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Regeneration and Repair of Damaged Trachea Epithelium following Brushing-induced Injury in Rabbits

Abstract—The repair process of airway epithelium involves cell migration, spreading, proliferation and re-differentiation. Objective: To investigate cellular and molecular responses to tracheal brush induced injury in a rabbit model. Methods: Seven male and eleven female New Zealand white rabbits were divided into uninjured and injured groups. After tracheal brushing, the animals were maintained in the laboratory before being sacrificed at given time points (1 hour, 12 hours, 3, 7, and 21 days). The trachea of each rabbit was retrieved and preserved before being subjected to haematoxylin and eosin staining and real time PCR. Results: After injury, the remained epithelial cells underwent an instant response by proliferating and migrating into the damaged site. This finding was in accordance with the proliferative and migration activity-related gene expression results of MMP-9, TIMP1, VIM, and ITG-β. The increased activity of these genes was crucial at the early time points, as it encouraged the remaining cells to repopulate the damaged area. Conclusions: Continuous regulation of MMP-9, VIM and ITG-β plays important roles in promoting cellular homing especially among cells bordering the lesion to migrate and repair the damaged extracellular matrix (ECM). Thus, this activation enhanced regeneration and repair of the damaged tracheal epithelium as early as 1 hour and completed at day 21 following injury.

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1 INTRODUCTION

The respiratory airway is constantly exposed to harmful foreign particles. To cope with this issue, the wall of the airway is lined with dynamic and distinctive defence mechanisms such as mucus production, ciliary movement, and a tight junction between epithelial cells. Under normal conditions, the cell proliferative activity of the airway is very low, with a turnover rate of less than 1% [1]. However, the cellular turnover rate increases in response to injury. To investigate the processes that occur in the airway during injury and repair, injury to this area must be provoked in an experimental model. A rabbit model has been previously used to study the effect of physically induced injury on repair and regeneration of tracheal epithelium [2, 3]. As the diameter of the rabbit bronchial lumen is smaller than that of larger animals such as sheep, the physical tools used to produce injury could only reach as far as the tracheal region. In addition, the absence of the submucosal gland in the rabbit tracheal airway makes the model suitable for studying mucus cell (i.e., goblet cell) production in response to tracheal airway injury without interference from the submucosal gland [4].

Regardless of the type of animal model used to study the regeneration and repair process, the following five stages of cellular repair are common to all [5]: 1) spreading and migration of cells to the damaged area; 2) cell proliferation and simple squamous epithelial formation; 3) cell proliferation and formation of multi-layered epithelial cells; 4) re-differentiation of terminally differentiated cells; and 5) ciliogenesis. However, the time needed to complete each of these stages differs depending on the animal model. For a small animal model, the time needed to gain a complete restitution of epithelium after injury was from five to seven days [6-10], whereas, larger animals such as sheep still showed repair progress after 7 days of bronchus injury [11]. In contrast, human airway regeneration takes longer time to complete. For example, in the human airway xenograft model, regeneration of the epithelial tracheal tissue takes

35 d to complete [12]. In other experiments, the pseudostratified epithelium was seen as early as day 25 and regeneration continued until day 35 [5, 13].

Although cellular mechanisms play large roles in recovering from airway injury, molecular aspects also are at work during the regeneration and repair process. During the repair process, changes in the expression level of genes occur simultaneously, with expression being either upregulated or down-regulated. As the healing process nears completion, the expression patterns return to those of the resting epithelium. Injury to the lung compromises epithelial barrier integrity, leading to pulmonary oedema, diffuse epithelial damage, and inflammation. Prominent genes markers involved in those process were aquaporin 1 (AQP1), aquaporin 5 (AQP5), chemokine (C-C motif) ligand 21 (CCL21), C-X-C Motif Chemokine Ligand 13 (CXCL13), interleukin 4 (IL4), surfactant protein A1 (SFTPA1), transforming growth factor beta 2 (TGF-β2), vascular endothelial growth factor (VEGF-α), cyclin dependent kinase 2 (CDK2), cyclin E, collagen type IV, matrix metallopeptidase 9 (MMP9), tissue inhibitor metallopeptidase inhibitor 1 (TIMP1), vimentin (VIM), integrin beta (ITG-β), forkhead box J1 (FOXJ1), and keratin 10 (KRT10).

