

ORIGINAL PAPER

Repair of the injury to respiratory epithelial cells characteristic of asthma is stimulated by *Allomyrina dichotoma* agglutinin specific serum glycoproteins

B. J. Patchell and D. R. Dorscheid

James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, University of British Columbia, Department of Medicine, University of British Columbia, Vancouver BC, Canada

Clinical and Experimental Allergy

Summary

Background The airway epithelium acts as a protective barrier, separating the external environment from the underlying tissue. Daily challenges result in damage to the epithelium that, in normal individuals, is quickly and effectively repaired. In respiratory diseases, such as asthma, this repair is compromised. Following injury to the epithelium, plasma leaks into the airway lumen acting as a protective protein cap. Carbohydrates are mediators of epithelial repair, however, the associated regulatory proteins remain unknown.

Objective To identify mediators of epithelial repair based on their carbohydrate moieties using an *in vitro* wound repair culture model of human airway epithelial cells (1HAEo⁻).

Methods Using the lectin *Allomyrina dichotoma* agglutinin (AlloA) as a tool, ligands essential in the repair of damaged epithelium were characterized. AlloA was subsequently used to purify and identify a glycoprotein associated with epithelial repair.

Results The addition of AlloA to the media of mechanically wounded monolayers inhibited repair. Fetuin, a highly glycosylated serum protein, was identified as a glycoprotein bound by AlloA. The addition of fetuin to serum starved monolayers stimulated wound closure.

Conclusion These results indicate that following mechanical injury to the epithelium, serum glycoproteins, not only provide a protective barrier, but also are involved in the initiation of wound closure.

Keywords airway epithelial repair, *Allomyrina dichotoma* agglutinin, asialoglycoprotein receptor, asthma, fetuin

Submitted 24 March 2005; revised 8 September 2005; accepted 29 September 2005

Correspondence:

Delbert R. Dorscheid, iCAPTURE Centre, St Paul's Hospital, Rm. 166, 1081

Burrard Street, Vancouver, BC, Canada V6Z 1Y6.

E-mail: ddorscheid@mrl.ubc.ca

Introduction

As a protective barrier for the underlying tissue the airway epithelium is routinely challenged resulting in loss of its integrity. It is also involved in the regulation of airway diameter [1] and mucociliary clearance [2]. Damage to the epithelium is a common pathological finding in asthma [3]. As barrier function is essential for preventing the penetration of noxious particles to the underlying tissue, epithelial repair occurs quickly, restoring the barrier's integrity [4]. Typically, bronchoalveolar lavage (BAL) fluid from asthmatic subjects contains creola bodies [5] and an increased presence of eosinophils, even when the clinical diagnosis of the disease is mild [6]. The accumulation of damage in asthma and the increased sloughing of epithelial cells suggest a defect in the repair mechanism or an increased susceptibility to injury. The chronic nature of

this damage results in the loss of barrier integrity and epithelial function. As such, remodelling of the airways and airway hyper-reactivity as seen in asthma, may be a result of this chronic and excessive damage [7]. These findings highlight the importance of efficient and effective repair of the epithelium following injury.

Following the loss of columnar epithelial cells, the subsequent repair involves the flattening and migration of neighbouring basal cells into the damaged area. This is followed by proliferation and differentiation, to restore the pseudo-stratified epithelium [8]. Cell culture models have been developed to study repair *in vitro*. Mechanically wounded monolayers of airway epithelial cells have been used to follow repair kinetics and to characterize the associated phenotype. Zahm et al. [9] have demonstrated that the cells adjacent to the wound migrated to repopulate the injured site. Increased proliferation was only seen

in cells distant from the wound [4]. This evidence suggests the initiation of repair is a tightly regulated process involving a specific subset of cells in proximity to the damaged area. Completion of the repair may require migration of cells from locations remote to the initial injury and subsequently replaced by proliferation. Models have demonstrated the kinetics of and some of the molecular elements that contribute to the morphological changes observed during epithelial wound repair [10–12].

The importance of carbohydrates in cellular functions has been well documented. Carbohydrate structures attached to the surface of cells via proteins or lipids are critical in the function of many cell types such as cell adhesion [13, 14], proliferation [15], growth potential [16] and migration. In epithelial cells, it has been shown that migratory cells express different cell-surface carbohydrates than stationary cells [17]. Cell-surface glycosylation profiles of airway epithelial cells were mapped and different cell types present a unique set of carbohydrates on their surfaces [18]. Subsequent work demonstrated that following mechanical injury, airway epithelial cell glycosylation profiles change coordinate with repair [19]. An essential role for carbohydrates in repair was identified using an *in vitro* model. Treatment of wounded monolayers with tunicamycin, an *N*-glycosylation inhibitor resulted in the loss of wound repair. Furthermore, the removal of terminal fucose prevented wound closure completely [20]. Adam et al. [21] demonstrated that the lectin, wheat germ agglutinin (WGA), binds the surface of airway epithelial cells and inhibits repair. The authors suggest that the lectin ligand plays a significant role in epithelial repair. In both of these studies, the work went no further as to identify to molecule(s) associated with the carbohydrate ligands.

Work *in vivo* has demonstrated the extravasation of bulk plasma into the airway lumen from the underlying microvessels following injury [22]. Plasma exudation occurs rapidly following epithelial loss and is coordinate with initial healing. This plasma-derived gel provides an immediate protective physical barrier. It is rich in fibronectin and other matrix molecules that may be involved in the stimulation of epithelial repair [23]. Plasma contains cytokines that may exert biological activity in airways flooded with exudates [24]. The repair of the damaged epithelium is initiated and proceeds much more promptly *in vivo* relative to repair described in cell culture experiments [25]. This suggests the presence of epithelial repair factors in the microcirculation. The role of serum glycoproteins in epithelial repair has not been described.

