Microparticle-associated endothelial protein C receptor and the induction of cytoprotective and anti-inflammatory effects

Margarita Pérez-Casal,[1] Colin Downey,[1] Beatriz Cutillas-Moreno,[1] Mirko Zuzel,[1] Kenji Fukudome,[2] and Cheng Hock Toh[1]

[1]Division of Haematology, University of Liverpool, UK and [2]Department of Immunology, Saga Medical School, Nabeshima, Japan

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Supplementary Design and Methods

Determination of endothelial gene expression by cDNA array

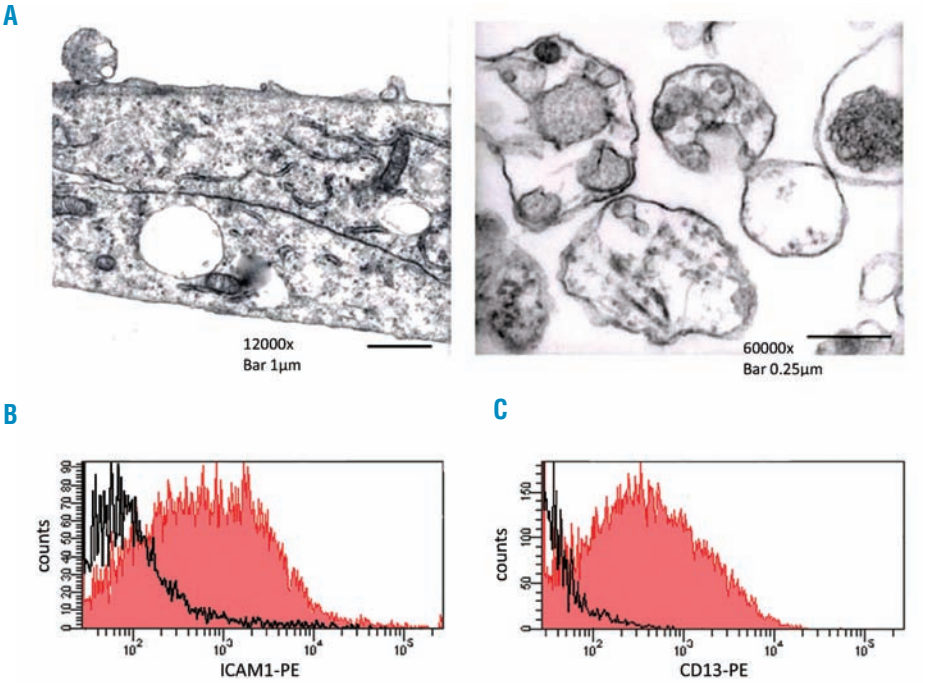
APC (40 nmol/L) on *in vitro*-generated MP-EPCR was used. As controls, both free and microparticulate-bound APC were incubated with anti-protein C (10 μg/mL) 1 h prior to incubation. In experiments involving microparticles, the possibility of APC becoming unbound to act as free APC was also considered. Buffer was removed at the end of the incubation period, centrifuged at 18,000 g for 30 min to pellet any residual microparticles and the supernatant checked with the chromogenic substrate for APC S2366. There was no specific APC signal when compared with the buffer-only control from HUVEC incubated under similar conditions. PAR1 was blocked either by a 30 min pre-incubation with the PAR1 antagonist T1 (50 μmol/L) or the PAR1 antibody ATAP2 (20 μg/mL). T1, ATAP2 and anti-protein C were also used on their own as controls for transcript levels. Total RNA was isolated from cells with RNEasy mini kits (QIAGEN, West Sussex, UK) according to manufacturer's instructions.

Gene expression profiling

A GEArray Q-Series human endothelial cell biology gene array (SuperArray, MD, USA) containing 112 tetra-spots of sequence-specific cDNA of different human genes, including five housekeeping genes and blank controls, was utilized. Gene expression was analyzed using the GEArray Expression Analysis Suite software v1.1 (*http://geasuite.superarray.com*). Four independent experiments were performed for each treatment with *in vitro* microparticles and array images transformed into raw data by the above software. Gene expression was corrected for background and normalized against the median value of each array. An average fold change of two or more in hybridization intensity between control and stimulated HUVEC signified a regulatory change. All data are MIAME compliant and have been submitted to the Gene Expression Omnibus (GEO) database (accession numbers GSE5660 and GSE5661).

