

Role of carbohydrates in repair of human respiratory epithelium using an *in vitro* model

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Summary

Background The epithelial layer in the conducting airway provides a primary protective barrier. Repair of this barrier normally occurs rapidly after damage, but is compromised in diseases such as asthma.

Objective We have developed a human *in vitro* model system to test our hypothesis that cell surface glycoconjugate-based interactions are required for the normal repair of damaged epithelium.

Methods Lectins having narrow carbohydrate specificities were used to identify and block specific carbohydrate moieties on human airway-derived epithelial cells in culture.

Results The lectin wheat germ agglutinin bound to *N*-acetyl glucosamine and inhibited the repair of epithelial damage while having little effect on cell viability. In contrast, other *N*-acetyl glucosamine binding lectins had no effect even when bound to the cell surface. The involvement of glycoconjugates was confirmed by pre-incubating the lectin with its specific sugar, preventing the inhibition of repair.

Conclusion These results indicate that lectin-binding sites are involved in epithelial repair and may be important in the repetitive cycles of injury and repair seen in asthma. This model system provides an insight into the role of glycoconjugates and will help to determine the function of specific carbohydrate groups in epithelial repair. These may present a target for therapeutic intervention in respiratory and other diseases.

Key words carbohydrate, cell culture, lectins, respiratory epithelia

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Introduction

The conducting region of the human respiratory system is lined by a primary protective barrier of epithelial cells at the interface between the underlying tissues and the environment. The architecture of this protective layer is maintained by tightly regulated cell–cell and cell–matrix adhesion [1]. When airway epithelium is damaged, the remaining basal airway epithelial cells respond by migrating to repopulate any damaged areas, subsequently proliferating and differentiating until epithelial integrity has been restored [2]. In certain disease conditions including asthma, a significant proportion of columnar epithelial cells are shed as Creola bodies [3], leaving a poorly differentiated ‘activated’ epithelium consisting largely of cells with a basal phenotype. This altered epithelium may allow a greater penetration of environmental agents including toxins, allergens and microorganisms, resulting in further damage and potentially initiating a cycle of prolonged and enhanced inflammatory responses [4]. As it is difficult to study epithelial repair processes *in vivo*, a number of studies have relied upon respiratory epithelial cell

cultures. Zahm et al. [5] have shown that to cover the area of damage of a mechanical wound, airway-derived epithelial cells closer to the wound migrated faster than those cells further away from the wound, and this continued until the wound had sealed over.

While neither the processes governing epithelial repair nor the changes in disease are fully understood, many adhesion molecules and cell surface receptors involved in defining and regulating these processes are glycoproteins. Other glycoconjugates, including glycolipids and glycosaminoglycans, also have an important influence on cell interactions [6].

More recent work has demonstrated the importance of protein–carbohydrate interaction on the surface of cells. Pace et al. [7] have established that the human lectin galectin-I induces apoptosis in human T cells by binding to a specific set of cell surface glycoproteins. They discovered that after galectin-I binding, the cells showed a dramatic redistribution of surface glycoproteins into microdomains, but this only occurred in those cells that subsequently died. The authors suggested that spatial redistribution of receptors is a necessary prerequisite for apoptosis. Subsequently, it has been shown that binding and cross-linking of lectins with glycoconjugates results in reorganization and aggregation of cell surface receptors, which have the effect of separating different receptor activities from each other [8].

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Lectins are naturally occurring glycoconjugate-binding molecules that can be isolated from a wide variety of plants and animals, and provide exquisitely selective tools to identify or block specific glycoconjugate motifs. While many lectins bind to a range of sugar structures, some are selective for particular sugars in particular linkages [9]. The lectin from the bacterium *Pseudomonas aeruginosa* (PAII) has been shown to bind to fucose residues on respiratory epithelium, resulting in the cessation of ciliary beating [10]. It is suggested that binding through this lectin could be a mechanism of attachment for the bacteria, which can then proliferate. The lectin from *Triticum vulgaris* (wheat germ agglutinin (WGA)) also bound to the apical surfaces of airway ciliated cells and again completely inhibited ciliary beating [11]. Although this lectin would not normally be encountered in the airways, it demonstrates the importance of blocking carbohydrate residues on physiological processes. Another lectin from *Agaricus bisporus* (ABA) reversibly inhibited epithelial cell proliferation without cytotoxicity, by binding to Gal β -1,3-GalNAc and also the sialylated form. In contrast, the lectin from *Arachis hypogaea* (PNA), which binds to the non-sialylated form of Gal β -1,3-GalNAc only, stimulated the proliferation of colonic epithelium, indicating that cell surface glycoproteins have diverse roles in regulating proliferation [12]. Using a lectin isolated from *Dolichos biflorus* (DBA), Sweatt et al. [13] demonstrated an increase of *N*-acetylgalactosamine in cell surface glycoconjugates at the site of wounding in pig corneas, postulating that this might influence the process of wound healing. Li et al. [14] have recently demonstrated that increased lectin binding can be detected in guinea-pig tracheal epithelial cells after damage, denoting an altered glycoconjugate profile as a result of damage, subsequent repair, or both.

