Journal of Cellular Biochemistry 86:194–201 (2002)

Differential Expression of a WD Protein During Squamous Differentiation of Tracheal Epithelial Cells

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Abstract The lining of the trachea consists of a pseudostratified, mucociliary epithelium that under a variety of conditions, such as vitamin A deficiency, toxic and mechanical injury, becomes a stratified squamous epithelium. Several in vitro cell culture models have been established to study the process of differentiation of airway epithelium. Such studies have indicated that mucosecretory differentiation of tracheal epithelial cells can be modulated by substratum. This study was undertaken to understand molecular mechanisms of squamous differentiation in tracheal epithelia. Primary cultured tracheal cells grown on uncoated filters were differentiated to single layer of squamous cells, whereas cells were grown as stratified columanar cells on collagen-I coated filters. The responses to secretagogues were altered according to culture conditions. DD-PCR revealed that FAK and a WD protein expression was increased in squamous tracheal epithelia. Expression of a WD protein was changed by the treatment of retinoic acid in various epithelial cells. These results indicated that squamous differentiation of tracheal cells changes the expression of a variety of genes, and that the experimental model for this study can be employed to study molecular mechanisms of squamous differentiation in airway epithelial cells. J. Cell. Biochem. 86: 194–201, 2002. © 2002 Wiley-Liss, Inc.

Key words: airway epithelial cells; squamous differentiation; secretagogues; WD protein

Tracheobronchial epithelium is pseudostratified and composed of three major cell types. Basal and secretory cells are known to divide, whereas ciliated cells are generally considered terminally differentiated. Ciliated cells have many important physiological roles in airway homeostasis, such as regulation of fluid and ion content in the airway lumen and mucociliary clearance of foreign particles [Jeffery, 1978; Sleigh, 1981]. Recent studies indicate that ciliated cells are easily damaged or lost after exposure to air pollutants such as nitrogen dioxide, formaldehyde, and smoke [Colizzo et al., 1992; Carson et al., 1993; Nowak et al., 1993]. The lining of the trachea consists of a pseudostratified, mucociliary epithelium that under a variety of conditions, such as vitamin A deficiency [Inayama et al., 1996], toxic and mechanical injury, becomes a stratified squamous epithelium. Functional differentiation in mammary epithelia requires specific hormones and local environmental signals. The latter are provided both by extracellular matrix [Hay, 1993] and by communication with adjacent cells [Streuli et al., 1991]. Several studies have indicated that the expression of the squamous differentiated phenotype and mucosecretory phenotype by cultured tracheal epithelial cells can be regulated by substratum and the presence of retinoic acid [Jetten et al., 1987; Rearick and Jetten, 1989; Fujimoto et al., 1993; Yoon et al., 1997]. Studies understanding the mechanisms that regulate hyperplasia and squamous metaplasia may provide insight into the processes that lead to squamous cell carcinomas [Jetten et al., 1992a,b].

In this study, we examined the effect of collagen coating on growth, function and gene expression of primary cultured rat tracheal epithelial cells. Primary cultured tracheal cells grown on uncoated filters were differentiated to

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Received 14 December 2001; Accepted 21 March 2002
DOI 10.1002/jcb.10201

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single layer of squamous cells, whereas cells were grown as stratified columnar cells on collagen-I coated. Differential display PCR shows that expression of a WD protein is increased differentially in squamous tracheal epithelial cells.

METHODS

Primary Cell Culture

Sprague Dawley rats (200–300 g) were anesthetized with intraperitoneal (i.p.) injection of pentobarbital sodium (120 mg/100 g). Primary cell culture was done by minor modification of the procedures described previously. Briefly, freshly excised tracheae were incubated at 4°C for 18–24 h in a Ca^{2+}-and Mg^{2+}-free, serum-free modified Eagle's minimum essential medium (MEM) containing 0.01% protease XIV (Sigma, St. Louis, MO). The epithelial cells were removed from the airways by scraping the epithelial surface of the tracheae, and the cells were then washed with fresh MEM medium containing fetal bovine serum (FBS) to neutralize the protease. After the final wash, the cells were resuspended in DMEM/Ham's F-12 mixture containing insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin E, epidermal growth factor, and 5% fetal calf serum (K1-10 medium), and a half of final cell suspension 5×10^5 cells/ml was added to each permeable filter support (12 mm SNAP-WELL; Costar, Cambridge, MA). For some experiments the filters treated with type I collagen (Sigma) were used. The cells were incubated in an atmosphere of 5% CO_2–95% air at 37°C in the K1-10 medium containing 5% FBS and 500 U/ml penicillin and 500 μg/ml streptomycin. Typically, at 4th or 5th day after seeding cells, transepithelial resistance measured by EVOM epithelial ohmmeter or by Ohm's law after steady-state voltage and current were recorded for a particular monolayer, was above 1,000 Ω/cm^2.

