Enhanced inhibition of nasal epithelial cell repair by innate stimulation in patients with allergic rhinitis

Zwiększone hamowanie naprawy komórek nabłonka nosa przez stymulację nieswoistą u pacjentów z alergicznym zapaleniem błony śluzowej nosa

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The study was supported by National Science Centre – grant No: 2012/05/B/NZ5/01859

Summary

Introduction. Impaired repair of airway epithelium may lead to persistence of inflammation and remodelling. Regeneration of injured epithelium is a complex phenomenon and the role of toll-like receptors (TLRs) and respiratory virus products in this process have not been established.

Aim of the study. In this study we aimed to test if wound repair in nasal epithelial cells is modulated by microbial products and if this process was different in patients with allergic rhinitis and in healthy subjects.

Materials and methods. Injured human nasal epithelial cells (hNECs) monolayers were incubated with the toll-like receptors agonists: poly (I:C) and lipopolisacharide (LPS); allergen Der p1, and supernatants from virus-infected epithelial cells. Regeneration of injured epithelium was assessed by measuring changes in the area of epithelial damage.

Results. Addition of either poly (I:C) or LPS induced a dose dependant inhibition of wound repair in hNECs monolayers. Supernatants from RV1b-infected cells decreased epithelial cell regeneration after mechanical injury only in allergic patients. At baseline conditions the dynamics of epithelial repair was similar in allergic and non-allergic epithelium. However, inhibitory effects of innate stimuli on epithelial repair was stronger in patients with allergic rhinitis as compared to healthy individuals.

Conclusions. This study showed that microbial products may affect regeneration of the nasal epithelium, and allergic patients are more susceptible to suppression of epithelial regeneration.

Keywords: *nasal epithelium, wound repair, TLRs; poly (I:C), LPS*

Streszczenie

Wprowadzenie. Upośledzenie regeneracji nabłonka dróg oddechowych może prowadzić do utrzymywania się stanu zapalnego i zapoczątkowywać przebudowę błony śluzowej. Proces regeneracji jest zjawiskiem złożonym i nie w pełni poznanym, a rola stymulacji receptorów toll-podobnych (TLR) w tym procesie nie została określona.

Cel pracy. Ocena wpływu produktów drobnoustrojów na regenerację nabłonka górnych dróg oddechowych i porównanie regeneracji u osób chorych na alergiczny nieżyt nosa i osób zdrowych.

Materiał i metody. Komórki nabłonka nosa pobrane od osób chorych na alergiczny nieżyt nosa i zdrowych hodowano do zlewności, a następnie uszkadzano mechanicznie. Do uszkodzonych hodowli dodawano agonistów receptorów toll-podobnych: poli (I:C) lub lipopolisacharyd (LPS), supernatant z komórek nabłonkowych zakażonych rinowirusem lub alergen Der p1. Regenerację komórek oceniano poprzez powierzchnię uszkodzenia.

Wyniki. Inkubacja uszkodzonych hodowli z poli (I:C) lub lipopolisacharydem powodowała hamowanie naprawy nabłonka. Dodanie nadsączu z hodowli zakażonych rinowirusem również hamowało regenerację u chorych na alergiczny nieżyt nosa. Silniejsze zahamowanie stwierdzono u osób chorych na alergiczny nieżyt nosa niż u osób zdrowych.

Wniosek. Produkty drobnoustrojów wywierały efekt modulujący regenerację nabłonka nosa, a osoby atopowe były bardziej podatne na zahamowanie regeneracji nabłonka.

Słowa kluczowe: *nabłonek nosa, regeneracja, receptory toll-podobne, poly (I;C), LPS*

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INTRODUCTION

Regeneration of the airway epithelium is a physiological process that occurs after injury in both healthy people and in patients with respiratory diseases either in upper or in lower airways [1, 2]. In parallel airway injury leads to inflammation and is associated with the release of mediators and enzymes by inflammatory and structural cells [3-5]. In healthy subjects, resolution of inflammation and tissue restoration is rapidly achieved, but in susceptible subjects persistence of inflammation and abnormal repair may contribute to delayed regeneration and tissue remodelling.

In asthma and in chronic rhinosinusitis, the barrier function of the airways epithelium is impaired [6, 7] and abnormal repair in epithelium may contribute to persistence of inflammation [8, 9]. It is not clear what factors cause persistence of inflammation and start structural changes. Initially, allergy was considered the main factor responsible for initiating and maintaining inflammation, and most research has focused on investigating the allergic pathways of chronic inflammatory airway diseases such as asthma, rhinitis and rhinosinusitis [10]. On the other hand, the limited remodelling in upper airways of patients with allergic rhinitis, in the context of a highly inflammatory milieu, supports the view that inflammation by itself, does not induce remodelling and that other factors must operate for structural changes to develop [11].

