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Original article

Epithelial-mesenchymal transition promotes reactivity of human lung adenocarcinoma A549 cells to CpG ODN

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EMT	epithelial-mesenchymal transition
ODN	oligodeoxynucleotides
TLR	Toll-like receptor

ABSTRACT

Background: Epithelial-mesenchymal transition (EMT) is reported to promote airway remodeling in asthmatics, which is the main histological change that causes complex and severe symptoms in asthmatics. However, little is known about whether EMT also plays a role in acute exacerbations of asthma evoked by respiratory tract infections.

Methods: A human lung adenocarcinoma line, A549, was incubated with TGF-β1 at 10 ng/ml to induce EMT. Then the cells were stimulated with CpG ODN. Expression of surface and intracellular molecules was analyzed by flow cytometry. IL-6, IL-8 and MCP-1 in the culture supernatant were measured by Cytometric Bead Assay, and the expression of mRNA was quantitated by real-time PCR. CpG ODN uptake was analyzed by flow cytometry.

Results: The culture supernatant levels of IL-6, IL-8 and MCP-1 and the expression of mRNA for these cytokines in CpG ODN-stimulated A549 cells that had undergone EMT was significantly higher compared to those that had not. Addition of ODN H154, a TLR9-inhibiting DNA, significantly suppressed the CpG ODN-induced production of those cytokines. However, flow cytometry found the level of TLR9 expression to be slightly lower in A549 cells that had undergone EMT compared to those that had not. On the other hand, CpG ODN uptake was increased in cells that had undergone EMT.

Conclusions: EMT induction of A549 cells enhanced CpG ODN uptake and CpG ODN-induced production of IL-6, IL-8 and MCP-1. These results suggest that EMT plays an important role in exacerbation in asthmatics with airway remodeling by enhancing sensitivity to extrinsic pathogens.

Introduction

Bronchial asthma is an allergic disease of the airways that is increasing throughout the world, with significant impacts on public health. Based on WHO estimates, 334 million people suffer from asthma,[1] and in Japan the prevalence rates of asthmatic symptoms and current asthma among Japanese adults have been reported to be as high as 10.1% and 4.2%, respectively.[2] While most asthmatics' symptoms can be controlled well with inhaled corticosteroids, symptoms of severe asthmatics remain difficult to control and can even become fatal, especially when acute exacerbation is evoked. The known potential causes of acute exacerbation include infections, allergens, occupational exposure, hormones, drugs, exercise, stress and air pollutants.[3] Among these, bacterial infection is reported to be frequently involved in asthma exacerbation in adults[4,5] as well as young children.[6]

Especially in severely asthmatic bronchi, airway remodeling is often identified as one of the main histological changes, together with airway inflammation. Histopathological findings of airway remodeling include goblet cell metaplasia/hyperplasia, subepithelial fibrosis, reticular basement membrane thickening and extracellular matrix deposition, increased airway smooth muscle mass and vascular changes.[7] Airway remodeling leads to irreversible airflow limitation[8] and airway hyperresponsiveness,[9] which are the main physiological changes seen in severe asthmatics. Indeed, the degrees of subepithelial layer thickening,[10] fibroblast accumulation and airway smooth muscle hypertrophy[11] are associated with the severity of asthma. One of the underlying causes of these histological changes is thought to be epithelial-mesenchymal transition (EMT).[12,13]

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EMT is a biologic process in which epithelial cells are phenotypically transformed into mesenchymal cells.[14,15] EMT is commonly classified into three types: type 1 is seen in organ development, type 2 is associated with tissue regeneration and organ fibrosis and type 3 occurs in tumor invasion and metastasis.[14–16] Recent studies suggest that type 2 EMT promotes airway remodeling in asthmatics[12,13] and that a potent *in vitro* EMT-inducer, TGF-β,[17] is associated with airway remodeling in asthma.[18] However, little is known whether or not, and how, changes in the characteristics of airway epithelial cells caused by EMT may be involved in acute exacerbation of asthma evoked by respiratory tract infections.

In the first line of defense against microbial pathogens, Toll-like receptors (TLR) recognize pathogen-associated molecular patterns leading to activation of the innate immune system.[19] It is widely accepted that unmethylated CpG motifs are recognized by TLR9 and act as pathogen-associated molecular patterns,[20–22] and synthetic oligodeoxynucleotides with species-specific CpG DNA motifs (CpG ODN) show the same effect as naturally-occurring CpG.[22] Therefore, we used CpG ODN in the present study and show that TGF-β1–induced EMT of a human lung adenocarcinoma line, A549, enhanced IL-6, IL-8 and MCP-1 production in response to CpG ODN.

Methods

Reagents

The following reagents were purchased as indicated: recombinant human TGF-β1 (PEPRO TECH, Rocky Hill, NJ, USA) (Sigma–Aldrich, St. Louis, MO, USA); ODN 2006 (B-class CpG ODN, 5′tcgtcgttttgtcgttttgtcgtt3′), control ODN (B-class ODN, 5′tgctgcttttgtgcttttgtgctt3′), ODN H154 (5′cctcaagcttgagggg3′)[22] and FAM-conjugated CpG ODN (Sigma–Aldrich); PBS and FBS (Thermo Fisher Scientific, Waltham, MA, USA); and D-MEM/Ham's F-12 medium (Wako Pure Chemical Industries, Osaka, Japan); and dexamethasone and ethanol (Wako Pure Chemical Industries).