Human nasal epithelial cells were cultured according to the method described by Jun et al. [2001]. Nasal specimens were obtained during surgery from inferior turbinate mucosa of patients suffering from septal deviation or nasal polyp.

Electrophysiology

Transepithelial electrophysiological measurements were performed in a modified Ussing chamber constructed to accept SNAPWELL filter. Short-circuit current (I_{sc}) was measured with a DVC-1000 voltage–current clamp with the voltage clamp mode. The filters above 1,000 Ω/cm^2 in transepithelial resistance measured by the EVOM epithelial ohmmeter were used for all experiments. The bath solution was a Kreb's bicarbonate Ringer's solution (KBR) that was composed of 140 mM NaCl, 2.3 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose (pH 7.4). Both mucosal and serosal bath solutions were subjected to constant recirculation, maintained at 37°C, and oxygenated gently with 95%/5%O_2/CO_2.

RNA Preparation and RT-PCR

Total cellular RNA was isolated from tracheal epithelial cells using RNazol B solution (Biotecx Lab., INC.) according to the manufacturer's recommendations. For differential display analysis, possible DNA contamination was removed by treating the obtained RNAs with DNase I (GenHunter) for 30 min at 37°C. Reverse transcribed into first stand cDNA using oligo dT primers, and amplified by 40 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 1 min) of PCR using 10 pmol of specific primers. Upon completion of the PCR, products were examined on a 2% agarose gel. β-Actin primers were used as an internal standard. The sequences of the primers are listed in Table I.

TABLE I. Sequence of Primers Employed in the PCR Amplification

Gene	Orientation	Sequence ($5' \rightarrow 3'$)
Human WD	Forward	AGAGATACGGCTATGTCGATCG
	Reverse	AATTATGGTTTCCAGAGCTTTAGG
Mouse WD	Forward	AGCGTTACCGCTATGTCGATCG
	Reverse	CACCGTAAAGGCTGACTTAAGG
Keratin 1	Forward	GGACATGGTGGAGGATTACCG
	Reverse	TGCTCTTCTGGGCTATATCCTCG
Keratin 13	Forward	CTGTTGCAGAGACGGAGTGC
	Reverse	GCCACCAGAGGCATTAGAGG
β-Actin	Forward	TTGTAACCAACTGGGACGATATGG
	Reverse	GATCTTGATCTTCATGGTGCTAGG

DDRT-PCR

Differential display PCR was performed using the RNAimage™ kit from GenHunter Co. following the manufacturer's instructions. Reverse transcription (RT) was done in three independent reactions using one-base-anchored H-T_{11}M primers. PCR reactions were performed using H-AP primer and H-T_{11}M primer. The amplified cDNAs were separated on a 6% denaturing polyacrylamide gels and autoradiographed with X-ray film. The excised dried band with attached Whatman paper was recovered. The band was amplified by PCR using the same reaction conditions described above. The amplified DNA fragments were cloned into the plasmid vector pGEM-T. DNA sequence analysis was performed according to the dideoxy method using the Sequenase 2.0 DNA Sequencing kit (USB) utilizing ^{35}S-dATP.

Southern Blot Analysis

RT-PCR products of mRNA were used in Southern blot analysis. Amplification products were electrophoresed on 2% agarose gels and then DNA was transferred to nitrocellulose membrane. cDNA probes were labeled with [^{32}P]dCTP by random priming and hybridized in 50% formamide, $5 \times$ SSC, $5 \times$ Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. After hybridization, filters were washed twice in $1 \times$ SSC for 15 min at room temperature and twice in $1 \times$ SSC and 0.1% SDS for 15 min each at 65°C. The washed filters were exposed to Hyperfilm-MP X-ray films.