Nasal epithelium constantly interact with pathogens such as bacteria and viruses via toll-like receptors , but the role of pathogen recognition receptors (PRRs) stimulation in nasal epithelium regeneration was not established. Despite the progress in understanding of regeneration mechanisms in lower airway epithelia [12-15], the mechanisms of nasal epithelial cells repair and remodelling are still poorly understood. In our previous study we documented for the first time that TLR agonist may modulate epithelial regeneration in monolayers of cultured cell line [16]. However, the effect of innate stimulation on regeneration of primary human epithelial cells have not been studied. In this study we aimed to test if wound repair in primary human nasal epithelial cells is modulated by microbial products and if this process differs in allergic and non-allergic subjects.

MATERIALS AND METHODS

Study subjects

Eight patients with allergic rhinitis (AR), diagnosed according to allergic rhinitis and its impact on asthma (ARIA) criteria, aged 23-40 years (three males, five females), were recruited Department of Clinical Immunology and Allergy, Medical University of Lodz. All eight patients had perennial nasal symptoms and five patients additionally reported seasonal exacerbations of nasal symptoms during tree and/or grass pollen season. Allergic sensitization was confirmed by skin prick testing (SPT) with a panel of inhalant allergens. Patient characteristics are presented in Table I. Patients received intranasal corticosteroids and oral anti-histamines, which were withdrawn 2 weeks before the sample collection. None of patients had bronchial asthma, chronic rhinosinusitis and nasal polyps. In patients with seasonal exacerbations, nasal sample collection was performed outside the relevant pollen season.

Seven non-atopic, healthy volunteers, aged 30–46 years (three males, four females), with negative skin prick tests to a panel of inhaled allergens were recruited from the University students and Department personnel. None of the study subjects was smoking or had had respiratory tract infection in the preceding 4 weeks. The study was approved by The Bioethical Committee at Nofer Institute of Occupational Medicine in Łódź (Poland) (decision number 12/2012). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Culture of epithelial cells

Nasal scrapings were performed using sterile cytology brushes (Daga Med, Gdansk, Poland) after topical anaesthesia with 10% lidocaine (Egis, Warsaw, Poland) administered on the nasal mucosa. Before the brushing, subjects blew their nose to remove mucus lining from the nasal cavity and the surface of the mid-part of the inferior turbinate was scraped. Epithelial cells obtained were transferred to bronchial epithelial cell growth medium (BEGM; Lonza, Cologne, Germany) containing 1% penicillin/streptomycin and cells were seeded into a 5 cm2 flask coated with colla-

Table I. Characteristics of allergic (A) and non-allergic (NA) patients

	A	**NA**
n	8	7
Sex		
Men/Women	3/5	3/4
Age (years)		
Mean	30	33
Min-Max	23-40	30-46
Allergic Rhinitis	8	0
Perennial and Seasonal	8	
Perennial only	5	

gen and fibronectin and cultured with hormonally supplemented BEGM at 37 °C with 5% CO2. Fresh medium was replaced every 48h until a monolayer formed. For the first two medium replacements, BEGM containing 1% of serum substitute (Ultroser G; Cytogen, Lodz, Poland) was used. Confluent cultures were trypsynized with 0,1% trypsin-EDTA solution (Sigma-Aldrich) and cells were splited into uncoated 35-mm-diameter Petri dishes with 2-mm square grids on the bottom surfaces (Invitrogen, Life Technologies, CA USA) for further experiments. The epithelial nature of cultured cells was confirmed by intracellular staining for anti-cytokeratin 19, assessed by cytofluorometry using a BD LSR Fortessa Analyzer (Becton Dickinson,Warsaw, Poland).

Wound repair assay

For assessment of monolayer regeneration, confluent cultures were mechanically scratched with a 10-μl pipette tip and washed with serum-free culture medium to remove damaged cells. Next, digital photos of identifiable squares of impaired cultures were taken at 0, 12h, and 24h after injury using an Olympus 3040Z digital camera with an Olympus CK2 inverted microscope (Olympus Optical, Middlesex, UK). The area of unpopulated cells was measured with image analysis software (CellSens Standard, Olympus). In each experiment, four squares on each plate were analysed and the mean was calculated. Each experiment was repeated at least three times.

In preliminary experiments, the wound area was analysed 3, 6, 12, 18 and 24h post injury to assess the time and rate of repair, and in subsequent experiments the damaged area 12h and 24h post injury was compared with the initial wound area.

