Tiotropium attenuates IL-13-induced goblet cell metaplasia of human airway epithelial cells

Loes E.M. Kistemaker[1,5], Pieter S. Hiemstra[2], I. Sophie T. Bos[1,5], Susanne Bouwman[1,5], Maarten van den Berge[3,5], Machteld N. Hylkema[4,5], Herman Meurs[1,5], Huib A.M. Kerstjens[3,5], Reinoud Gosens[1,5]

Online Data Supplement

Methods

Culture of human airway epithelial cells

The immortal human bronchial epithelial cell line 16HBE14o− was cultured in minimum essential medium (ME)), supplemented with 10% heat-inactivated foetal bovine serum, 20 U/ml penicillin and 20 μg/ml streptomycin and 2 mM L-glutamine (Gibco, Grand Island, NY). Cells were collected for RNA analysis.

Primary human airway epithelial (HAE) cells were obtained from healthy lung transplant donors and isolated by enzymatic digestion. Cells from passage 2 were grown submerged on collagen-coated Transwell inserts until confluence, after which they were cultured at an air-liquid interface (ALI) and differentiated for two or three weeks in B/D medium (1:1 mixture of DMEM [Gibco, Grand Island, NY] and bronchial epithelial growth medium [BEGM; Lonza, Walkersville, MD, USA], supplemented with 0.4% [w/v] bovine pituitary extract [BPE], 0.5 ng/ml epidermal growth factor [EGF], 5 μg/ml insulin, 10 μg/ml transferrin, 1 μM hydrocortisone, 6.5 ng/ml T3, 0.5 μg/ml epinephrine [all from Lonza], 15 ng/ml retinoic acid [Sigma Chemical, St. Louis, MO], 1.5 μg/ml bovine serum albumin [Sigma Chemical, St. Louis, MO], 0.5 mM sodium pyruvate [Gibco, Grand Island, NY], 20 U/ml penicillin and 20 μg/ml streptomycin [Gibco, Grand Island, NY]). Cells were exposed to IL-13 (1-5 ng/ml; Peprotech, Rocky Hill, NJ), tiotropium (10 nM; provided by Boehringer Ingelheim) or the combination of IL-13 and tiotropium that was added to the basal medium during the differentiation process. ALI cultures were maintained for 14 or 21 days at 37°C in a humidified atmosphere of 5% CO_2. Medium and stimuli were refreshed three times per week. The apical side of the epithelial cells

was washed with PBS at the same time. At the end of the experiment, cells were collected for RNA analysis or fixed for histochemistry, and basal medium was collected for cytokine analysis.

Real time PCR

Real time PCR was performed with denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 10 minutes at 72°C. Real-time PCR data were analyzed using the comparative cycle threshold (Ct: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA. The specific forward and reverse primers used are listed in Table E1.

Tables

Table E1: Primers used for qRT-PCR analysis. CHT1: high-affinity choline transporter 1; CTL1: choline transporter-like protein 1; CTL4: choline transporter-like protein 4; CarAT: carnitine acetyltransferase; M_1R: muscarinic M_1 receptor; M_2R: muscarinic M_2 receptor; M_3R: muscarinic M_3 receptor; AChE: acetylcholinesterase; BChE: butyrylcholinesterase; FoxA2: forkhead box protein A2; FoxA3: forkhead box protein A3; SPDEF: SAM pointed domain containing ETS transcription factor.

Gene	Primer sequence	NCBI accession number
MUC5AC	Forward – ATTTTTTCCCCACTCCTGATG Reverse – AAGACAACCCACTCCCAACC	XM_006718399.1
Tektin	Forward – TGGGCTGAAGGATACAAAGG Reverse – GATGGCCTTTTCAAGAGCTG	NM_053285.1
CHT1	Forward – TGCAGTATCTCTGCCCTGTG Reverse – AGCTGGGGGAAGATAACGAT	NM_021815.2
CTL1	Forward – TAGCCGACGGTTATGGAAAC Reverse – CCTTGTAAAAGCCACCCGTA	NM_080546.4
CTL4	Forward – GGGATCAGCGGTCTTATTGA Reverse – GGCGCAGAAGCAAGATAAAC	NM_025257.2
CarAT	Forward – AAGAAGCTGCGGTTCAACAT Reverse – GGGCTTAGCTTCTCCGACTT	BT006801.1
M_1R	Forward – CCGCTACTTCTCCGTGACTC Reverse – GTGCTCGGTTCTCTGTCTCC	NM_000738.2
M_2R	Forward – TACGGCTATTGCAGCCTTCT Reverse – GCAACAGGCTCCTTCTTGTC	NM_001006630.1
M_3R	Forward – GGTCATACCGTCTGGCAAGT Reverse – AGGCCAGGCTTAAGAGGAAG	NM_000740.2

AChE	Forward – CCTCCTTGGACGTGTACGAT Reverse – CTGATCCAGGAGACCCACAT	NM_001282449.1
BChE	Forward – AAGCTGGCCTGTCTTCAAAA Reverse – CCACTCCCATTCTGCTTCAT	NM_000055.2
FoxA2	Forward – ACTACCCCGGCTACGGTTC Reverse – AGGCCCGTTTTGTTCGTGA	XM_006723562.1
FoxA3	Forward – CTTCAACCACCCTTTCTCCA Reverse – GGGAATAGAGGCCCTGGTAG	NM_004497.2
SPDEF	Forward – ATGAAAGAGCGGACTTCACCT Reverse – CTGGTCGAGGCACAGTAGTG	XM_005248988.2
18S	Forward – CGCCGCTAGAGGTGAAATTC Reverse – TTGGCAAATGCTTTCGCTC	NR_003286.2