Allomyrina dichotoma agglutinin (AlloA) is a C-type lectin specific for α -D-galactose containing oligosaccharides similar to the binding specificity of human lectins such as the asialoglycoprotein receptor (ASGPR). We have demonstrated that, following mechanical injury, exposure to AlloA prevented wound closure of airway epithelial cells. The AlloA-specific glycoprotein responsible for ef-

fecting *in vitro* wound closure is the serum protein fetuin. The addition of fetuin to serum deprived cell cultures stimulated the initiation of wound closure. This study demonstrates the role that plasma and serum glycoproteins have in the repair of damaged airway epithelium.

Materials and methods

Cell culture

Normal human airway epithelial cells (1HAEO⁺) were a gift from Dr D. Gruenert, University of Burlington, VT [26]. 1HAEO⁺ cells are SV40-transformed normal human airway epithelial cells that have been characterized previously [27, 28] and express multiple surface carbohydrate markers of primary basal airway epithelial cells [18]. They were grown in culture for lectin histochemistry, wound repair kinetics and protein extraction as previously described [18]. Cells were collected by incubation in 0.25% trypsin and 1.0 mM EDTA prior to lifting for protein collection.

Monolayer wound creation and lectin cytochemistry

Monolayers were grown to confluence and small circular wounds (1.0–2.0 mm²) were created using a small rubber dental GUM[®] Stimulator (Sunstar Butler, Guelph, Canada). Lectin cytochemistry was carried out as described previously [20]. At timepoints 0, 6, 12 and 24 h after the creation of the wound, cells were fixed with 4% paraformaldehyde at room temperature (RT) for 4 h. Monolayers were incubated with biotinylated lectins AlloA and *Griffonia simplicifolia* agglutinin (GS α -1, 5 μ g/mL, EY Laboratories Inc., San Mateo, CA, USA) in HEPES buffer without BSA. Microscope images were photographed with a Sony Iris charge-coupled device camera (Sony, Rolling Meadows, IL, USA) on a Nikon Diaphot inverted stage microscope. Video images were digitized with a Macintosh computer and Apple Video Player software (Apple Computer, Cupertino, CA, USA).

Monolayer wound repair assays

This assay was carried out as has been described previously [11, 29, 30]. Briefly, cells were grown to confluence in six-well dishes and circular wounds (~2.0 mm²) were created using a small rubber stylet (four wounds per well). After wounding, cells were washed with medium without FBS. Treatment of the cells included an unstimulated control incubated in the medium without FBS. The remaining five wells were incubated in medium with 10% FBS and each with 15 ng/mL human epidermal growth factor (EGF) (Invitrogen, Burlington, Canada) and unconjugated AlloA (EY Laboratories Inc.) at 0, 10, 25, 50 and 100 μ g/mL. Wounds were imaged at $t=0, 6, 12, 18$ and

24 h after wounding using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Missiauga, Canada, Coolpix E995. Wound area was calculated by manual tracing and area calculation software (ImagePro Plus 32). As a control, GS α -1 lectin was added to the culture media at the same doses as AlloA to determine the effect of a non-specific lectin on wound closure. A similar protocol was performed with serum protein stimulation of wound repair. 1HAEo⁻ cells were grown to confluence and serum starved for 24 h. Fetuin (Sigma, Oakville, Canada) was added in increasing doses (0.03–1.0 mg/mL) to the serum-free medium. Wounds were imaged at $t=0$, 6, 12 and 24 and closure rates were determined as described above.

Lectin precipitation assay

1HAEo⁻ cells were grown to confluence, a wound was created in the monolayer of cells using a cell scraper (Sarstedt, Montreal, Canada). The wound size was large enough to remain open at 18 h. Wounded monolayers were washed with phosphate-buffered saline (PBS). DMEM plus 10% FBS was added. After 18 h in culture, the growth medium was removed and monolayers were washed with PBS to remove any remaining media. Protein lysis buffer (1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate (Na-DOC), 150 mM sodium chloride (NaCl), 1 mM ethyleneglycotetraacetic (EGTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μ g/mL aprotinin, 1 μ g/mL pepstatin, 1 μ g/mL leupeptin, 1 mM sodium vanadate (Na₃VO₄), and 1 mM sodium fluoride) was added to the monolayer, the dish was scraped and the suspension was transferred into 1.5 mL microcentrifuge tube and tumbled for 15 min at 4 °C before centrifugation at 12 000 $\times g$ at 4 °C for 10 min. Protein concentrations were determined for each of the lysates using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein (200 μ g) was diluted into 250 μ L final volume with lectin buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.2.) with protease inhibitors (Sigma, P-8340). Before specific lectin precipitation, the non-specific proteins were cleared from the protein lysates by the addition of 60 μ L of avidin-agarose beads (Sigma, S-1638). Following incubation for 1 h at 4 °C, the beads were precipitated at 12 000 $\times g$ and the supernatant was transferred to a 1.5 mL microcentrifuge tube. Twenty micrograms of biotinylated lectin was added to the supernatant and incubated overnight at 4 °C. To precipitate the lectin specific-proteins, the supernatant was incubated with 60 μ L of 60:40 avidin-agarose beads slurry for 1 h. The beads were precipitated and the pellet was washed twice with lectin buffer. Precipitated proteins were denatured in SDS buffer.

Protein purification and sequencing

Precipitated proteins were separated by SDS-PAGE. The gel was stained with Coomassie R-250 and destained

overnight in 30% methanol, 10% acetic acid. Selection of bands for sequencing was based on visual assessment. The purified band was excised from the gel and sequenced via MALDI-TOF MS analysis (The University of Victoria – Genome British Columbia Proteomics Centre, www.proteincentre.com). Briefly, excised protein was subjected to tryptic digestion and spotted onto MALDI-TOF plates with α -cyno-4-hydroxycinnamic acid (CHCA) matrix using conditioned C18 ZipTips (Millipore, Nepean, Canada). ZipTips were conditioned using successive washes of; 100% acetonitrile, 50% acetonitrile 0.1% trifluoroacetic acid (TFA), and 0.1% TFA. Ten microliters of the digested sample was then loaded onto the ZipTip. The bound peptides were then washed 3 $\times$ with 0.1% TFA. The peptides were then eluted directly onto a MALDI target plate using 1 μ L of a 10 mg/mL solution CHCA in 50% acetonitrile, 0.1% TFA. Peptide mass fingerprints were obtained using MALDI-TOF mass spectrometry (Applied Biosystems Streetsville, Canada, Voyager DE-STR MALDI-TOF). Spectra were acquired in positive ion mode using the ion reflector at an accelerating voltage of 20 kV. A total of 100 scans were averaged to create the final spectrum. The grid voltage was set to 65% of the accelerating voltage and a delay time of 80 ns was used. Peptide mass data was submitted to MASCOT database for protein identification (<http://www.matrixscience.com/>).