Cellular differentiation genes such as FOXJ1 and KRT10 were employed to determine their involvement in cellular development towards functional airway epithelium. Generation of pseudostratified epithelial cells in airway indicates an ongoing process during airway epithelium homeostasis and also in response to injury. In animal models, FOXJ1 is one of the most well characterized transcription factors involved in ciliated cell differentiation [14], whereas KRT10 is expressed in a differentiated epithelial cell from a single basal cell [15]. The genes participate in cell cycle, signal transduction, metabolism, and transportation [16]. As the healing is almost completed, the expression patterns are similar with the resting epithelium. Cell migration is an essential activity in a dynamic repair process. Following removal of epithelium, remaining cells have to move into denuded area in order to replace the lost cells and start to regenerate the normal epithelium [8]. Locomotion of the cells involves attraction and breakage of the adhesive contacts between cells and extracellular matrix (ECM), which acts as a floor for the cells to adhere and subsequently exert the attraction force that enables cell to migrate [17]. Matrix metalloproteinases (MMPs) represent a group of enzymes involved in the degradation of most of the components of the ECM and therefore participate in pathological remodelling situations such as acute lung injury [18]. MMP9 has been regarded as a crucial component in epithelium repair in many studies been conducted [19]. MMP9 specifically degrades gelatinase b, which is also known as type IV collagen of ECM. MMP9 is strongly expressed by human bronchial epithelial cells during repair process [20]. Inflammation is a hallmark of lung diseases. Local activation of resident cells in the airway guides to elaboration of several pro-inflammatory cytokines and chemokines, the signals from these cells lead to up-regulation and down-regulation of gene expression and biosynthesis of pro-inflammatory mediators [21]. When airway niche is exposed to inflammatory agonists, proinflammatory cytokines such as CCL21 will attract neutrophils and lymphocytes to the injury site.

Our previous study investigated the effect of tracheal brushing to the epithelium structure following 30 min, 1 hour, 6, 12, and 24 hours of injury. The result demonstrated that repair process occurred as early as 1 hour after perturbation and longer time period is required to observe the regeneration of whole epithelium. Thus, in this study, we increased the time point of histological observation up to 21 days and also investigated the genes involved in tracheal epithelium remodelling and repair. Understanding these biological responses (both cellular and molecular) of the airway epithelium to physically induced injury offers further promise in developing a novel airway injury model.

2 METHODS

2.1 Experimental design

Seven male and eleven female New Zealand white rabbits (n = 18), aged 3-6 months weighing; 2 to 4 kg (2.7 ± 0.6 kg) were used in this experiment. The rabbits were grouped into uninjured (n = 3) and injured treatments group. They were housed individually under standard conditions prior and during injury treatments. The rabbits were sacrificed at different time points (n = 3 for each): 1 hour, 12 hours, 3 days, 7 days and 21 days after the given injury. The uninjured rabbits were served as negative control group (no brushing was given). The study protocol was approved by the Animal Ethics Committee of the Universiti Sains Malaysia (USM/Animal Ethics Approval/2010/63/258). The tracheal brushing induced-injury was conducted following a

technique described previously [2]. After brushing was completed, each rabbit was put back into its housing before being euthanized at the designated time point. Euthanization was performed by pentobarbital (Dolethal, France) injection (1ml/kg) intravenously.

2.2 Histological staining

After euthanization, the trachea was removed, fixed in 10% formalin, embedded using paraffin before sectioning the tissue to 3 µm thickness. The sections were then subjected to standard haematoxylin and eosin staining (H&E staining) (both from Labstain, Malaysia) to analyse the injury and its repair process. The sections were viewed under a light microscope (Olympus, Japan) and captured using image analyser software (Soft Imaging System Olympus, Japan).