Taken together, these studies indicate that cell surface carbohydrates are centrally involved in regulating epithelial repair processes. Here we describe the effect on repair of blocking specific carbohydrate-based interactions using a model system, which allows us to dissect out the role of specific cell surface carbohydrates in the repair of mechanical damage to a human airway epithelial layer using different lectins. We demonstrate that some lectins binding to the cell can affect the rate of repair without altering cell viability, while others bind to the cell but do not affect the repair process. These observations have important implications for not only understanding the epithelial injury–repair cycle but also identifying targets for novel drug development in such diseases as asthma.

Materials and methods

Tissue culture

The lung carcinoma epithelial cell line NCI-H292 (ECACC, Salisbury, UK) [15] was maintained in RPMI 1640 medium (Life Technologies Ltd., Paisley, UK) with 10% fetal calf serum (FCS), 1 mM glutamine and 1% of a penicillin/streptomycin solution (10 000 U/mL and 10 000 μ g/mL, respectively) at 37 °C in a 5% CO₂ environment. For assessment of damage repair the cells were grown in uncoated 35 mm diameter Petri dishes with 2 mm square grids

embossed on the bottom surfaces (Life Technologies Ltd., Paisley, UK) at an initial density of 1.0×10^6 cells per dish. After 3 days, when the cells were fully confluent, they were damaged mechanically by scoring with a pipette tip. Cultures were examined on an Olympus IMT2 inverted microscope (Olympus Optical Co. (UK) Ltd., Middlesex, UK), and photographs of identifiable squares of the damaged areas were taken at time 0 and after 24 and 48 h using Kodak Technical Pan film (Kodak Ltd., Hemel Hempstead, Herts, UK) (Figs 1a and b). Each experiment was repeated at least

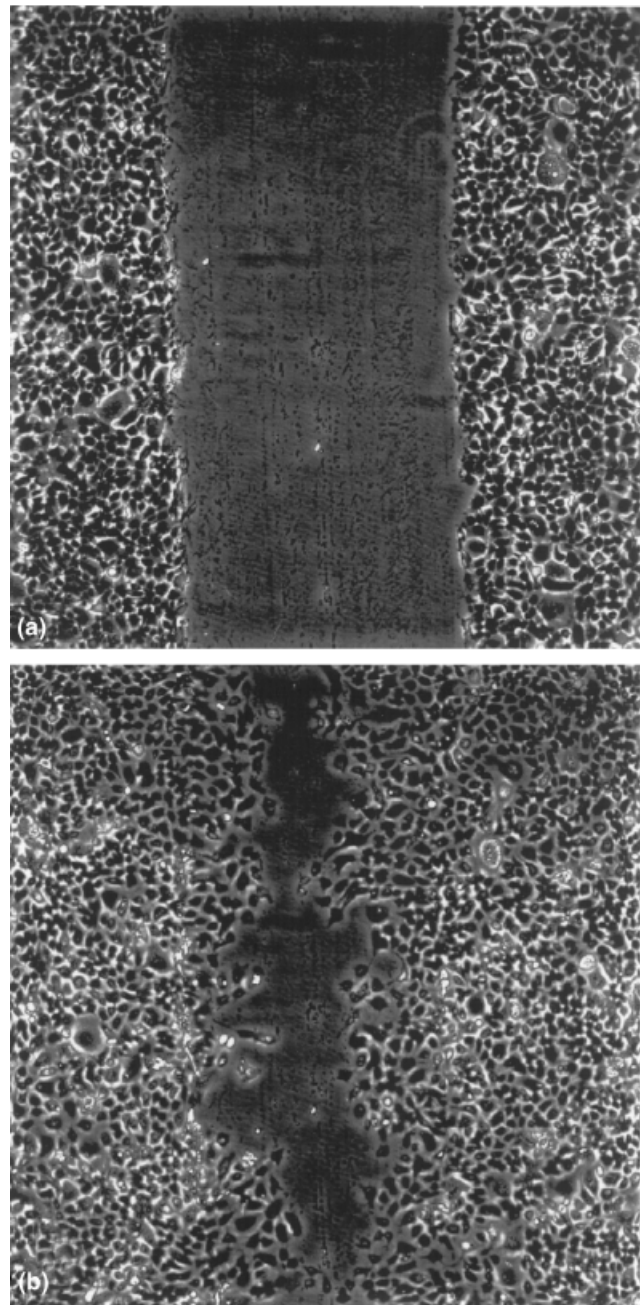


Fig. 1. Epithelial cells repair after experimental damage. H292 cells were grown to confluence in RPMI 1640 medium and mechanically damaged by scoring with a pipette tip. An area of cultured cells immediately after mechanical damage (a) can then be compared to the same area 24 h later (b) showing substantial movement of the cells to repair damage. Magnification $\times 200$.