Cloning and Sequencing of cDNAs

The reamplified cDNA bands were cloned into plasmid pGEM-T easy vector (Promega, Madison, WI) according to the manufacture's instructions. Plasmid DNAs were purified using QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA sequencing of cloned cDNAs with either T7 or SP6 primer was carried out using the Sequenase v 2.0 DNA sequencing kit (Biochemicals, Cleveland, OH) following the manufacture's instructions or using the ABI PRISM™ Dye Terminator cycle sequencing ready reaction kit protocol with Automated DNA sequencer (Perkin Elmer ABI PRISM™ model). Comparison of DNA homology and Protein homology with GenBank and the EMBL databases was performed using BLAST and FASTA.

cDNA Library Screening

To obtain full cDNA clones, the rat lung 5′-strech cDNA library (Clontech Lab, Inc.) was screened by the probes to be obtained from DDRT-PCR. Positive phages were isolated, and phage DNAs were digested with EcoR I. The band was excised, then melted at 65°C. Ligation reactions were performed using phosphatase-treated, EcoR I-digested pBluescript (Stratagene; ~0.2 μg/ml), and T4 DNA ligase (Pharmacia). The reaction was incubated at 15°C for 16–18 h and then used to transform competent *Escherichia coli* DH5α(BRL). Transformants were analyzed by sequencing and plasmid DNA was prepared using standard plasmid preparation techniques. Plasmid DNA sequencing of cloned cDNAs with T3 and T7 primer was carried out using the Sequenase 2.0 DNA Sequencing kit (USB).

Retinoic Acid Treatment

HaCaT cells, BCC cells, Hepa1-c1c7 cells, and nHNE cells were grown to subconfluenced then treated with 10^{-6} M *all-trans*-retinoic acid for 5 days. The medium was replaced every 48 h. At the end of the treatments, cells were washed twice with phosphate-buffered saline (PBS) solution and for studies of WD gene expression, RNA was isolated from cells.

RESULTS

Morphology of RTE Cells According to Culture Condition

To examine effect of collagen coating to SNAP well filter on growth pattern of rat tracheal epithelial (RTE) cells, RTE cells were grown on collagen-coated filters or uncoated filters. Figure 1 is a photograph of tracheal cells grown on uncoated filters (Fig. 1A) or collagen I-coated filters (Fig. 1B). When cultured on collagen I-coated filters, the cells were grown as multilayers of cuboidal cells at the 5th day after seeding cells, whereas cultures on uncoated filters consisted of single layer of squamous cells. Tracheal cells grew more rapidly on collagen I-coated filters than on uncoated filters. There was a significant difference between them in the basal transepithelial potential difference (11.7 ± 2.8 mV and 31.7 ± 4.8 mV, respectively) after establishment of tight junctions, although the basal I_{sc} is similar (13.2 ± 2.1 μA/cm^2 and 15.1 ± 3.1 μA/cm^2, respectively).

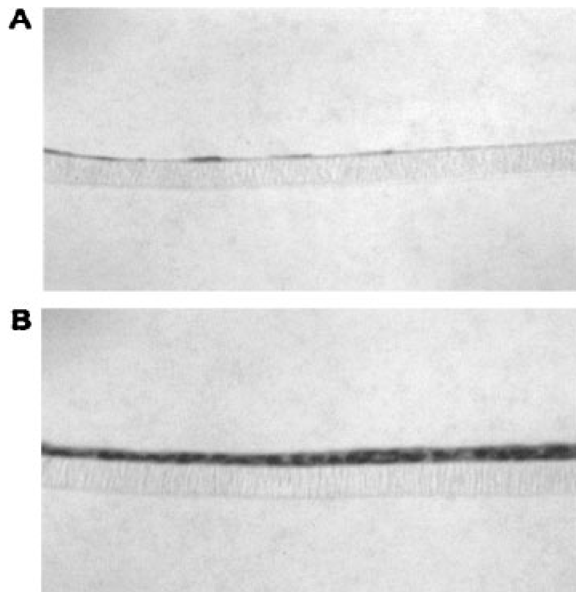

Fig. 1. Morphology of tracheal epithelia grown on uncoated filters (**A**) or collagen I-coated filters (**B**). Monolayers were removed from the TRANS well filters at the 5th day after seeding, fixed by paraformaldehyde and stained with hematoxylin-eosin.

These results indicate that monolayers grown on collagen I-coated filters had higher trans-epithelial resistance (TER) than that on uncoated filters.