Quantitative real-time polymerase chain reaction

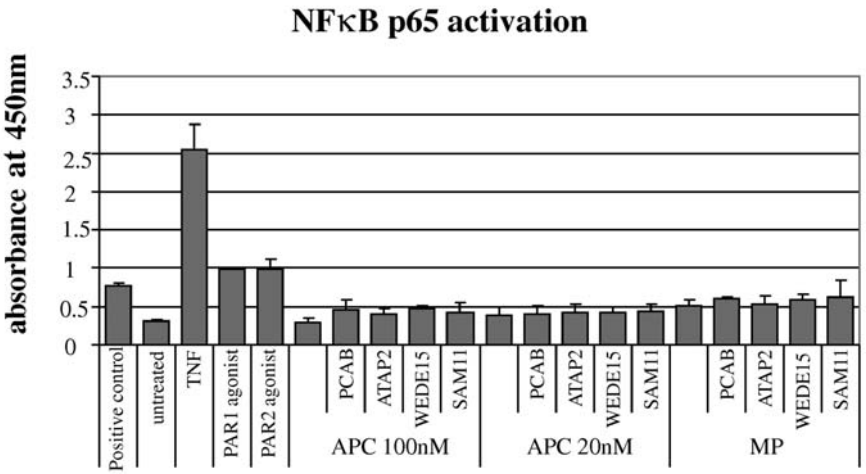
qRT-PCR confirmation of array data involved 0.5 μg RNA as the source for cDNA synthesis using the First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Diagnostics, UK). Equal aliquots were used for amplifying the genes of interest and the housekeeping gene GAPDH using the LightCycler FastStart DNA Master Plus SYBR Green I (Roche Diagnostics, UK). Primers were designed with ProbeFinder v2.32 software (*https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp*) for real-time amplification; primer sequences are detailed in *Online Supplementary Table S2*. Cycling conditions were initial



Supplementary Figure S1. Electron microscopy and flow cytometric characterization of microparticles. (A) Endothelial cells were incubated with APC 100 nmol/L overnight at 37°C, then fixed and viewed by transmission electron microscopy. The microphotograph on the left follows the release of a microparticle from the endothelial surface, within the 1 μm range in size. The microphotograph on the right shows the isolated microparticle pellet. (B) Microparticles isolated by sequential centrifugation from the plasma of septic patients undergoing rhAPC treatment were labeled with anti-ICAM1-phycoerythrin and (C) anti-CD13-phycoerythrin. In both cases, the specific fluorescence is overlaid over the isotypic control.

Supplementary Table S1. Gene expression after free-APC or microparticle (MP) treatment on HUVEC with measurements normalized and expressed as fold difference to untreated values.