Statistics

Values are presented as means $\pm$ SE. The significance of differences between means was assessed by analysis of variance followed by the Tukey-HSD test, with the level of significance set at $P < 0.05$.

Results

Lectin staining of wounded airway epithelial cell monolayers. AlloA staining of 1HAEo⁻ cells accumulates following mechanical injury (Fig. 1a–d). Before mechanical wounding, there is negligible AlloA staining, representing baseline (Fig. 1a). Monolayers fixed 6 h after wounding demonstrated AlloA-positive staining only on cells in proximity to the wounded area (Fig. 1b) relative to the baseline (Fig. 1a). Monolayers fixed at 12 and 24 h after wounding had a dramatic accumulation of AlloA-positive-stained cells inside the wound area (Figs 1c and d). Lectin staining with GS α -1, a lectin with similar carbohydrate specificity to AlloA, demonstrated a completely different staining pattern than that observed using AlloA (Figs 1e and f). Similar to AlloA staining, prior to mechanical injury, GS α -1 staining is absent (Fig. 1e). Following mechanical injury and 24 h repair, there was very little change in GS α -1-positive staining cells relative to the corresponding AlloA staining (Fig. 1d).

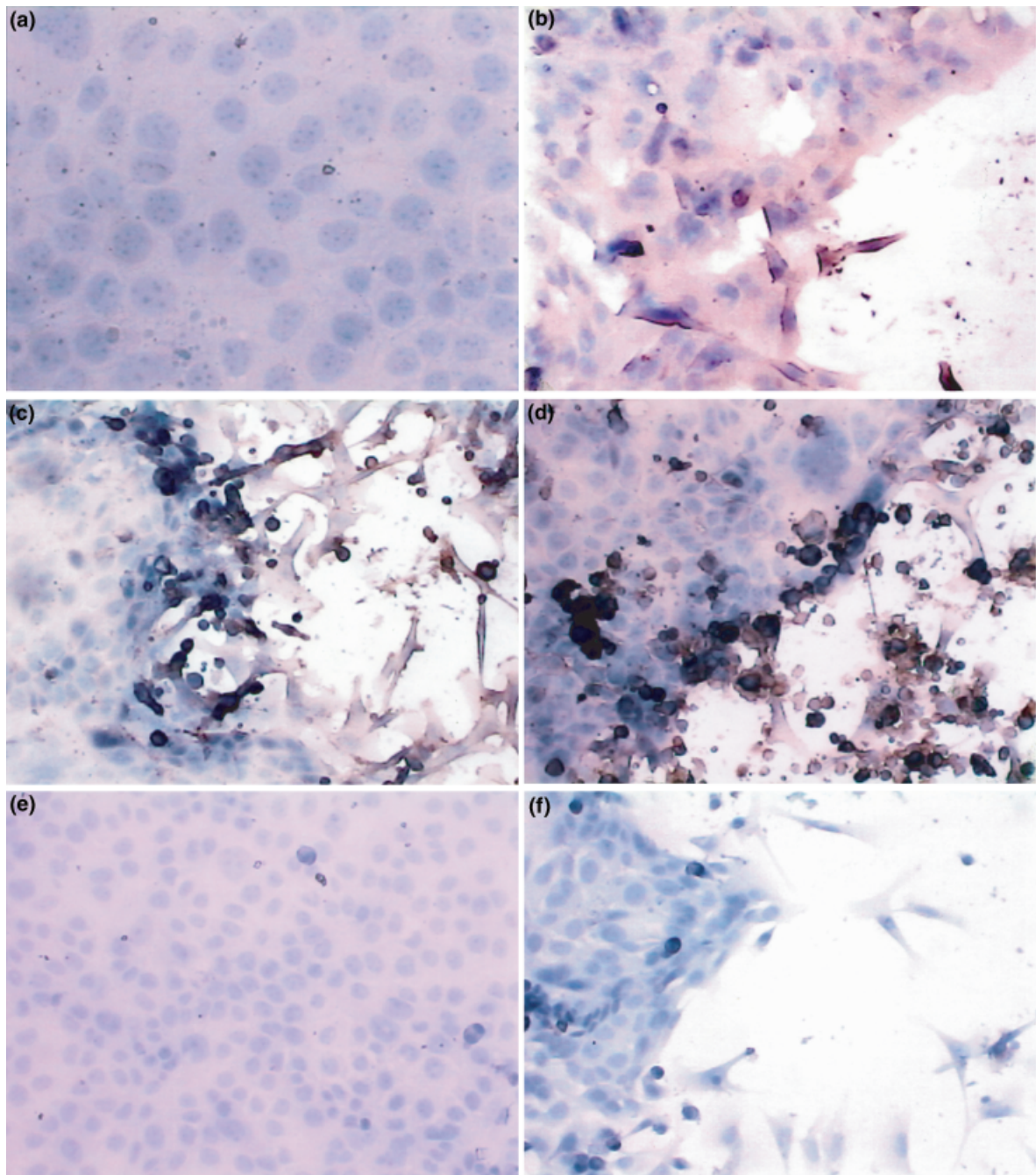


Fig. 1. AlloA and GSx-1 lectin binding on the surface of human airway epithelial cells (1HAEo⁻) monolayers. AlloA-positive staining cell numbers increased over time and accumulate in the disrupted site following mechanical injury. (a) No mechanical injury demonstrates the negative baseline pattern of AlloA staining, (b) 6 h repair, (c) 12 h repair and (d) 24 h repair demonstrate a pattern of increasing AlloA staining in proximity to the damaged site. GSx-1 lectin staining of 1HAEo cells results in negative baseline in both (e) confluent and (f) 24 h repair monolayers. Original magnification $\times 200$.