2.3 Gene expression analysis using real time PCR

After brushing was performed, rabbits were euthanized at the respective time points and the trachea was collected and preserved at –20ºC. The tissue was then subjected to RNA extraction following the RNeasy mini kit standard protocol (Qiagen, Germany). Our analysis focused on the genes related to inflammation, proliferation, ECM/cell adhesion, and cell specific genes. Twenty six genes (Table I) were selected based on these five target groups. Four housekeeping genes were used as controls (β-actin, Glyceraldehyde-3-phosphate dehydrogenase, Peptidylprolyl isomerase A, and 18s). PCR primers were custom-designed and optimised using the Custom Taqman PCR array (Applied Biosystems, US). Reverse transcription-PCR reactions were performed with the StepOnePlus™ Real Time PCR system (Applied Biosystems, US) starting at 95 °C for 2 min for polymerase activation, followed by 40 cycles of 95 °C for 10 s and finished by 60 °C for 20 s. All calculations were performed using StepOne Software v2.3 (Applied Biosystems, US).

3 RESULTS

3.1 Time dependent histological changes

The normal trachea lining consisted of three layers: mucosa, submucosa, and cartilage (Fig. 1A). Blood vessels were located in the submucosa layer. The tracheal pseudostratified epithelium layer was a part of the mucosa layer, which faces the lumen. The brushing-induced injury resulted in removal of intact epithelium layer. The injury had resulted in damaged areas in some parts of the trachea with a few epithelial cells remained intact on the basement membrane (Fig. 1B-C). At 12 h after injury, epithelial cells started to proliferate and migrate to cover the damaged area (Fig. 1D). On day 3, epithelial cells started to differentiate to form flattened epithelial cells (Fig. 1E-G).

Table I: List of genes included in qRT-PCR analysis

Target	Gene symbol	Gene name
Inflammation	AQP1	aquaporin 1
	AQP5	aquaporin 5
	CCL21	chemokine (C-C motif) ligand 21
	CXCL13	chemokine (C-X-C motif) ligand 13
	IFN-γ	interferon gamma
	IL-4	interleukin-4
	IL-8	interleukin-8
	SFTPA1	surfactant protein A1
	TGF-β2	transforming growth factor beta 2
	TNF	tumour necrosis factor
	VEGF-α	vascular endothelial growth factor alpha
Proliferation	CDK2	cyclin-dependent kinase 2
	Cyclin A	
	Cyclin B	
ECM/Cell adhesion	COL4A3	collagen type IV
	MMP9	matrix metallopeptidase 9
	TIMP1	tissue inhibitor metallopeptidase inhibitor 1
	VIM	vimentin
	ITG-β	integrin beta
Cell specific marker	FOXJ1	forkhead box J1
	KRT10	keratin 10
	SCGB1A1	secretoglobin, family 1A, member 1
Stem cell	CD38	CD38 molecule
	CD59	CD59 molecule
	HGF	hepatocyte growth factor
	POU5F1	POU class 5 homeobox 1

This finding was similarly observed on day 7 where the cells started to re-populate the damaged area (Fig. 1H). On day 21, the damaged area was fully covered with a single layer of flattened cells (Fig. 1I-K). These flattened cells were in continuous contact with the epithelium.