The following antibodies were purchased as indicated: PE-conjugated mouse anti-human E-cadherin mAb (IgG1κ, clone 67A4) (BD Biosciences, San Jose, CA, USA); APC-conjugated mouse anti-human N-cadherin mAb (IgG1κ, clone 8C11), APC-conjugated rat anti-human TLR9 mAb (IgG2aκ, clone eB72-1665), APC-conjugated mouse anti-human CD14 mAb (IgG1κ, clone 61D3), PE-conjugated mouse IgG1κ (clone P3.6.2.8.1), APC-conjugated mouse IgG1κ (clone P3.6.2.8.1) and APC-conjugated rat IgG2aκ (clone eBR2a) (eBioscience, San Diego, CA, USA); PE-Cy7-conjugated mouse anti-human CD205 mAb (IgG1κ, clone HD30) (Miltenyi Biotec, Bergisch Gladbach, Germany); and PE-Cy7-conjugated mouse IgG1κ (clone MOPC-21) (BioLegend, San Diego, CA, USA).

Cell line and culture

A human lung adenocarcinoma line, A549, was cultured in D-MEM/Ham's F-12 medium containing 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO_2 until the experiments.

For induction of EMT, A549 at a cell density of 5×10^4 cells/ml was seeded with TGF-β1 at 10 ng/ml in D-MEM/Ham's F-12 medium containing 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated for 3 days at 37 °C, 5% CO_2.

Flow cytometric analysis

Expression of cell-surface E-cadherin, N-cadherin, CD14 and CD205 was analyzed by flow cytometry. After incubation with or without TGF-β1, as previously described, 1×10^5–10^6 cells were washed with FACS buffer (PBS containing 3% FBS), blocked with human IgG at 1 μg/ml and incubated with 25 μg/ml of PE-conjugated mouse anti-human E-cadherin mAb/APC-conjugated mouse anti-human N-cadherin mAb or isotype control in FACS buffer on ice for 30 min. After washing with FACS buffer, the cells were resuspended in FACS buffer and analyzed by FACSVerse (BD Biosciences). For each sample, at least 10,000 events were collected, and dot plots and histograms were generated using FlowJo (Tree Star Inc., Ashland, OR, USA).

Expression of intracellular TLR9 was analyzed by flow cytometry. After incubation with or without TGF-β1, 1×10^5–10^6 cells were washed with FACS buffer and blocked with human IgG at 1 μg/ml, followed by fixation and permeabilization using Fixation/Permeabilization Solution (BD Biosciences) according to the manufacturer's protocol. The cells were washed and then incubated with 20 μg/ml of APC-conjugated rat anti-human TLR9 mAb or isotype control in Perm/Wash buffer on ice for 30 min. Then the cells were resuspended in the FACS buffer and analyzed by FACSVerse.

To analyze the cellular uptake of CpG, EMT-induced cells were incubated with FAM-conjugated CpG ODN or unconjugated CpG ODN at 10 μg/ml in D-MEM/Ham's F-12 medium for 24 h at 37 °C and shielded from light. Then 1×10^5–10^6 cells were washed with FACS buffer and analyzed by FACSVerse.

Quantitation of A549-derived cytokines

EMT-induced cells in D-MEM/Ham's F-12 medium were incubated with CpG ODN or control ODN at 1, 3, 10 and 30 μg/ml for 48 h for concentration analysis, or at 10 μg/ml for 6, 12, 24 and 48 h for time-course analysis. The levels of IL-6, IL-8 and MCP-1 in the culture supernatants were measured by Cytometric Bead Assay (CBA; BD Biosciences) according to the instructions of the manufacturer. The supernatants were stored at −80 °C until assay. For inhibition assay, EMT-induced cells were incubated with CpG ODN at 10 μg/ml and ODN H154 at 0, 1, 3, 10, 30 and 100 μg/ml or dexamethasone at 0, 1, 10, and 100 pM for 24 h, and the culture supernatants were subjected to CBA.

Real-time quantitative PCR analysis

EMT-induced cells were incubated with CpG ODN or control ODN at 10 μg/ml for 4 h. Then the total RNA was extracted from the cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted mRNA was reverse–transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed using primers and Taqman probes designed by Thermo Fisher Scientific, Inc. Data were calculated by the ΔΔCt method, using the cDNA and, as a reference, β-actin cDNA. The relative quantitation (RQ) values were calculated using the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

Statistics

Statistical analysis was performed using an unpaired two-tailed t-test for pair-wise comparisons or by ANOVA with Tukey's test for multiple comparisons. Statistical significance was defined as a P value of less than 0.05. All data are expressed as the mean ± SD of 3–6 independent experiments.

Results

TGF-β1 induced EMT in A549 cells

EMT induction by TGF-β1 in A549 alveolar epithelial cells has been established.[16] Using our materials, after incubation with TGF-