Electrophysiological responses of various secretagogues were tested in the cells grown on uncoated filters or on collagen I-coated filters. As shown in Figure 2, there were significant increases in the Ca^{2+}-mediated responses by carbachol and ATP, not in cAMP-mediated response by forskolin, in the monolayers grown on collagen I-coated filters.

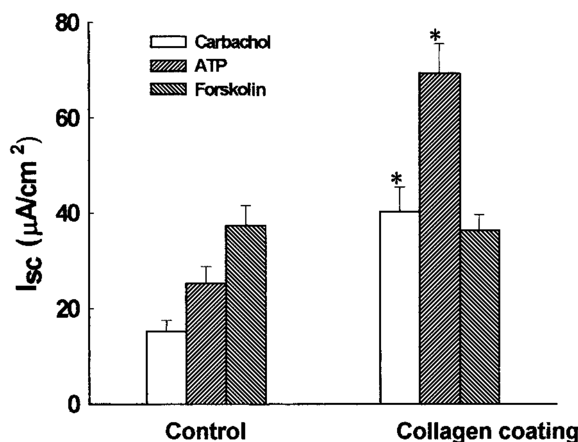

Fig. 2. Effects of secretagogues on I_{sc} in cultures grown on uncoated (Control) or collagen-coated filters. ATP (10 μM), carbachol (10 μM), or forskolin (1 μM) were added to the serosal solution. Values shown are maximal changes (I_{sc} was the change in I_{sc} between pretreatment and stimulated values) and are means ± SE for four experiments. * $P < 0.01$ compared with the control values.

DDRT-PCR

To identify differentially expressed genes between tracheal epithelia grown on collagen-coated filter and uncoated filter, DDRT-PCR method was used. Three different arbitary primers were used for synthesis of the initial cDNA. For PCR amplification, the combination of eight primer sets was applied. Amplified cDNA fragments were separated on denaturing polyacrylamide gel, a representative example of differential display results is shown in Figure 3. The marker (*) points to a band where was distinctly specific PCR amplification of mRNAs isolated from rat tracheal epithelia grown on uncoated or collagen I coated filters.

The 10 bands to show differential expression between two culture conditions were recovered from the dried denaturing polyacrylamide gel and reamplified by PCR with the same sets of primers. PCR products were subcloned into the plasmid vector pGEM-T and sequenced by

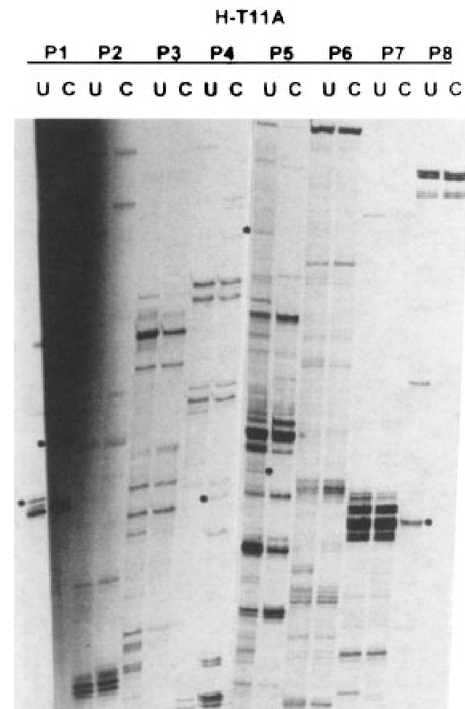

Fig. 3. A representative gel showing differential display of total RNAs from rat tracheal epithelia grown on uncoated (U) or collagen I coated filters (C). Anchor primers and arbitrary primers were indicated. Bands that showed differential expression according to culture condition are marked with *.

TABLE II. Summary of Tracheal Differential Display Clones

Clone	Identity/homology	Effect of squamous differentiation
rTEC 1	Unknown	↑
rTEC 2	Unknown	↓
rTEC 3	Protein disulphide isomerase	n.d.
rTEC 4	Elongation factor (EF-Tu)	n.d.
rTEC 5	Unknown	n.d.
rTEC 6	Cytochrome C reductase	n.d.
rTEC 7	Unknown	n.d.
rTEC 8	Ribosomal protein L44	n.d.
rTEC 9	WD-repeat proteins	↑
rTEC 10	Unknown	↑

n.d., not determinded.