Effect of AlloA on wounded airway epithelial monolayer repair. Representative images of wound closure for serum-free (Control) and EGF + serum stimulated monolayers demonstrate closure over time (Fig. 2). Control serum-free monolayer wounds closed slowly over a 24 h period, while closure in the presence of serum and EGF progressed to near completion after 24 h. Representative images of AlloA treatments highlight the wound closure

inhibition capability of AlloA. Low dose AlloA (10 $\mu\text{g/mL}$) demonstrates very little effect with respect to inhibiting wound closure. Increasing the concentration of AlloA (100 $\mu\text{g/mL}$) resulted in large wound areas remaining after 24 h of repair. Wounds in the presence of GSx-1 at the same dose (100 $\mu\text{g/mL}$) closed completely over the same 24 h period. Mean initial wound area for each of the wounds used in this study was $2.38 \pm 0.15 \text{ mm}^2$. Initial

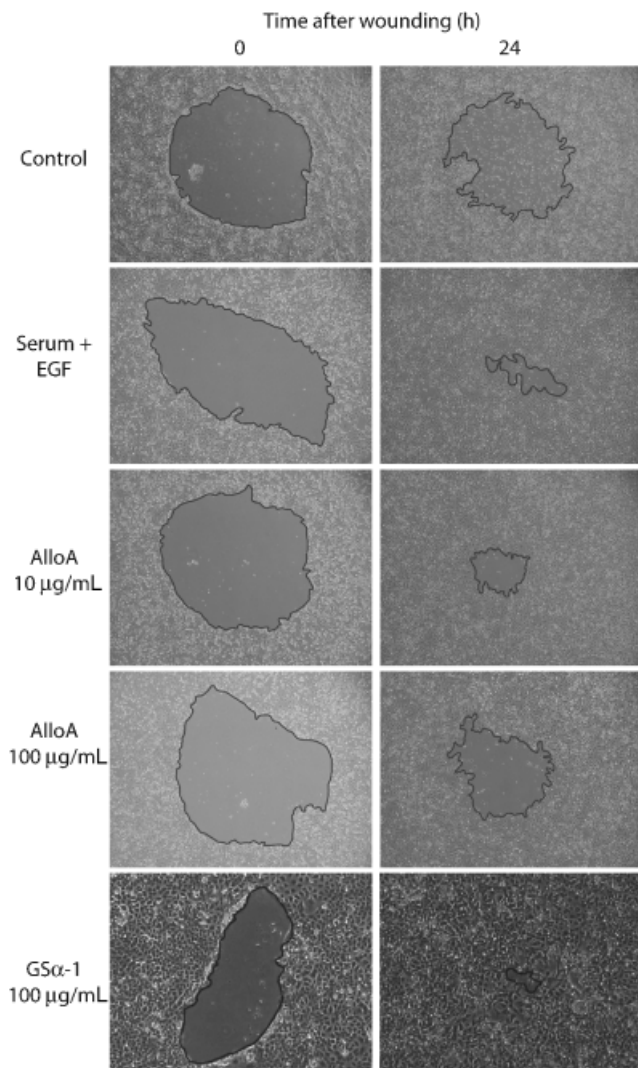


Fig. 2. Representative phase-contrast images of wounded human airway epithelial cells (1HAEo⁻) and wound closure inhibition caused by *Allomyrina dichotoma* agglutinin (AlloA) at the indicated times. Control (serum free), serum+epidermal growth factor (EGF) (10% FBS, 15 ng/mL EGF), 10 and 100 µg/mL AlloA and 100 µg/mL GSα-1 images are shown. The inhibition of repair after AlloA addition to wounded 1HAEo⁻ monolayers in culture is dose dependent. This effect is not shared with GSα-1, as its addition added to the culture media resulted in no wound closure inhibition. Manual traces are included for illustrative purposes. Original magnification $\times 40$.

wound areas for monolayers for both AlloA and GSα-1 treatment were equivalent and consistent. Treatment of several wounds each with varying amounts of the AlloA lectin, revealed inhibition of wound closure in a dose-dependant manner (Fig. 3a). Wounds grown in 10% FBS with 15 ng/mL EGF closed substantially over the 24 h period ($9.2 \pm 1.3\%$ initial wound area remaining) relative to unstimulated serum-free control wounds ($61.4 \pm 3.3\%$). After 24 h, wounds treated with 10 µg/mL AlloA had very little inhibition of wound closure ($14.7 \pm 3.0\%$). Increasing the dose of AlloA to 25 and 50 µg/mL resulted in