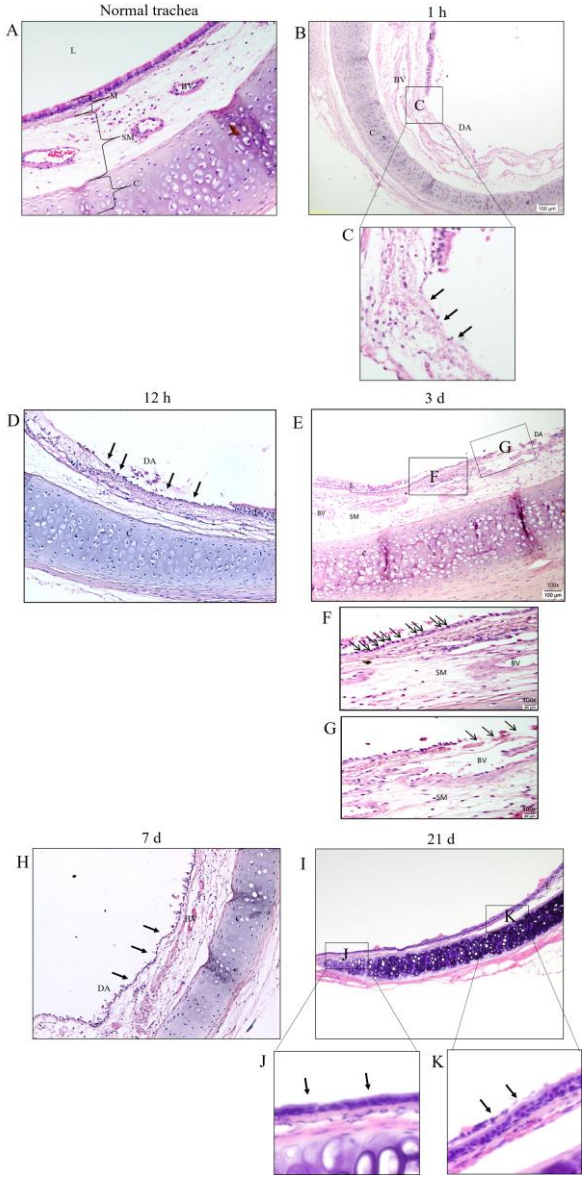

Figure 1 (A) Normal trachea epithelium. (B-C) At 1 h following injury, the damaged area exhibits complete loss of epithelium. The remaining epithelial cells started spreading to cover the damaged area following (D) 12 h to (E-G) 3 d of injury. (H) The cells actively proliferating and almost covering the whole damaged area. (I-K) 21 days following injury, epithelial cells differentiated into two layers of flattened cells which had closed the whole damaged area. (E = epithelium; BV = blood vessels; DA = damaged area; M = mucosa; SM = sub-mucosa; C = cartilage) (Scale bar A, B, D, E, H, I = 100μm).

3.2 Changes in gene expression after tracheal airway injury

Assessment of the individual fold-changes of gene expression indicated that there were variations in expression of inflammatory genes throughout the process of trachea repair (Fig. 2A). In this study, expression of AQP1 was higher than that of AQP5 throughout the experiment, which indicated that AQP1 played an important role in fluid transportation to reduce airway damage and pulmonary oedema. Anti-inflammatory genes of IL-4 upregulations were only seen at 21 days, whereas TGF-β2 was consistently upregulated from day 1 until day 21. In this model, recruitment of inflammatory cells was led by CCL21, which resulted in up-regulation of the genes throughout the time point. The injury also altered alveolar epithelial barrier as proven by the upregulation of both SFTPA1 and VEGF-α gene at 1 d following injury. However, the capability of airway to self-repair had improved the alveolus surface tension and absence of lung collapse that caused the SFTPA1 expression to decrease on day 21. An increased fold change in VEGF-α at day 1 until day 14 was associated with endothelial cell repair. VEGF-α promoted endothelial cell proliferation, capillary formation and survival of newly formed blood vessels, and its regulation was found to be down-regulated after 21 d.

In this study, CDK2 and cyclin E expression were upregulated throughout the experiment (Fig. 2B). These findings implicate that airway damage is characterized by enhanced cellular proliferation and apoptosis both being regulated by complex interactions of CDK2 and cyclin E.

Most of the ECM/cell adhesion genes in this group were upregulated at all-time points (MMP-9, TIMP1, VIM, and ITG-β) (Fig. 2C). Both MMP-9 and TIMP1 production was one of the airway injury features involved in degrading all components of the ECM including collagen type IV. In this study, MMP-9 and TIMP1 expression was expressed at all time points with higher level of MMP-9 as compared to TIMP1. VIM activity was associated with ITG-β expression. In this study, VIM and ITG-β were up-regulated throughout the airway epithelium repair and remodelling process.