Functional group/ Gene name			PC blockade		PAR1 (antagonist T1)		PAR1 (ATAP2)		GeneBank N.
	APC	MP	APC	MP	APC	MP	APC	MP	
Cell activation									
Annexin V	12.7	5.04	0.90	0.85	0.67	1.009	0.72	0.94	NM_001154
TF	-2.3	-1.3	1.01	0.76	1.09	0.74	1.31	0.50	NM_001993
Pro-apoptosis									
ADAM17	4.19	1.02	1.1	0.65	0.78	0.65	0.96	0.63	U92649
Bax	-4.85	-2.66	1.07	0.78	0.67	0.90	0.65	0.55	L22474
Caspase 1	2.57	2.09	1.02	0.95	1.66	1.74	1.38	0.16	M87507
Caspase 3	2.8	1.9	0.9	0.75	1.06	1.13	0.59	1.11	NM_004346
Caspase 6	8.45	2.45	0.89	0.69	0.81	0.82	0.63	0.91	U20537
Caspase 10	3.9	1.8	1.04	0.66	1.01	0.81	1.04	0.18	NM_001230
CRADD	3.39	1.61	1.03	0.85	3.6	1.52	3.37	1.28	NM_003805
Anti-apoptosis									
Bcl-xL	31.2	11.6	1.1	1.12	1.06	0.98	1.15	0.12	Z23115
Bcl-2	-4.22	-1.8	0.95	0.41	1.3	1.02	0.49	0.58	NM_000633
BFL1	6.2	1.7	1.02	0.98	0.65	0.81	0.43	1.12	NM_004049
CASPER/FLIP	2.09	2.032	0.58	1.3	0.64	1.11	1.13	1.12	NM_003879
Cox2	5.13	1.85	1.3	0.89	1.38	0.81	0.60	0.90	NM_000963
A20	2.9	1.7	1.3	0.94	0.73	1.02	0.7	0.45	NM_006290
Fibronectin	2.9	5.55	1.08	0.95	0.49	1.2	1.16	1.47	X02761
Angiogenesis									
Angiopoietin 1	5.04	4.8	3.6	3.15	2.44	1.8	1.66	1.44	NM_001146
Angiopoietin 2	8.55	7.2	0.21	0.22	1.3	0.96	0.74	0.96	NM001147
FGF2	-2.32	-1.09	0.72	0.44	0.78	0.84	0.85	0.46	NM002006
KDR	7.4	7.02	1.02	1.1	7.9	6.00	5.93	2.17	AF035121
MMP1	14.15	6.7	1.32	1.24	0.98	7.9	0.41	1.03	X05231
MMP2	36.15	28.3	1.13	1.26	0.94	0.98	1.04	0.84	J03210
MMP9	-6.3	-5.4	0.66	1.38	1.03	1.04	1.71	1.38	J05070
VEGF	-2.32	-2.04	1.13	0.62	1.21	0.97	0.62	0.59	NM_003376
VEGFR1	-2.3	-1.7	0.81	0.54	0.96	1.3	0.42	1.34	NM_002019
VEGFR3	3.2	2.1	1.15	1.2	0.91	0.83	1.13	1.20	NM_002020
Adhesion									
ICAM1	9.8	11.4	0.97	0.83	1.3	1.09	0.79	0.9	NM_000201
ICAM2	14.3	8.03	0.92	2.16	0.93	1.3	1.03	1.02	NM_000873
ICAM3	3.03	2.6	1.01	0.85	0.72	0.79	0.13	0.72	NM_002162
Integrin a5	8.69	7.06	0.64	0.98	2.57	1.75	1.94	1.95	X06256
Integrin aV	8.5	7.1	1.4	1.02	0.96	0.77	0.98	1.11	NM_002210
Integrin beta1	4.24	5.8	1.24	1.13	1.21	1.01	1.27	1.13	NM002211
PECAM1	15.1	8.4	1.05	1.14	1.26	0.69	0.78	0.93	NM000442
PSGL1	6.15	7.39	0.85	0.96	1.65	1.98	1.85	1.29	NM_003006
Cytokine production									
MCP1	4.3	5.6	1.19	0.72	1.01	0.85	0.47	0.72	X14768
MIP-3a/SCYA20	-2.0	-1.7	0.15	0.53	1.36	2.98	1.56	1.49	NM_004591
SCYA5/RANTES	5.4	4.7	0.12	0.39	0.69	1.03	0.95	0.93	NM_002985
GM-CSF	3.8	4.06	0.85	1.11	0.78	0.6	0.57	0.59	M11734
G-CSF	4.75	4.5	0.78	0.6	0.5	0.18	0.11	1.03	NM_000759
SCYD1	2.4	1.91	0.71	1.3	0.39	0.57	0.60	0.39	NM_002996
IL1B	5.39	7.5	0.34	0.16	1.2	-0.14	1.28	1.45	M15330
IL8	6.02	6.4	1.18	0.72	0.87	1.2	0.39	1.20	M17017
IFN-b1	3.3	2.4	2.6	2.1	0.58	1.19	0.57	0.32	NM_002176
Vessel Tone									
PLA2G4C	3.6	3.8	1.3	1.2	1.7	2.9	1.44	1.96	NM_003706