Table E2: Components of the non-neuronal cholinergic system expressed in epithelial cells. Expression of the components was analyzed by real-time qPCR in submerged 16HBE14o- and submerged HAE cell cultures, in freshly isolated HAE cells and in ALI-cultured HAE cells. Data represent means ± s.e. of the mean, n=3. * $p < 0.05$; *** $p < 0.001$ compared to submerged culture, # $p < 0.05$; ## $p < 0.01$ compared to HBE culture, $$$ $p < 0.001$ compared to freshly isolated cells. CHT1: high-affinity choline transporter 1; CTL1: choline transporter-like protein 1; CTL4: choline transporter-like protein 4; CarAT: carnitine acetyltransferase; M_1R: muscarinic M_1 receptor; M_2R: muscarinic M_2 receptor; M_3R: muscarinic M_3 receptor; AChE: acetylcholinesterase; BChE: butyrylcholinesterase.

	16HBE14o- cells (±sem)	**Submerged-cultured HAE cells (±sem)**	**Freshly isolated HAE cells (±sem)**	**ALI-cultured HAE cells (±sem)**
CHT1	0.01 (±0.00)	1.00 (±0.60)	22.39 (±5.22)$^{\#\#}$	0.13 (±0.07)$^{***, \$\$\$}$
CTL1	5.49 (±1.22)*	1.00 (±0.09)	0.50 (±0.20)$^{\#}$	2.74 (±1.04)$^{\#}$
CTL4	0.01 (±0.00)	1.00 (±0.06)	34.03 (±13.10)	98.31 (±49.62)
CarAT	1.26 (±0.59)	1.00 (±0.27)	2.22 (±1.06)	0.97 (±0.46)
AChE	0.02 (±0.01)	1.00 (±0.16)	0.62 (±0.51)	0.02 (±0.01)
BChE	0.14 (±0.09)*	1.00 (±0.09)	0.47 (±0.34)	0.15 (±0.07)*
M_1R	1.65 (±1.07)	1.00 (±0.56)	4.17 (±1.83)	0.15 (±0.02)
M_2R	0.18 (±0.06)	1.00 (±0.41)	4.09 (±2.10)	0.23 (±0.06)
M_3R	0.01 (±0.01)	1.00 (±0.92)	4.37 (±3.39)	3.19 (±1.09)

Table E3: Effect of IL-13 on the expression of the non-neuronal cholinergic system. Data represent average Ct values corrected for 18S (i.e. highest values represent lowest expression levels), n=4 donors. CHT1: high-affinity choline transporter 1; CTL1: choline transporter-like protein 1; CTL4: choline transporter-like protein 4; CarAT: carnitine acetyltransferase; M_1R: muscarinic M_1 receptor; M_2R: muscarinic M_2 receptor; M_3R: muscarinic M_3 receptor; AChE: acetylcholinesterase; BChE: butyrylcholinesterase.

	CTR (±sem)	**IL-13 (±sem)**
CHT1	24.4 (±1.0)	24.7 (±0.5)
CTL1	13.1 (±0.4)	13.0 (±0.2)
CTL4	11.5 (±0.2)	11.2 (±0.1)
CarAT	14.3 (±0.4)	14.6 (±0.2)
M_1R	20.4 (±0.4)	20.3 (±0.4)
M_2R	22.5 (±0.6)	22.4 (±0.7)
M_3R	18.1 (±0.1)	18.4 (±0.1)
AChE	21.7 (±0.4)	21.6 (±0.3)
BChE	20.2 (±0.9)	21.2 (±0.4)

Table E4: Effect of IL-13 and tiotropium on inflammatory cytokine release. Cytokine levels were measured by a Luminex assay on the basal medium. No significant differences were observed. Data represent mean (pg/ml) ± s.e. of the mean, n=8 donors. The following cytokines were below the detection limit of 3.2 pg/ml: IFNγ, IL-1 α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-15, IL-17A, MIP-1 α, TNF-α, TNF-β.

	CTR	**IL-13**	**Tiotropium**	**IL-13 + tiotropium**
Eotaxin	22.3 (±2.2)	25.6 (±2.3)	22.0 (±2.3)	25.2 (±2.9)
G-CSF	378.4 (±262.7)	415.7 (±208.6)	503.7 (±259.9)	206.4 (±118.3)
GM-CSF	48.2 (±13.9)	61.8 (±21.3)	30.0 (±6.6)	70.8 (±26.5)
IFNα2	38.2 (±7.2)	39.4 (±7.0)	36.5 (±7.8)	39.9 (±8.2)
IL-7	12.5 (±2.5)	15.3 (±2.4)	11.7 (±2.2)	15.8 (±3.1)
IL-8	1740.5 (±276.7)	2065.5 (±123.8)	1737.9 (±236.4)	1822.9 (±190.9)
IP-10	24.9 (±6.4)	41.5 (±12.4)	26.9 (±5.8)	59.2 (±17.5)
MCP-1	30.7 (±18.6)	77.6 (±56.9)	35.2 (±18.5)	142.9 (±87.5)
MIP-1β	11.0 (±0.1)	11.3 (±0.2)	11.1 (±0.1)	11.2 (±0.2)