dideoxy method using the Sequenase 2.0. A computer search against GenBank and EMBL DNA databases revealed that four clones did not have any homology with known cDNA, and six clones were identical with known cDNAs (Table II). It was difficult to obtain amount of RNA sufficient for Northern blot analysis from cells grown on permeable filters. Therefore, to confirm differential expression of genes between two culture conditions, we determined mRNA expression of clones in uncoated or collagen I coated filters by Southern blot analysis against RT-PCR products of mRNAs. RT-PCR was done with the same primers, which were used for DDRT-PCR. PCR products were transferred into nylon membrane and probed with clone rTEC1, rTEC2, rTEC9, and rTEC10. The results of mRNA expression of clones were shown in Figure 4. Expression of rTEC1, rTEC9, and rTEC10 was higher in cells grown on the absence of collagen coating than the remaining clone rTEC2 yielded no signal.

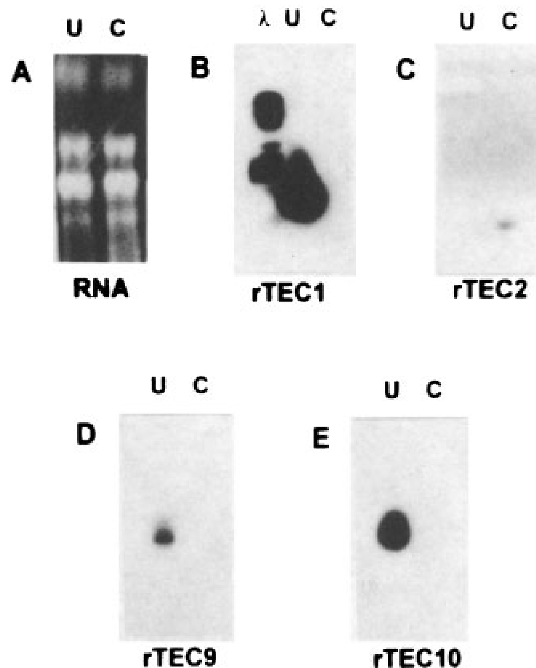

Fig. 4. mRNA expression of clones in rat tracheal epithelia grown on uncoated or collagen I-coated filters. Total RNAs were isolated from tracheal cultures grown on uncoated or collagen I-coated filters at the 4th day after plating. RT-PCR was done with the same primers, which were used for DDRT-PCR. PCR products were transferred into nylon membrane and probed with clone rTEC1 (**B**), rTEC2 (**C**), rTEC9 (**D**), and rTEC10 (**E**). (**A**). Photograph of RNA gel. Two μg of total RNA isolated from uncoated filters (U) or collagen I-coated filters (C) was electrophoresed in formaldehyde-agarose gel.

cDNA Library Screening

To elucidate identities of unique cDNA, a rat lung 5′-stretch cDNA library was screened with clone that did not show any apparent homoly with known cDNAs. Five positive plaques were isolated. Phage DNA with the longest insert was cloned into pBluescript KS (−) and analyzed by dideoxy sequencing. From the library screening, we found that rTEC1 is identical with focal adhesion kinase. We obtained a clone from library screening using rTEC9 probe. However, we could not find the first ATG in the clone,

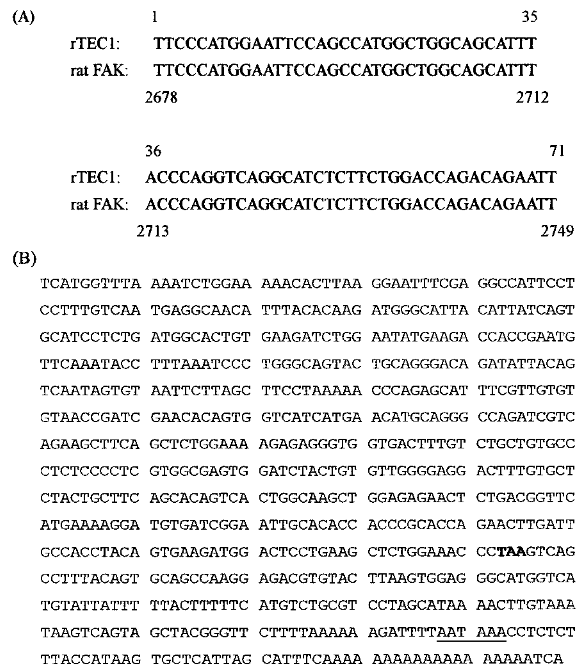

Fig. 5. **A**: Partial sequences of clone rTEC1. Alignment of the nucleotide sequence of the clone rTEC1 with that of rat focal adhesion kinase. **B**: Partial nucleotide sequences of 3′-end of rTEC9 cDNA. Polyadenylation signal was underlined and stop codon was bold in the sequences.

