrombin for 24 h. The results presented represent the mean ± SD from at least 3 different experiments (*p= 0.0001, compared with HUVECs)

with confluent HUVECs cultured on collagen-coated 6-well culture plates in M199 containing 5% FBS and 1% penicillin-strepromycin. Cells were activated with different thrombin concentrations ranging from 1 to 8 U/mL for various time intervals up to 24 h. The membrane ICAM-1 expression was determined as mentioned above.

2.7 Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed using the SPSS 20 program (SPSS Inc., Chicago, IL, USA). Independent-samples t-test and paired-sam-

ples t-test were conducted where appropriate. Statistical significance was defined as $p< 0.05$.

3. Results

3.1 Effect of thrombin on the membrane expression of ICAM-1 on HUVECs

Thrombin induced the membrane expression of ICAM-1 on HUVECs in a dose- and time-dependent manner (**Figure 1A, B**). Firstly, we determined the concentration of thrombin that induced the maximum expression of ICAM-1 at 24 h of incubation (**Figure 1A**). Then, we conducted time-dependent experiments using the concentration of 8 U/mL thrombin in which we observed the maximum ICAM-1 expression. Thrombin-induced ICAM-1 expression reached its maximum levels after 24 h of incubation (**Figure 1B**). Thus, the maximum expression of ICAM-1 on HUVECs was observed after 24 h of incubation at a thrombin concentration of 8 U/mL.

3.2 Effect of thrombin on the membrane expression of ICAM-1 on OECs

Based on the above results, we then determined the effect of thrombin on ICAM-1 expression on OECs using 8 U/mL thrombin and 24 h incubation activation time. As it is shown in **Figure 2**, thrombin significantly increased by 3.3-fold ICAM-1 expression on OECs, after 24 h of incubation.

3.3 Comparison of ICAM-1 membrane expression on HUVECs and OECs at resting and activation state

Finally, we investigated the possible differences on ICAM-1 expression on HUVECs and OECs in the resting state, as well as after their activation induced by thrombin. In the resting state, the ICAM-1 expression (% gated $CD31^+/CD54^+$) on OECs was significantly higher by 2.1-fold compared with HUVECs (**Figure 3**). By contrast, the % increase of ICAM-1 expression following activation with thrombin was significantly lower on OECs compared with HUVECs (**Figure 4**).

4. Discussion

In the present study we show for the first time that the membrane expression levels of ICAM-1 on non-activated OECs is significantly higher compared with mature endothelial cells (HUVECs). We may suggest that the increased expression of this adhesion molecule may facilitate the adhesion of OECs to the arterial wall, a prerequisite step for their maturation into endothelial cells thus leading to the regeneration of the arterial wall endothelium. This suggestion needs to be supported by further studies. Furthermore, the increased membrane expression of ICAM-1 may represent a marker of endothelial immaturity among circulating endothelial progenitor cells. The present study further demonstrates for the first time that the ICAM-1 expression in thrombin-activated OECs is significantly lower compared with mature endothelial cells. This result suggests that either the concentration of intracellular preformed ICAM-1 is low due to its increased expression in the plasma membrane on OECs. Alternatively, the intracellular signaling pathway leading to ICAM-1 formation following activation with thrombin, primarily mediated through PAR-1[3], may be still immature on OECs or the PAR-1 expression levels in these cells are lower compared with HUVECs. This hypothesis reserves further investigation.

The maximum ICAM-1 expression on HUVECs and OECs induced by thrombin is observed at 24 h at a thrombin concentration of 8 U/mL. This is probably due to the fact that the mRNA transcription and protein biosynthesis are processes that need several hours to be completed.[38, 39] Our results concerning the effect of thrombin on HUVECs are in agreement with previous studies showing that ICAM-1 membrane expression reaches a peak after 24 h of incubation.[22, 40, 41]

In conclusion, we showed for the first time that ICAM-1 expression on OECs at baseline and after thrombin activation is different compared with HUVECs. The biochemical basis of this difference, as well as its pathophysiological significance remain to be established in future studies.

Conflict of Interest

All authors declare no conflict of interest.

Acknowledgments

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Περίληψη

Επίδραση της θρομβίνης στα πρόδρομα ενδοθηλιακά κύτταρα

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Εισαγωγή: Η θρομβίνη αποτελεί τη σερινοπρωτεάση κλειδί του καταρράκτη της πήξης εμφανίζοντας παράλληλα μη αιμοστατικές κυτταρικές δράσεις, οι οποίες διαμεσολαβούνται κυρίως από τον υποδοχέα που ενεργοποιείται από πρωτεάσες -1 (protease-activated receptor-1, PAR-1).
Σκοπός: Στην παρούσα μελέτη διερευνήθηκε η επίδραση της θρομβίνης στη μεμβρανική έκφραση του μορίου προσκόλλησης ICAM-1 σε προχωρημένης ωρίμανσης πρόδρομα ενδοθηλιακά κύτταρα (late-outgrowth endothelial cells, OECs) προερχόμενα από $CD34^+$ κύτταρα, καθώς και σε ώριμα ενδοθηλιακά κύτταρα από ομφάλιο λώρο (human umbilical vein endothelial cells, HUVECs).
Μέθοδοι: $CD34^+$ κύτταρα απομονώθηκαν από μονοπύρηνα κύτταρα από ομφάλιο λώρο ανθρώπου χρησιμοποιώντας το CD34 Microbead Kit και καλλιεργήθηκαν υπό κατάλληλες συνθήκες προς σχηματισμό OECs. OECs 4ης και HUVECs 3ης γενιάς τα οποία είχαν καλύψει το πλακίδιο επίστρωσης κατά 70-90% επωάστηκαν με 1-8 U/mL θρομβίνης για διάφορες χρονικές στιγμές μέχρι 24 h και μελετήθηκε η μεμβρανική έκφραση του ICAM-1 (anti-CD54-PE) με τη μέθοδο της κυτταρομετρίας ροής. Ως δείκτης των ενδοθηλιακών κυττάρων χρησιμοποιήθηκε το μόριο προσκόλλησης PECAM-1 (anti-CD31-FITC).
Αποτελέσματα: Η θρομβίνη (8 U/mL) αύξησε την έκφραση του ICAM-1 στα OECs κατά 3,3 φορές μετά από 24 h επώαση. Σε κατάσταση ηρεμίας, η έκφραση του ICAM-1 ήταν σημαντικά αυξημένη κατά 2,1 φορές στα OECs σε σύγκριση με τα HUVECs. Αντίθετα, η % αύξηση της έκφρασης του ICAM-1 μετά από ενεργοποίηση με θρομβίνη ήταν σημαντικά χαμηλότερη στα OECs, σε σχέση με τα HUVECs.
Συμπεράσματα: Η θρομβίνη αποτελεί διαμεσολαβητή της έκφρασης του ICAM-1 στα πρόδρομα ενδοθηλιακά κύτταρα και η έκφραση αυτή τόσο σε κατάσταση ηρεμίας, όσο και μετά από ενεργοποίηση με θρομβίνη, διαφέρει σε σχέση με εκείνη στα HUVECs. Η βιοχημική βάση αυτής της διαφοράς, καθώς και η παθοφυσιολογική της σημασία, μένει να διερευνηθεί.

Λέξεις ευρετηρίου: θρομβίνη, υποδοχέας που ενεργοποιείται από πρωτεάσες -1, πρόδρομα ενδοθηλιακά κύτταρα, ICAM-1

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