Cell Stimulation and Treatment of ECs with Conditioned Media from Infected Cells

Damaged and not-damaged cultures (controls) were stimulated with the TLR agonists: poly (I:C) (Sigma-Aldrich) (0.1 μg/mL, 1 μg/mL, 10 μg/mL) and LPS (Sigma-Aldrich) (1 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL) for 60 min; the allergen Der p 1 (Indoor Biotechnologies Ltd., Cardiff, UK) and conditioned media from virus-infected epithelial cells.

Preparation of Conditioned Media from RV1b-Infected Cells

Confluent EC cultures were infected with RV1b at 0.1 MOI. After 1 h of incubation with RV1b, supernatants were removed and the cells were cultured in MEM supplemented with 2% FCS for 24, 48, and 72h. At these three time points, supernatants were collected and prepared for further experiments by centrifugation, filtration, and inactivation with UV irradiation for 30 min.

Statistical analysis

Statistical analysis was performed with Statistica version 10 PL (Statsoft Polska, Krakow, Poland). Data are displayed as means and standard errors of the means. Changes in mediators release and mRNA expression were analysed using Wilcoxon's ranked-pairs analysis for paired data. Data normality was assessed using the Shapiro-Wilk test. For comparisons of mediator release between AS and AT patients the nonparametric Mann-Whitney U test was used preceded by evaluation of normality. A *p-value* lower than 0,05 was considered as statistically significant.

RESULTS

Viability of injured hNECs cells

Following injury, cell cultures were observed for 48h under light microscope and we did not observed neither detachment of cells nor cell lysis. We were able to record cell proliferation and migration toward injury surface. When monolayers were stained with crystal violet, no disruption of the cell layer was observed 24h and 48h post injury. Viability of cells in injured cultures assessed with MTT was not differ from not injured cultures.

Wound Repair in hNECs from allergic and non-allergic patients

In the preliminary experiments the repair response was recorded 3, 6, 12, 18 and 24h after injury and the complete closure of injured area was observed between 20 to 24h after injury (Fig. 1).

There was no differences in repair response to injury between allergic and non-allergic patients when recorded at 12h and 24h post injury.

Effect of poly (I:C) and LPS on wound repair in hNECs from allergic and non-allergic subjects

When injured hNECs monolayers were incubated with poly (I:C) (0,1μg/ml, 1μg/ml, 10μg/ml) a dose depended inhibition of wound repair was observed (data not shown). In further experiments, to compare the effect of poly (I:C) on wound repair in allergic and non-allergic subjects, the highest concentration of poly (I:C) (10μg/ml) was used. At 24 hours after injury the area of unhealed epithelium

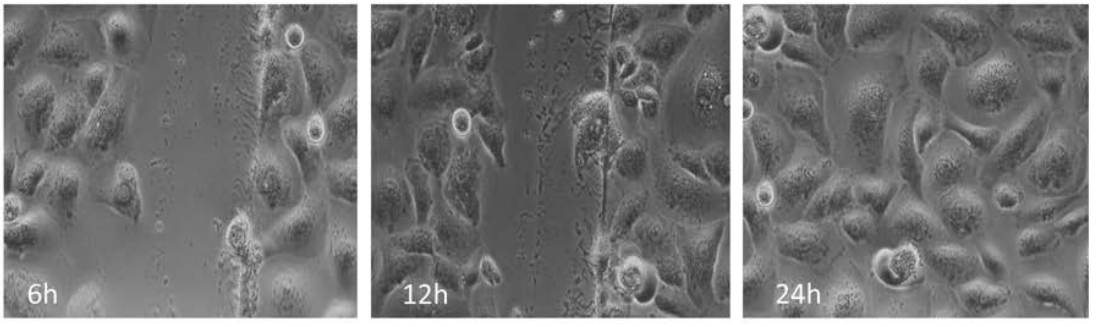

Fig 1. Regeneration of human nasal epithelial cells 6, 12, 24h post injury (magnification 100X)

was significantly higher ($p<0.05$) in hNECs from allergic patients (mean remaining wound area was 49,4±15nm^2) as compared to cells from non-allergic subjects (mean remaining wound area was 27,1±12,4nm^2) (Fig. 2).

Similarly to poly (I:C), preincubation of damaged epithelial cells monolayers with LPS resulted in a dose dependant decrease in wound repair in both allergic and non-allergic epithelium. LPS (10μg/ml) inhibited epithelial regeneration induced by mechanical injury to higher extend in allergic (mean remaining wound area was 68,2±24nm^2) as compared to non-atopic patients (mean remaining wound area: 35±17nm^2) (Fig. 3).