although a typical polyadenylation signal (AATAAA) was located downstream from the first in-frame stop codon (Fig. 5). From blast search, we found that the rTEC9 clone belongs to a member of WD protein family and has homology with *C. elegans smu-1* gene product.

Effects of Retinoic Acid on Gene Expression

We found a member of WD protein that is differentially expressed in airway epithelial cells that have squamous morphology. The addition of retinoic acid inhibits squamous differentiation in human nasal epithelia [Jun et al., 2001] and some epithelial cell lines [Choi and Fuchs, 1990; Denning and Verma, 1994], and modulates cytokeratin gene expression [Jetten et al., 1989; Breikreutz et al., 1993]. Therefore, we examined whether retinoic acid affects expression of cytokeratin and the WD gene in cultured HaCaT (a keratinocyte cell line), Hepa-1c1c7 (a hepatoma line), BCC (basal cell carcinoma cell line in skin, [Chiang et al., 1994]) and nHNE (human nasal epithelial cells). When the cells were confluent, culture media were changed to media not containing or containing 10^{-6} M *all-trans*-retinoic acid for 5 day. The expression of cytokeratin and the WD gene was analyzed by RT-PCR with total RNA extracted from cultured cells. The treatment of retinoic acid down-regulated the expression of K1 and K13 genes in nHNE and HaCaT cells (Fig. 6A) and the WD gene expression in HaCaT, BCC, and nHNE cells, not in Hepa-1c1c7 cells (Fig. 6B).

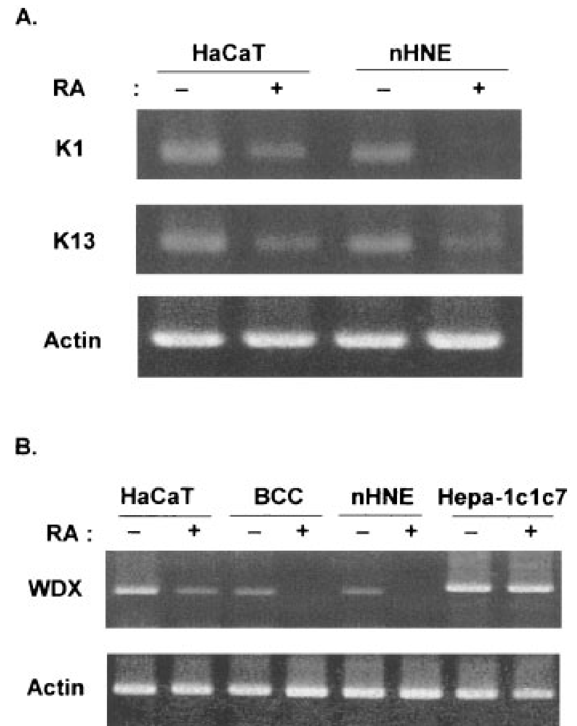

Fig. 6. Effects of retinoic acid on expression of cytokeratin and WD genes in cultured cells. HaCaT, Hepa-1c1c7, BCC, and nHNE cells were treated with 10^{-6} M retinoic acid for 5 days. The expression of cytokeratin (**A**) and the WD gene (**B**) was determined by RT-PCR. β-Actin band was used as loading control.

DISCUSSION

The lining of the trachea consists of a pseudo-stratified, mucociliary epithelium that under a variety of conditions, such as vitamin A deficiency, toxic and mechanical injury, becomes a stratified squamous epithelium. Studies using in vitro cell culture models indicated that the mucosecretory differentiation in tracheal epithelial cells could be modulated by substratum [Jetten et al., 1987; Rearick and Jetten, 1989]. Morphological and biochemical differentiation requires a combination of environmental cues including cell interaction with extracellular matrix proteins [Fujita et al., 1986]. In this study, primary cultured tracheal cells grown on uncoated filters were differentiated to single layer of squamous cells, whereas cells were grown as stratified columnar cells on collagen-I coated filters. Furthermore, the responses of tracheal epithelial cells to secretagogues are different between cells grown two culture conditions. The data in this study showed that presence of collagen I during culture of tracheal epithelial cells increased TER, which may result in the increases in I_{sc} responses to some secretagogues in cells grown on collagen-coated filters. However, the forskolin-mediated I_{sc} in contrast with Ca^{2+}-mediated I_{sc} responses was not increased in monolayers grown on collagen I-coated filters. These results indicate that the presence of collagen substratum during culture affect on some physiological functions as well as morphological differentiation in tracheal epithelia.