Supplementary Figure S2. Effect of APC in free and microparticulate form on NFκB activation. Nuclear extracts prepared from endothelial cells were used in an ELISA-based transcription factor assay. Along with the positive control within the commercial kit, cells were stimulated with TNF-α, PAR1 and PAR2 agonists as further controls. The bar chart shows the levels of absorbance at 450 nm.

denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 10 s and extension at 72°C for 12 s, with acquisition of fluorescence at the end of each extension. For each sample and gene, a threshold cycle (C_T) value was recorded.[1] In brief, the C_T value of the gene of interest was subtracted from that of GAPDH to generate a ΔC_T value; all results were normalized to the untreated control. The relative values of gene amplification were calculated by the following equation: $2^{-\Delta\Delta CT}$, with -ΔΔCT as the normalized ΔCT value of the target gene generated through subtracting the ΔCT of each sample from that of the untreated control. For further confirmation of amplicon size, PCR products were loaded on 2% agarose gels and visualized under UV light.

Transmission electron microscopy

Endothelial cells grown on monolayers were fixed by immersion in 2.5% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4) for 16-24 h. Each sample was then cut into 1-mm thick slices and washed in cacodylate buffer followed by secondary fixation in 1% osmium tetroxide for 1 h. Samples were processed according to standard procedures,[2] by dehydration in ethanol followed by infiltration and embedding in Epon resin before polymerization at 60°C for 16 h. Ultrathin sections (75 nm) were cut and mounted on copper grids before double staining with uranyl acetate/lead citrate and examined with an EM10 C electron microscope (Carl Zeiss GmbH, Oberkochen, Germany).

Nuclear factor kB transcription factor assay

EAhy926 cells were grown to confluency in T75 cm^2 flasks, incubated with and without APC (20 and 100 nmol/L) or MP-APC (20 nmol/L) for 2 h in the absence and presence of protein C (PCAB) blockade, PAR1 (ATAP2 and WEDE15) and PAR2 (SAM11) blockade. As positive controls, PAR1 agonist (10 μmol/L), PAR2 agonist (100 μmol/L) and tumor necrosis factor-α (10 ng/mL) were used. Nuclear Extract Kit (Active Motif, CA, USA) was used to prepare nuclear extracts according to the manufacturer's instructions. The protein concentration of the extract was calculated with a Bio-Rad Protein Estimation Assay. Levels of NFκB-p65 activation were obtained using a TransAM NFκB Transciption Factor Assay kit (Active Motif, CA, USA). In brief, 20 μg of nuclear extract were added to each sample-well in an ELISA plate coated with oligonucleotide containing the consensus site for NFκB. Upon binding, antibody to activated NFκB-p65was added. Horseradish peroxidase-conjugated secondary antibody was used for colorimetric detection by absorbance at 450 nm in a Spectramax plate reader (Molecular Devices).

Supplementary Table S2. Sequences of the primers used for qRT-PCR.

Gene	Sequence	Amplicon size	GenBank N.
A20	F: TGCACACTGTGTTTCATCGAG R: ACGCTGTGGGACTGACTTTC	76	NM_006290
Bax	F: ATGTTTTCTGACGGCAACTTC R: ATCAGTTCCGGCACCTTG	104	L22474
Bcl-xL	F: GCACAGCAGCAGTTTGGAT R: CCGGTACCGCAGTTCAAA	97	Z23115
IL-8	F: TACTCCAAACCTTTCCACCC R: AACTTCTCCACAACCCTCTG	157	M17017
MCP-1	F: AGTCTCTGCCGCCCTTCT R: GTGACTGGGGCATTGATTG	93	X14768
GM-CSF	F: TCTCAGAAATGTTTGACCTCCA R: GCCCTTGAGCTTGGTGAG	98	M11734
KDR	F: GCACATTTGTCAGGGTCCA R: GGTACTTCGCAGGGATTCTG	108	AF035121
GAPDH	F: AGCCACATCGCTCAGACA R: GCCCAATACGACCAAATCC	66	M33197

Supplementary References

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2. Hetzel U, König A, Yildirim AO, Lämmler Ch, Kipar A. Septicaemia in emerald monitors (Varanus prasinus Schlegel 1839) caused by Streptococcus agalactiae acquired from mice. Vet Microbiol 2003;95:283-93.