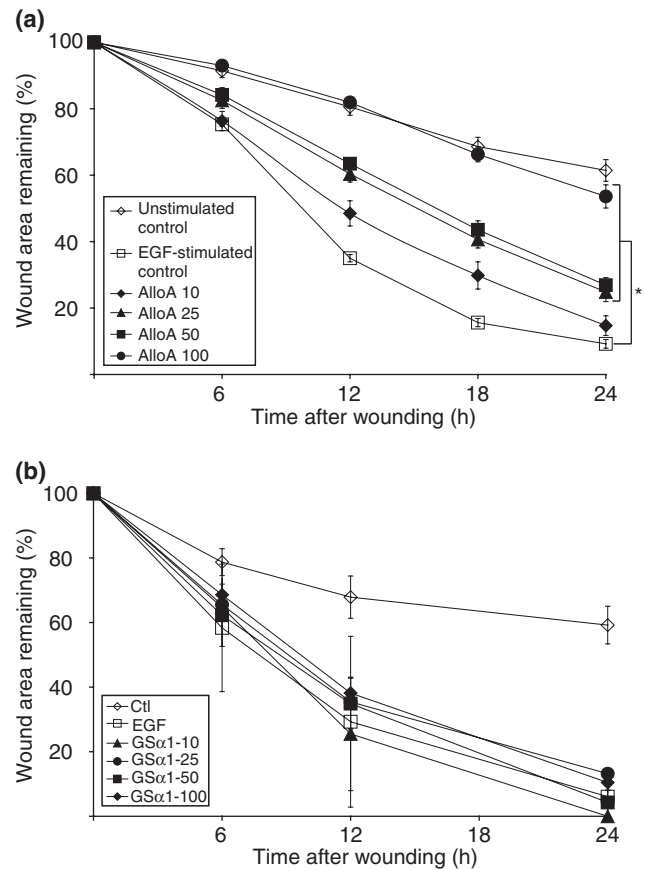


Fig. 3. Closure of 1HAEo⁻ wounds in the presence of lectins. (a) *Allomyrina dichotoma* agglutinin (AlloA) in increasing amounts (10–100 µg/mL) disrupted the kinetics of wound closure in a dose-dependent manner. Controls included negative unstimulated (Control) and stimulated (serum+ epidermal growth factor (EGF)) monolayers. After 24 h repair (a) a dose response is shown as a result of the AlloA treatments. A marked reduction in wound repair was demonstrated at the 100 µg/mL level. This effect was lost as the lectin concentration was reduced such that there is no statistical difference in wound closure of the 10 mg/mL AlloA relative to the serum+EGF-stimulated control. Treatment with (b) GSα-1 resulted in no wound closure inhibition over the same range of lectin doses. $N \geq 3$ for all experiments, $*P < 0.05$ relative to EGF-stimulated repair.

increased inhibition of wound closure ($24.8 \pm 2.9\%$ and $26.9 \pm 2.3\%$ respectively). The addition of AlloA at 100 µg/mL produced significant inhibition of serum-stimulated wound closure ($53.6 \pm 3.4\%$). There was no statistical difference between repair closure rates for the unstimulated serum-free monolayers and the 100 µg/mL AlloA-treated cell monolayers ($61.4 \pm 3.3\%$ vs. $53.6 \pm 3.4\%$). In a dose-dependant manner, the addition of AlloA inhibited stimulated wound closure to rates similar to the unstimulated control ($n \geq 4$ for all experiments).

The lectin GSα-1 was used as a control for the AlloA experiment to demonstrate the inhibition was lectin specific. Despite similar carbohydrate specificity, GSα-1 lectin does not bind basal cells of the intact epithelium

[18] nor does it bind the surface of 1HAEo⁻ cells during wound closure (Fig. 1). Using GS α -1 at the same doses as AlloA (10–100 μ g/mL), wound closure was followed for 24 h (Fig. 3b, $n \geq 3$ for all experiments). GS α -1 demonstrated no inhibition, at any time-point, of the serum-stimulated control wound closure. The closure rates for the independent controls of the AlloA and GS α -1 were similar. These findings suggest that AlloA is binding a carbohydrate on a structure critical in the effective repair of damaged airway epithelial cells.

Purification of AlloA-specific glycoproteins. Differences were observed in the protein profiles generated by lectin-specific precipitations from each of the cell lysates (Fig. 4). A protein band that was enriched in the AlloA precipitates of wounded monolayers relative to the confluent monolayers and absent from the GS α -1 lectin precipitation was selected for further analysis. The 62 kDa band was present only in the AlloA lectin precipitations (Fig. 4a) and absent from GS α -1 precipitations (Fig. 4b). By MALDI-TOF sequencing, this protein band was identified as bovine fetuin. As fetuin is a component of FBS, this bovine protein originated from the culture medium. Fetuin was present in our protein extracts despite a thorough washing of the monolayer before protein collection. Considering our AlloA lectin staining of 1HAEo⁻ monolayers and the precipitation of fetuin with AlloA suggested fetuin may

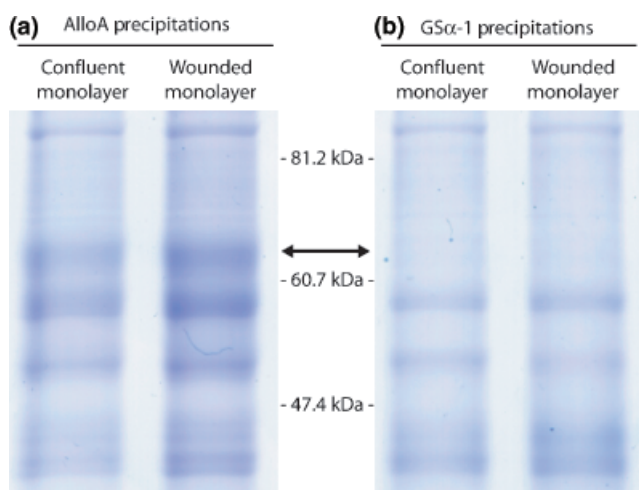


Fig. 4. Lectin precipitation profiles of human airway epithelial cells (1HAEo⁻) monolayers protein extracts. Protein extracts from confluent and wounded monolayers were incubated with biotinylated lectins (15 μ g/mL) and avidin agarose beads as described in 'Materials and methods'. Precipitated proteins were separated by 10% SDS-PAGE and the gel was stained with Coomassie R-250. Lectin controls were included in the gel to identify the lectin. Differences were observed between *Allomyrina dichotoma* agglutinin (AlloA) (a) and GS α -1 (b) precipitations. One protein band of 62 kDa was present only in the AlloA precipitations and appeared to be enriched in the wounded monolayer preparations. The 62 kDa AlloA-specific band (two-headed black arrow) was identified as bovine fetuin by protein sequencing.

bind a cell surface receptor whose presentation on the cell surface increases following mechanical injury. As AlloA specifically bound fetuin, increasing competition for binding by AlloA with the human cellular receptor for fetuin may be responsible for the observed dose-dependant AlloA inhibition of wound closure. Furthermore, the glycosylated structure is a likely and important component of this interaction. Bovine and human fetuin are both highly glycosylated and similar in structure. As such they potentially bind to the same human lectin or receptor.