Effect of supernatants from RV1b infected airway epithelial cells on wound repair in NECs

Incubation of injured hNECs monolayers with supernatants from RV1b infected cells, resulted in decreased repair response 24h post injury only in allergic patients. In cultures from non-allergic subjects, addition of supernatants from RV1b infected cells caused slight decrease in regeneration, but not statistically significant. (Fig. 4).

In contrast incubation of injured epithelial monolayers cells with Der p1 did not influenced epithelial cell regeneration from either atopic or non-atopic subjects (Fig. 5).

DISCUSSION

In the present study, we have investigated for the first time regeneration of primary nasal epithelial cells cultured as monolayers from patient with allergic rhinitis, and we demonstrated that toll-like receptor (TLR3 and TLR4) agonists: may differentially modulate repair of nasal epithelial cells from allergic and in non-allergic subjects.

To investigate the epithelial cell injury and regeneration the previously described experimental model of the primary human nasal epithelial cells cultures was used [17, 18]. The injury of epithelial monolayer was induced by mechanical scratching and assessed by a validated methodology [16, 17].

We observed that a complete regeneration of injured hNECs monolayers occurred within 20- 24 hours in all subjects . There was no difference in the dynamics of spontaneous healing of the nasal epithelial cell cultured from patients with allergic rhinitis and healthy subjects, suggesting that underlying inflammation does not affect basic mechanisms involved in epithelial cell regeneration in patients with perennial allergic rhinitis . In contrast recent study documented that the mean wound repair rates were significantly slower in NEC monolayers from patients with chronic rhinosinusitis with nasal polyps (CRSwNP) as compared to control subjects [19]. Although both allergic rhinitis and CRSwNP, are associated with mucosal inflammation, the immunological mechanism underlying chronic inflammatory response in CRSwNP is different [20, 11] and result in impairment of baseline repair processes in nasal epithelium.

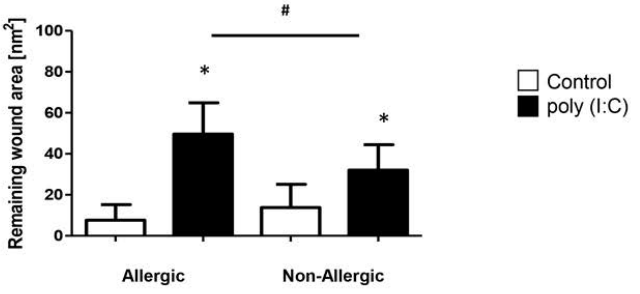

Fig 2. Effect of poly(I:C) on wound repair in allergic and non-allergic subjects, 24h post injury, *p<0,05; # p<0,05; * statistical difference between regeneration in non-stimulated and stimulated with poly (I:C) cultures, # statistical difference between poly (I:C) induced regeneration in allergic and non-allergic subjects

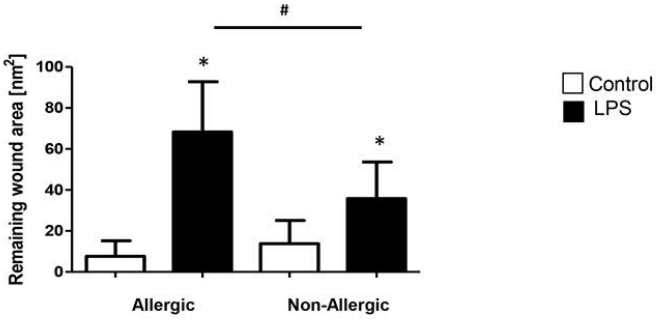

Fig 3. Effect of LPS on wound repair in allergic and non-allergic subjects, 24h post injury, *p<0,05; # p<0,05; * statistical difference between regeneration in non-stimulated and stimulated with LPS cultures, # statistical difference between LPS induced regeneration in allergic and non-allergic subjects

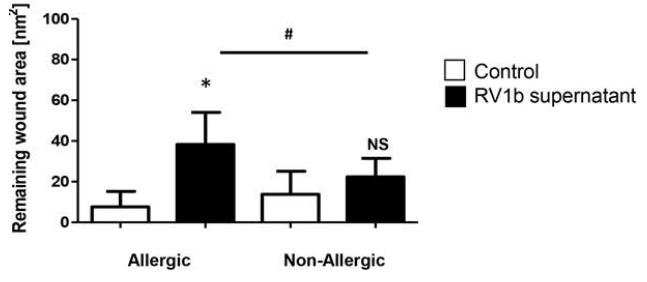

Fig 4. Effect of RV1b supernatant on wound repair in allergic and non-allergic subjects, 24h post injury, *p<0,05; # p<0,05; * statistical difference between regeneration in non-stimulated and stimulated with RV1b supernatant cultures, # statistical difference between RV1b supernatant induced regeneration in allergic and non-allergic subjects