To understand molecular mechanisms of these phenomena, we employed a differential display polymerase chain reaction (DD-PCR) technique. Using DD-PCR, we have identified differentially expressed genes in tracheal epithelial cells shown in squamous phenotype. Among them, two genes attract special interests: one is focal adhesion kinase, and the other is a WD protein.

Focal adhesion kinase (pp125FAK) is present at sites of cell/extracellular matrix adhesion and has been implicated in the control of cell behavior [Otey, 1996]. In particular, as a key component of integrin-stimulated signal transduction pathways, pp125FAK is involved in cellular processes such as spreading, motility, growth, and survival [Akasaka et al., 1995; Sankar et al., 1995; Xu et al., 1996]. In addition, a number of reports have indicated that pp125FAK may be up-regulated in human tumor cells of diverse origin such as breast, colon, thyroid, and prostate [Glukhova et al., 1995; Owens et al., 1995; McCormack et al., 1997]. In this experiment, increase of FAK expression in squamous tracheal epithelia may have correlation with increase in surface area of cells in squamous phenotype.

In this study, expression of a WD protein, which has homology with *C. elegans smu-1* gene product, was increased in tracheal epithelial cells with squamous phenotype. Proteins in the WD-40 family contain four to sixteen conserved WD-40 repeats characterized by a variable length region followed by a core sequence delimited by two characteristic dipeptides, GH and WD [Neer et al., 1994; Smith et al., 1999; Neer and Smith, 2000]. WD-40 motifs, that were first discovered in the beta subunit of a retinal GTP-binding protein, are found in over 100 proteins with diverse cellular functions. WD-repeat proteins have been identified as regulatory components of multi-protein complexes that govern splicing, transcriptional activation or repression, chromatin modification, pre-mRNA and pre-rRNA processing, RNA export, cell cycle entry and exit, ubiquitination, cell death, signal transduction, microtubule assembly, and protein transport to, from and across membranes [Duronio et al., 1992; van der Voorn and Ploegh, 1992; Neer et al., 1994; Smith et al., 1999]. Although the full cDNA sequence of the WD protein that was identified in this experiment was registered in the Genbank Database, its physiological functions are unknown. Benedetto et al. [2001] reported that the rat homolog of this protein (bwd) is expressed in restricted areas of adult rat brain, suggesting some roles of the protein in brain functions. Recently, SMU-1, a *C. elegans* homolog of the WD protein, may involve in regulation of the alternative splicing of some RNAs [Spike et al., 2001]. It has been known that WD proteins have structural domains that involve in interaction with several cellular proteins [Smith et al., 1999]. Therefore, further studies will be required for determining whether regulation of splicing process of some genes by the protein in mammalian cells results in squamous differentiation of epithelial cells or whether other functions of the protein involve in the process.

This study showed that the treatment of retinoic acid inhibited the expression of the WD gene in human nasal epithelial cells, human keratinocyte cell line, and a cancer cell line originated from skin, not in liver cell line. Several reports have shown that retinoic acid inhibits squamous metaplastic changes in epithelial cells [Jetten et al., 1992a; Fujimoto et al., 1993; Jun et al., 2001] and modulates cytokeratin gene expression [Jetten et al., 1989; Breikreutz et al., 1993]. The data in this experiment also showed that retinoic acid down-regulated K1 and K13 gene, markers of squamous differentiation [Mendelsohn et al., 1991; Tomie-Canie et al., 1996]. Therefore, retinoic acid-induced inhibition of the WD gene as well as K1 and K13 genes further supports the possibility that the protein may involve in differentiation of tracheal epithelial cells. These results indicated that squamous differentiation of tracheal cells changes the expression of a variety of genes, and that the experimental model for this study can be employed to study molecular mechanisms of squamous differentiation in airway epithelial cells.

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