Effect of serum glycoproteins on wound closure. Serum glycoproteins may participate in repair by augmenting closure rates. We tested fetuin in our model of monolayer wound repair (Fig. 5). Fetuin was identified as a protein that AlloA binds to inhibit wound repair. Mean initial wound area for the wounds used in this study was 1.27 ± 0.32 mm². Initial wound areas for monolayers within each experimental series were equivalent and consistent. Considering the percentage remaining of the initial wound area, control serum-free wounds closed slowly over a 24 h period ($65.1 \pm 3.3\%$), whereas wounds grown in 10% FBS closed such that the remaining wound was a fraction of its original size over the same 24 h period ($8.9 \pm 3.5\%$). The addition of fetuin (0.03–1.0 mg/mL) to the culture media-stimulated wound closure in a dose- and time-dependant manner relative to untreated control wounds (Fig. 5). Following 12 h of repair, the percentage of the initial wound area remaining relative to untreated control monolayers was significant only in those treated

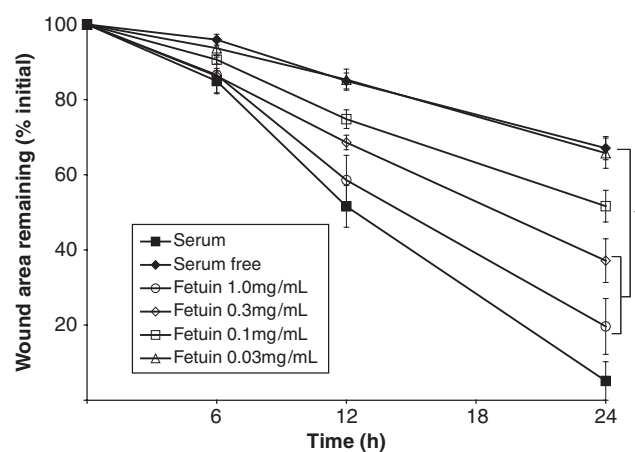


Fig. 5. Wound closure stimulation by serum glycoproteins. Cells were grown to confluence and serum starved for 24 h. Small circular wounds were created. Following wounding, the negative control was incubated in serum-free medium and the positive control was incubated in serum-containing medium (10% FBS). Treatment monolayers were wounded and incubated in serum-free media with the glycoprotein fetuin added. Wound closure was followed using time-lapse videomicroscopy. The serum glycoprotein fetuin stimulates wound closure of serum deprived human airway epithelial cells monolayers. $n = 4$ for all experiments, $P < 0.05$ relative to the unstimulated serum-free control.

with 1.0 mg/mL fetuin ($58.6 \pm 13.2\%$ vs. $79.1 \pm 3.1\%$ for control, $N=4$). Following 24 h of repair, closure of wounds treated with both 0.3 and 1.0 mg/mL was significantly simulated relative to the untreated serum-free control wounds ($37.1 \pm 4.7\%$ and $19.6 \pm 4.7\%$, respectively vs. $65.1 \pm 6.8\%$ for control). Despite an additional 10-fold increase in the concentration of fetuin (10 mg/mL), wound closure rates were still not equivalent to that effected by 10% FCS (data not shown). Fetuin alone may only be one of many required mediators to the initial events of repair.

Discussion

Asthma is a disease of the lower airways, described as reversible airflow obstruction because of inflammation and airways hyper-reactivity. For this reason, most therapeutic and research approaches have focused on the symptomatic control of the disease through anti-inflammatory steroids and bronchodilation. However, pathological studies of patients who died as a result of severe asthma had phenotypic changes in their airways including chronic damage to the airway epithelium [7] suggesting a defect in the mechanism of repair. The mediators of this repair remain unknown. Using a model of epithelial wound repair, we hoped to identify novel mediators that could be applied to future studies. In this study we have shown that the addition of fetuin, a serum glycoprotein, stimulated 1HAEo⁻ wounds in culture. Furthermore ASGPR, a serum protein receptor and human lectin is expressed by airway epithelial cells in our model of wound repair (data not shown). The data suggests that serum glycoproteins play a role in the stimulation of airway epithelial wound repair. Glycoproteins appear to be an important component in this wound repair model, and may act through a galactose binding serum protein receptor such as the ASGPR. The role for serum glycoproteins and human lectins in disease is a growing field that requires further study.

Our wound repair model results expand on work done previously that revealed the importance of carbohydrates in airway epithelial repair as well the presence of serum glycoproteins in the airways as a result of damage to the epithelium. Work by White et al. [31] has shown that lectins can be added to the culture media to competitively inhibit the receptor–ligand interaction ultimately inhibiting cellular functions. The authors in this study demonstrated that the addition of WGA following mechanical injury competitively inhibits α -dystroglycan binding of laminin. WGA is a lectin that binds *N*-acetylglucosamine containing oligosaccharides. Conversely, AlloA is a C-type lectin that binds terminal galactose containing oligosaccharides [32] and would therefore associate with a unique group of ligands compared with WGA. In our repair model, the addition of AlloA resulted in the inhibition of wound closure, despite

stimulation with EGF and the presence of serum in the culture media. These findings suggest that AlloA effects inhibition of wound closure by two possible mechanisms. AlloA may bind a membrane glycoprotein such as a receptor, inhibiting its critical function. Conversely AlloA in the culture media is binding essential glycoproteins found within the media, blocking the processes of cell surface binding required for the downstream signalling and cellular activation. AlloA has previously been identified as a mitogenic factor of mature T cells [33]. These findings were cell type specific, as the same effect was not seen in B cells and may explain the differences in effect that we observed in airway epithelial cells.