Cell specific markers of FOXJ1 and KRT10 were used to identify certain cells on the epithelium (Fig. 2D). Expression of FOXJ1, a marker for ciliated cells, was upregulated from day 1 to day 14 and then decreased on day 21,

whereas, expression of KRT10, a marker for basal cells increased progressively until day 21.

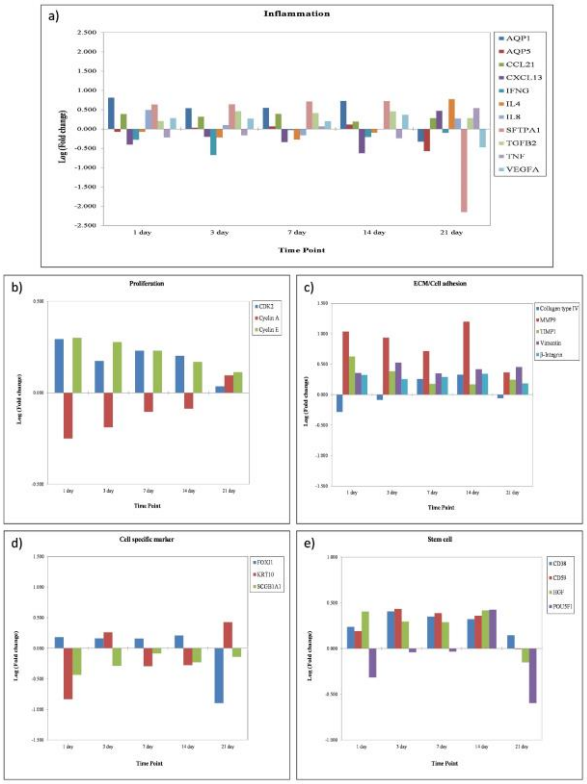

Figure 2 (A) Majority of inflammatory genes were upregulated in response to the injury. (B) Expression of the cell proliferation genes of CDK2 and cyclin E genes upregulated throughout the whole time points. (C) In ECM/cell adhesion genes, MMP9 and VIM expression upregulated to inhibit the collagen type IV expression, whereas VIM and ITG-β genes were upregulated to promote cell attachment and migration into the injury site. (D) In gene specific cell markers, FOXJ1 expression was upregulated until day 14, whereas KRT10 expression was upregulated only on day 1 and 21.

4 DISCUSSION

In the normal state, cells of the airway epithelium have a lower turnover rate (< 1%) [8, 9]. However, when the epithelium has to repair itself, the rate can increase to 30-fold higher than normal [8, 9]. Any disturbance of this layer compromises the physiological function of the trachea. In the present study, the brushing technique was used to inflict injury on the epithelium layer to provoke responses of the cells located at the border of the lesion as well as circulating cells (i.e., blood and/or bone marrow-derived cells). As a response to this stimulus, the cells of the area bordering the lesion responded by migrating and spreading to the damaged area. The cells then proliferated and differentiated in order to reconstitute the disrupted epithelium and re-established the normal epithelium layer [22, 23].

The airway epithelium that undergoes denudation due to acute inflammatory airway diseases can also effect changes in lung vascular and epithelial permeability. Acute lung injury is most frequently accompanied by an inflammatory response, elevations in pro-inflammatory mediators, alteration of the alveolar capillary barrier, and increased extravascular lung water content [24]. When injury occurs, pro-inflammatory genes were activated with release of the cytokines and chemokines. As the inflammatory reaction progresses, inhibitory gene (anti-inflammatory) products also appear, that suppress pro-inflammatory activation.

In this study, CCL21 plays prominent role as pro-inflammatory chemokine that recruits localization of inflammatory cells into injury site. IL-4 and TGF-β2 paradoxically has important immunosuppressive and anti-inflammatory activities, which supposedly can inhibit the CCL21 gene. TGF-β2 activity was also prominent compared to IL-4 since IL-4 expression only upregulated after 21 days of injury. TGF-β2 mediated remodelling following airway injury thus was highly expressed throughout the repair process. Evidence of increased permeability of the alveolar capillary membrane is also one of the main features in acute lung injury. During the genesis of airway injury, the selective barrier function of the pulmonary endothelium and epithelium were lost due to injury or dysfunction.

