Article

Acetate Decreases PVR/CD155 Expression via PI3K/AKT Pathway in Cancer Cells

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MATERIALS AND METHODS

Colorectal cancer lines and human CD8⁺ T cells

HCT116 (HLA-A2-positive) was cultured in RPMI1640 media containing L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin (all from GIBCO) at 37 ℃ 5% CO_2 condition. HCT116 was purchased from ATCC. Human $CD8^+$ T cells (HLA-A2-positive) were purchased from STEMCELL. The cells were expanded by a 1:10 ratio of beads from the human T cell activation/Expansion kit (Miltenyi Biotec). For activation of $CD8^+$ T cells, the T cells were treated with beads from the human T cell activation/Expansion kit at a 1:2 ratio for three days. Colorectal cancer lines were co-cultured with activated $CD8^+$ T cells at a 1:1 ratio for two days(23-25).

Short-chain fatty acid (SCFA) treatment

Acetate, butyrate, and propionate were diluted by distilled water with sodium acetate (S5636, Sigma Aldrich), sodium butyrate (P5436, Sigma Aldrich) and sodium propionate (B5887, Sigma Aldrich) then filtered by 0.22 μm syringe filter. The treatment of acetate (25, 50, and 100 mM), butyrate and propionate (10,20 and 40mM) exogenously into HCT116, was performed for one day. The change of immune-suppressive molecules by short-chain fatty acid was analyzed by flow cytometry, and real-time PCR. Relative quantification of target genes was performed by qPCR and the $2^{-\Delta\Delta Ct}$ method. Target gene expression was normalized to the GAPDH mRNA level.

Flow cytometry measurement

The cell death of HCT116 was measured with AnnexinV-APC (Biolegend) and 7AAD (BD Biosciences). To test the effector response of $CD8^+$ T cells, activated $CD8^+$ T cells were co-cultured with HCT116 cells which is pre-treated with (25 or 50mM, for 24hrs) for 48hrs. Intracellular staining was performed according to the manufacturer`s instructions (BD Biosciences). Briefly, $CD8^+$ T cells were treated with Golgi stop (BD Biosciences), phorbol 12-myristate 13-acetate (Invivogen)

and Ionomycin (Sigma-Aldrich) for 6 hr. For flow cytometric analysis of $CD8^+$ T cells, anti-human granzyme B-FITC (GB11, BD Pharmingen), CD8a-PE (SK1), IFNγ-APC (4S.B3) and perforin-PerCP-cy5.5 (B-D48) antibodies (all from Biolegend) were used. Cancer cells were stained with anti-human CD112-PE (TX31) or CD155-APC (SKII.4) antibodies (all from Biolegend). All samples were analyzed by flow cytometry (CytoFlex, Beckman Coulter) and FlowJo software (Tree Star).

The measurement of the viability of HCT116

The viability change of HCT116 by acetate was measured with CCK8 (DOJINDO). Absorbance was measured at 450 nm using a microplate reader (GloMax® Discover Microplate Reader, Promega).

Measurement of mRNA level using qPCR

Total RNA of acetate treated cells or siRNA treated samples were extracted using Trizol (Thermo Fisher Scientific). cDNA was synthesized using SuperScript VILO™ Master Mix (Invitrogen). RT-qPCR was performed with TB Green Premix Ex Taq™ (TaKaRa). Primers listed below were synthesized by Bioneer Inc. (Daejeon, Korea) (Table 1).

Table 1. Primers sequence information for real-time PCR

Genes		Sequence (5'-3')	Size (bp)
hPDL1	Forward	AAATGGAACCTGGCGAAAGC	104
	Reverse	GATGAGCCCCTCAGGCATTT	
hPDL2	Forward	GTCTTGGGAGCCAGGGTGAC	99
	Reverse	TGAAAAGTGCAAATGGCAAGC	
hCD112	Forward	TCCGGCTATGATGACAACTG	193
	Reverse	TGCAGACGAAGGTGGTATTG	
hCD155	Forward	CCAACATGGAGGTGACGCAT	210
	Reverse	GGCAGGTGTAGTTGCCTTCA	

hCEACAM1	Forward	GAGTAGTGGCCCTGGTTGCTC	85
	Reverse	CGCTGGTCGCTTGCCCT	
hGalectin-9	Forward	CTTTCATCACCACCATTCTG	91
	Reverse	ATGTGGAACCTCTAAGCACTG	
hGAPDH	Forward	GAAGGTGAAGGTCGGAGTC	172
	Reverse	GAAGATGGTGATGGGATTT	

Inhibition Assay

For inhibition of CD112 or CD155 on HCT116, anti-human CD112 (TX31) or CD155 (SKII.4) antibodies were treated to HCT116 for 6 hours. After PBS washing, activated $CD8^+$ T cells were co-cultured for two days. The cell death of HCT116 was measured by flow cytometry. For inhibition of NF-κB signaling, anacardic acid or LY294002 (all from Abcam) 10 μM were treated into HCT116 for two days. The change of CD112 and CD155 was measured by flow cytometry. For inhibition of CD155 using small interfering RNA (siRNA), siRNA targeting gene of interest or control siRNA (Bioneer) were transfected using the Neon™ system (Invitrogen). All the knockdown was carried out for 48 hours and the knockdown efficiency was confirmed by qPCR. siRNAs were purchased from by Bioneer Inc. (Daejeon, Korea).

Protein analysis

To confirm the protein expression of signaling proteins, the cells were disrupted by RIPA buffer containing protease inhibitor cocktail (Abcam). The amount of total protein in the supernatant was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific). Samples were boiled at 100 °C for 5 min, separated by gradient gel (Bio-rad) and transferred onto a polyvinylidene difluoride (PVDF) (Bio-rad) followed by blocking with 10% BSA in TBST (TBS with 0.1% Tween 20) at room temperature for 1 hour. Target signaling proteins, for example, anti-pP65(S536 (93H1)), P65(D14E12 8242S), pAkt(T308, 4056S)), Akt(9272S), ,

pERK(T202/Y204), ERK(135F5, 4695S) (all antibodies from Cell Signaling), Beta-actin (Santa Cruz Biotechnology) antibodies were incubated with various concentration of BSA in TBST at room temperature for 1 hour. The immunoblots were visualized by Amersham™ ECL™ Prime Western blotting detection reagent and then analyzed by LAS-3000 (Fujifilm).

Statistical Analysis

All experiments were performed independently at least three times. Values are expressed as mean ± SD. Significance was analyzed using a two-tailed, unpaired *T*-test. A *p*-value of less than 0.05 was considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).