The glycoprotein bound by AlloA was identified by lectin precipitations and protein sequencing as fetuin. Fetuin was present in the AlloA precipitations and absent in the GS α -1 precipitations. As the media was completely removed and the cells were washed before protein extraction, the presence of fetuin in the protein extracts, and subsequently the AlloA precipitations, can only be explained if the fetuin is bound by a cell surface receptor on the airway epithelial cells. The binding of fetuin to the cell surface is supported by our AlloA lectin histochemistry results where the AlloA-positive staining on the cell surface is attributed to bound fetuin.

The presence of serum glycoproteins in the airway was first observed in the work of Erjefalt et al. [22]. Almost immediately after the epithelium is damaged, cells in the wound margin phenotypically change to basal-like cells and flatten out to restore the physical barrier lost in the shedding of epithelial columnar cells [8, 34]. Following mechanical injury to guinea-pig trachea, plasma from the microcirculation exudes into the region of damage and provides an immediate cover of the denuded basement membrane [22]. In our culture model, bovine fetuin present in the media is responsible for the initial stimulation of closure. Recently in human lungs, proteomic analysis of oedema fluid and BAL fluid from acute lung injury (ALI) patients had an increase in the α_2 -Heremans-Schmid-glycoprotein (human fetuin) [35]. The presence of human fetuin in the luminal space of the lung following injury corresponds with our findings. Damage to the airway epithelium would facilitate the binding of serum glycoproteins to their cellular receptor and signal that migration and repair are needed.

The addition of fetuin to the culture media resulted in a stimulation of wound closure in our model. Over the full range of fetuin treatments (0.03–1.0 mg/mL), there was a dose-dependent increase in wound closure relative to the unstimulated control (Fig. 5). Interestingly, with a 10-fold increase in fetuin (10.0 mg/mL) wound repair was not fully restored to the level of serum-stimulated closure (data not shown). We suggest that fetuin plays a role in the complex repair processes, however, it is likely one of several serum factors necessary for repair. The addition of fetuin alone

initiates closure in conjunction with physical barrier re-establishment. Subsequent factors present in the plasma that exudes into the airway would be required for complete repair. In the presence of serum, wound closure continues to progress at a rate much greater than that in the presence of fetuin alone. Its addition alone, in the absence of the additional factors present in serum, is insufficient to restore epithelial repair completely. As a result, dramatically increasing the fetuin concentration had no cumulative effect on wound closure stimulation.

Fetuin is a highly glycosylated protein whose function is not well understood. It has been implicated in the deactivation of macrophages through binding to its only known receptor, the ASGPR [36]. The ASGPR is a C-type lectin that is similar to AlloA, binds galactose-containing glycoproteins. ASGPR has primarily been studied in the liver and is responsible for the clearance and endocytosis of serum glycoproteins [37]. It has been detected in non-hepatic tissues, however lung was not included [38]. The ASGPR is expressed in our *in vitro* model of airway epithelial repair and may be one of the potential fetuin receptors responsible for the fetuin stimulation of wound closure. Future studies should also consider the expression and function of ASGPR, other serum protein receptors and human lectins in normal and asthmatic tissue.

Complex interactions and signalling pathways are responsible for the level of organization required to perform repair. In the epithelium, this involves cell migration, proliferation and differentiation. Holgate et al. [39] has suggested that the asthmatic epithelium is locked in a chronically repairing phenotype acting as a continuous source of pro-inflammatory products and growth factors that drive airway remodelling. We have highlighted the importance of serum glycoproteins in the processes of epithelial repair. In other inflammatory conditions, the released cytokines are known to affect the glycosylation phenotype [40]. If the inflammatory environment of the asthmatic airway alters glycosylation such that the receptor interaction with serum proteins is affected, this may result in the accumulation of epithelial damage as the initiation of repair would be impaired. Cell surface glycosylation in asthmatics has been demonstrated as abnormal [41]. Our results add to the previous findings that serum glycoproteins provide not only a protective barrier to sites of injury on the airway epithelium, but also actively stimulate airway epithelial repair. This study also demonstrates that glycosylated structures are an important part of this repair process and may provide new insight for research and therapeutic approaches in diseases where the presence of damage to the epithelium is a common observation.

Acknowledgements

This work was supported by Canadian Institutes of Health Research (CIHR Grant 20R90480). The authors gratefully

thank Dr Gurpreet K. Singhera, Kimberly R. Wojcik, and Dr Steven R. White for their technical assistance and Gillian Kent for editorial assistance.

References

- 1 Fedan JS, Yuan LX, Chang VC, Viola JO, Cutler D, Pettit LL. Osmotic regulation of airway reactivity by epithelium. *J Pharmacol Exp Ther* 1999; 289:901–10.
- 2 Wanner A. Mucociliary clearance in the trachea. *Clin Chest Med* 1986; 7:247–58.
- 3 Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 1985; 131:599–606.
- 4 Zahm JM, Kaplan H, Herard AL et al. Cell migration and proliferation during the *in vitro* wound repair of the respiratory epithelium. *Cell Motil Cytoskeleton* 1997; 37:33–43.
- 5 Oddera S, Silvestri M, Balbo A et al. Airway eosinophilic inflammation, epithelial damage, and bronchial hyperresponsiveness in patients with mild–moderate, stable asthma. *Allergy* 1996; 51:100–7.
- 6 Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137:62–9.
- 7 Beasley R, Roche WR, Roberts JA, Holgate ST. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989; 139:806–17.
- 8 Erjefalt JS, Erjefalt I, Sundler F, Persson CG. *In vivo* restitution of airway epithelium. *Cell Tissue Res* 1995; 281:305–16.
- 9 Zahm JM, Pierrot D, Chevillard M, Puchelle E. Dynamics of cell movement during the wound repair of human surface respiratory epithelium. *Biorheology* 1992; 29:459–65.
- 10 Puddicombe SM, Polosa R, Richter A et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000; 14:1362–74.
- 11 Kim JS, McKinnis VS, Nawrocki A, White SR. Stimulation of migration and wound repair of guinea-pig airway epithelial cells in response to epidermal growth factor. *Am J Respir Cell Mol Biol* 1998; 18:66–74.
- 12 White SR, Dorscheid DR, Rabe KF, Wojcik KR, Hamann KJ. Role of very late adhesion integrins in mediating repair of human airway epithelial cell monolayers after mechanical injury. *Am J Respir Cell Mol Biol* 1999; 20:787–96.
- 13 Takada A, Ohmori K, Yoneda T et al. Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Res* 1993; 53:354–61.
- 14 Yoshida-Noro C, Heasman J, Goldstone K, Vickers L, Wylie C. Expression of the Lewis group carbohydrate antigens during *Xenopus* development. *Glycobiology* 1999; 9:1323–30.
- 15 Martinez JA, Torres-Negron I, Amigo LA, Banerjee DK. Expression of Glc3Man9GlcNAc2-PP-Dol is a prerequisite for capillary endothelial cell proliferation. *Cell Mol Biol (Noisy-le-grand)* 1999; 45:137–52.
- 16 Pili R, Chang J, Partis RA, Mueller RA, Chrest FJ, Passaniti A. The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. *Cancer Res* 1995; 55:2920–6.