VEGF gene promotes endothelial cell proliferation, capillary formation, and the survival of newly formed blood vessels [25]. It is known to recruit and retain circulating endothelial progenitor cells for neovascularization. Our results showed, expression of VEGF-α was upregulated during repair and down-regulated following 21 days, indicating that the endothelial cells released VEGF-α to promote neovascularization for airway repair. The permeability of the alveolar capillary membrane initiates leakage of protein-rich fluid from the vascular to the interstitial and/or alveolar space which is also known as oedema. As shown in rats with acute lung injury [26], the decreased lung AQP1 and 5 expression is related to pulmonary oedema development. Thus, these results showed that at least three naturally produced factors (TGF-β2, VEGF-α, and AQP1) were increased during the airway injury to contain the inflammatory response.

The CDK2-cyclin E complex is important in the transition of the G1/S phase in regulation of the cell cycle [27]. The up-regulation of these genes consistent with histological staining suggested that the proliferation occurred from day 1 until day 21 after injury. These findings also implicated that airway damage is characterized by enhanced cellular proliferation and apoptosis both being regulated by complex interactions of CDK2 and cyclin E. Migration is closely associated with the ECM component and cellular adhesion [17, 20, 28]. ECM components such as collagen IV provide an important foundation to support the cells [28]. Meanwhile, VIM and ITG-β are cellular adhesion proteins that form an anchor on the ECM on which the cells can firmly attach [28, 29], and facilitate the process of the cellular migration. Matrix metalloproteinases of MMP9 and TIMP are capable of degrading all components of the ECM such as collagen type IV following injury process [30]. These ECM components have been shown to regulate each other during the complex process of migration [17, 19, 20, 28, 29].

In this study, most of the genes associated with the ECM/cell adhesion were upregulated at most of the time points which suppressed collagen type IV production. During migration, VIM facilitated the exert traction of the focal adhesion between cells and basement membrane and led to cells motility [31]. Once cells had traction on the basement membrane, MMP9 hydrolysed collagen IV to facilitate the cells to migrate [32]. MMP9 activity is under the influence of TIMP. TIMP specifically binds to MMP9, which leads to the inhibition of MMP9 activity [17]. In our study, expression of MMP9 was always higher than that of TIMP throughout the experiment. When inflammation is present, MMP9 was released into the tissue mainly by neutrophils and other inflammatory cells such as macrophages, mast cells, and lymphocytes [33]. Secreted MMP9 facilitated epithelial cells migration. At the same time, MMP9 created a positive feedback loop by recruiting more inflammatory cells to the tissue. Thus, once injury occurred, MMP9 is constantly expressed to recruit inflammatory cells to the injured tissue [34]. This suggests the continuous migration of both cell types (i.e., inflammatory and epithelial cells) to the injured area, which supported by the histological appearance of cells on day 21 (i.e., the presence of flattened cells in the damaged area).

During the later stage of the regeneration process, differentiation of progenitor cells gives rise to a different type of epithelial cell in order to complete regeneration of the epithelium [35, 36]. KRT10, which is an intermediate filament in the basal cells, is functionally important in the basal cells differentiation process [37]. In the present study, KRT10 expression was down-regulated on day 14 but up-regulated on day 21. Pertinent to the finding that the expression of migration related genes (i.e., ITG-β and VIM) remained high from the early time points until day 21. VIM activity was associated with ITG-β expression, it is suggested that both genes were required for cell migration activity. Both genes played roles in increasing cellular adhesion which helped cells to migrate to the injured site from the early phase after injury until day 21.

5 CONCLUSIONS

In this study, we demonstrated that the early response to injury of the rabbit tracheal airway exhibited an increased expression of genes related to proliferation and migration, which allowed the epithelial cells to migrate and repopulate the damaged area.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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