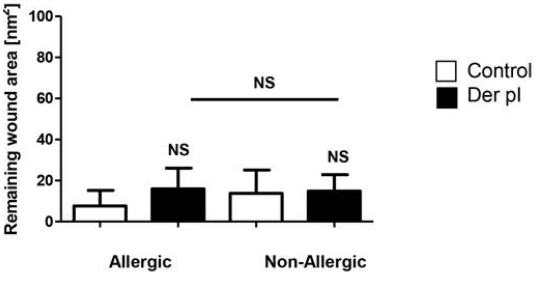

Fig 5. Effect of Der pI on wound repair in allergic and non-allergic subjects, 24h post injury, NS - not significant

Nasal epithelium is continuously exposed to environmental factors such as pathogens, allergens and irritants which are known to initiate mucosal inflammation, but could also play an important role epithelial repair. We hypothesized that TLRs which participate in the innate immune responses to pathogens, may modulate regeneration of epithelial cells during respiratory infection [21]. To assess the role of TLR stimulation, we used the TLR3 agonist poly (I:C) and the TLR4 agonist-LPS. Addition of poly (I:C) to mechanically damaged monolayers of NECs significantly decreased repair rates. Since TLR3 recognizes double-stranded (ds)RNA, which is the nuclear material of many viruses [22] our data suggest that inhibition of epithelial cell regeneration may be an important mechanism associated with injury of epithelial cells observed during viral infection in the airways of patients suffering from asthma [23]. Similar a dose-dependent decrease in epithelial monolayers repair was observed after cell stimulation with LPS a TLR4 agonist. Since LPS is a bacterial product and TLR4 may be activated with other bacterial products as well [24], one can speculate that bacterial infections may, similarly to viruses, modulate airway epithelial cell repair occurring in response to injury induced by environmental factors. These observations are in line with our previous study demonstrating that LPS and poly(I:C) inhibited the regeneration of injured bronchial epithelial cell monolayers (BEAS-2B) [16].

In the next step of the study, we assessed the potential, indirect effect of respiratory viral infection on epithelial regeneration, by exposing hNECs supernatants from virus infected airway epithelial cell. Incubation of hNECs cultures with supernatants from RV1b-infected hNECs decreased the regeneration 24 hours post injury in allergic patients to similar degree as observed with TLR3 agonist. This observation suggest that products released by virus-infected airway epithelial cells (e.g. danger-associated molecular patterns such as ATP, HMGB1, and S100 proteins) may negatively affect repair process in pathogen-injured epithelium [25-27]. Previous studies have documented airway epithelial barrier disruption by different factors, such as cigarette smoke [28, 29], allergens [30], and viral infection [31, 32]. However, there are only few studies referring to the role of viral infection in epithelial regeneration [33, 34], and our is the first one describing the involvement of toll-like receptors in inhibition of nasal epithelial regeneration.

Inhibition of regeneration by innate stimuli (TLR agonists and virus-infected cell supernatants) was significantly stronger in allergic patients as compared to non-allergic subjects. Our allergic patients suffered from perennial symptoms, thus underlying inflammation could be responsible for increased susceptibility to innate stimulation. It has been previously documented that virus proliferation rates and the immune response profile are different in nasal epithelial cells from patients with allergic rhinitis compared to healthy individuals, which could be potentially related to ongoing inflammation [18]. Furthermore previous studies documented that TLRs expression may be related with allergic rhinitis [35-38], suggesting that differential TLR expression or response to innate stimulation, could be responsible for enhanced inhibitory effect on epithelial cells regeneration.

It has been shown previously that allergens might have nonspecific action on respiratory epithelia via enzymatic activities. For example, the mite allergen Der p1 is able to disrupt epithelial tight junctions and this increases the epithelial permeability [39, 40]. In our study, incubation of injured NECs cultures with Der p1 did not affect epithelial cell regeneration neither in atopic nor in non-atopic subjects.

In summary, this study documented for the first time, that innate stimuli (TLR agonists or virus-infected cells products) slow down regeneration of injured human nasal epithelium and this process is differentially modulated in allergic patients in healthy individuals. This observation may contribute to a better understanding of the relationship between pathogens and allergic inflammation in airway remodeling.

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