- 17 Gipson IK, Riddle CV, Kiorpes TC, Spurr SJ. Lectin binding to cell surfaces: comparisons between normal and migrating corneal epithelium. *Dev Biol* 1983; **96**:337–45.
- 18 Dorscheid DR, Conforti AE, Hamann KJ, Rabe KF, White SR. Characterization of cell surface lectin-binding patterns of human airway epithelium. *Histochem J* 1999; **31**:145–51.
- 19 Xiantang L, Dorscheid DR, Wojcik KR, White SR. Glycosylation profiles of airway epithelium after repair of mechanical injury in guinea pigs. *Histochem J* 2000; **32**:207–16.
- 20 Dorscheid DR, Wojcik KR, Yule K, White SR. Role of cell surface glycosylation in mediating repair of human airway epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2001; **281**:L982–92.
- 21 Adam E, Holgate S, Fildew C, Lackie P. Role of carbohydrates in repair of human respiratory epithelium using an in vitro model. *Clin Exp Allergy* 2003; **33**:1398–404.
- 22 Erjefalt JS, Erjefalt I, Sundler F, Persson CG. Microcirculation-derived factors in airway epithelial repair in vivo. *Microvasc Res* 1994; **48**:161–78.
- 23 Persson CG, Erjefalt JS, Greiff L *et al*. Plasma-derived proteins in airway defence, disease and repair of epithelial injury. *Eur Respir J* 1998; **11**:958–70.
- 24 Grainger DJ, Kemp PR, Metcalfe JC *et al*. The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis. *Nat Med* 1995; **1**:74–9.
- 25 Zahm JM, Chevillard M, Puchelle E. Wound repair of human surface respiratory epithelium. *Am J Respir Cell Mol Biol* 1991; **5**:242–8.
- 26 Cozens AL, Yezzi MJ, Kunzelmann K *et al*. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994; **10**:38–47.
- 27 Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol* 1995; **268** (Part 1):L347–60.
- 28 Cozens AL, Yezzi MJ, Yamaya M *et al*. A transformed human epithelial cell line that retains tight junctions post crisis. *In Vitro Cell Dev Biol* 1992; **28A**:735–44.
- 29 Kim JS, Rabe KF, Magnussen H, Green JM, White SR. Migration and proliferation of guinea pig and human airway epithelial cells in response to tachykinins. *Am J Physiol* 1995; **269** (Part 1):L119–26.
- 30 Kim JS, McKinnis VS, White SR. Migration of guinea pig airway epithelial cells in response to bombesin analogues. *Am J Respir Cell Mol Biol* 1997; **16**:259–66.
- 31 White SR, Wojcik KR, Gruenert D, Sun S, Dorscheid DR. Airway epithelial cell wound repair mediated by alpha-dystroglycan. *Am J Respir Cell Mol Biol* 2001; **24**:179–86.
- 32 Sueyoshi S, Yamamoto K, Osawa T. Carbohydrate binding specificity of a beetle (*Allomyrina dichotoma*) lectin. *J Biochem (Tokyo)* 1988; **103**:894–9.
- 33 Kimura S, Umetsu K, Yamashita T, Suzuki T, Arai S, Sendo F. T cell mitogenicity of a novel beta-D-galactoside-specific lectin from the beetle, *Allomyrina dichotoma* (allo A). *Immunopharmacology* 1987; **13**:181–8.
- 34 Erjefalt JS, Sundler F, Persson CG. Epithelial barrier formation by airway basal cells. *Thorax* 1997; **52**:213–7.
- 35 Bowler RP, Duda B, Chan ED *et al*. Proteomic analysis of pulmonary edema fluid and plasma in patients with acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**:L1095–104.
- 36 Wang H, Zhang M, Bianchi M, Sherry B, Sama A, Tracey KJ. Fetuin (alpha2-HS-glycoprotein) opsonizes cationic macrophage-deactivating molecules. *Proc Natl Acad Sci USA* 1998; **95**:14429–34.
- 37 Stockert RJ. The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol Rev* 1995; **75**:591–609.
- 38 Park JH, Cho EW, Shin SY, Lee YJ, Kim KL. Detection of the asialoglycoprotein receptor on cell lines of extrahepatic origin. *Biochem Biophys Res Commun* 1998; **244**:304–11.
- 39 Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF. The bronchial epithelium as a key regulator of airway inflammation and remodelling in asthma. *Clin Exp Allergy* 1999; **29** (Suppl. 2):90–5.
- 40 Yang X, Lehotay M, Anastassiades T, Harrison M, Brockhausen I. The effect of TNF-alpha on glycosylation pathways in bovine synoviocytes. *Biochem Cell Biol* 2004; **82**:559–68.
- 41 Kauffmann F, Frette C, Pham QT, Nafissi S, Bertrand JP, Oriol R. Associations of blood group-related antigens to FEV1, wheezing, and asthma. *Am J Respir Crit Care Med* 1996; **153**:76–82.