Table 1. Lectins used together with their inhibitory carbohydrates

Lectin	Abbreviation	Common name	Inhibitory carbohydrate with preference ()
<i>Triticum vulgare</i>	WGA	Wheat germ	<i>N</i> -Acetyl glucosamine (<i>N</i> -acetylchitotriose)
<i>Phytolacca americana</i>	PWM	Pokeweed	<i>N</i> -Acetyl glucosamine (variable with iso-lectins)
<i>Lycopersicon esculentum</i>	LEA	Tomato	<i>N</i> -Acetyl glucosamine (3+ α -(1,4)-linked GlcNAc repeats)

three times. Additions of propidium iodide to the cultures (5 µg/mL) were used to determine cell viability, counting the number of fluorescent (non-viable) nuclei after 1 h, and these results were used to calculate the percentage of viable cells. Viability was also assessed using an MTS assay. A single cell suspension was prepared using a non-enzymatic cell dissociation solution (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) for 30 min. The cells were washed in medium devoid of FCS and the lectin from *Triticum vulgare* (WGA) (Sigma-Aldrich Co. Ltd.) was added at a concentration of 5, 10, 15 or 20 µg/mL of medium. Known concentrations of cells containing WGA were then aliquoted into a 96-well plate and incubated for 24 h at 37 °C in 5% CO₂. After this time the viability was determined using a non-radioactive cell proliferation assay kit (Promega, Southampton, UK). The assay utilizes a tetrazolium compound (MTS), which is bio-reduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan can then be measured. After addition of the MTS and a further 2 h incubation, the optical density of the wells in the plate was measured at a wavelength of 492 nm on a Multiskan Ascent plate reader (Thermo Labsystems, Altrincham, UK).

Lectin addition

All experimental cultures had the medium changed to medium without FCS immediately after damage and prior to lectin addition. WGA was used as it was known to bind to the surfaces of respiratory epithelium and to alter ciliary beating [11]. Initially, the lectin WGA (Sigma-Aldrich Co. Ltd.) was added to the cultures at a range of concentrations (5, 10, 15 and 20 µg/mL) in order to determine the optimal concentration for lectin addition taking into account cytotoxicity. Subsequently, all lectins were added at a final concentration of 20 µg/mL, which proved to be the optimum lectin concentration where damage repair was inhibited and the viability of the cells remained above 90%. *Lycopersicon esculentum* (LEA) lectin (Vector Laboratories, Peterborough, UK), which was known to bind to the cell membranes, and pokeweed mitogen (PWM) (lectin) (Sigma-Aldrich Co. Ltd.), which did not bind to the cell membranes, were also used. Details of the carbohydrate specificities of these lectins are given in Table 1. The PWM used in this study consisted of a minimum of five isolectins. Media in control cultures were changed at the same time points as the treated cultures, but received no lectin additions to the medium. Lectin specificity control experiments were undertaken by pre-incubating the lectins with specific sugars (0.3 M) (Sigma-Aldrich Co. Ltd.) for 30 min prior to addition to the culture medium. The final concentration of the sugar after addition to the culture medium was 12 mM. Additions of the appropriate sugar alone were also made to culture medium.

Lectin cytochemistry

To determine whether the lectins bound to the surfaces of the cells, lectins directly conjugated to fluorescein-isothiocyanate (FITC) (Vector Laboratories) at a concentration of 5 µg/mL were added to cultures of cells grown on glass coverslips for 30 min at room temperature. After rinsing the cells in phosphate-buffered saline (PBS) at pH 7.4, the coverslips were mounted in a Mowiol Citifluor solution. Binding was assessed by viewing the cells with an Olympus BH2 fluorescence microscope using a 488 nm excitation filter and a 540 nm emission filter. Binding specificity was ascertained by pre-incubating the lectins for 30 min with their specific sugars prior to incubation with the cells as described above. Photographs were taken using an Olympus PM10 photomicrography unit with Kodak TMax 400 film.

Estimation of growth

The images were digitized from the negatives (taken as described above) using a video camera connected to an image analyser (Imaging Associates Ltd., Thame, UK). The image analyser was then used to calculate the areas of initial damage in the cultures by drawing round the damage. In subsequent measurements the remaining areas of damage were calculated in a similar manner. Subtraction of the area of damage remaining after 24 and 48 h from the original area of damage at time 0 allowed the area covered by cells as a result of wound repair to be derived. The initial length of the wound for each measurement was always the same relating to the squares marked on the Petri dish (any at diagonals to the marked grid were rejected). As the width of the damage varied somewhat, the calculation of the area of regrowth was the most accurate way of expressing the results. Each experiment was repeated a minimum of three times. Statistical comparisons were made using the Student's *t*-test.

Results

Lectin addition

WGA When varying concentrations of the lectin WGA were added to the culture medium of confluent H292 cells after damage, a clear dose response was demonstrated. Figure 2 shows a typical response after 24 h incubation with WGA. The control culture and concentrations of WGA at 5 µg/mL and less showed a high level of damage recovery within 24 h. WGA additions at the higher level of 10 µg/mL showed a marked reduction in recovery in comparison to the control, and WGA additions at 20 µg/mL showed only a very low level of repair when compared with the control.

Measurements of wound repair were taken after both 24 and 48 h in the initial experiments described above; however

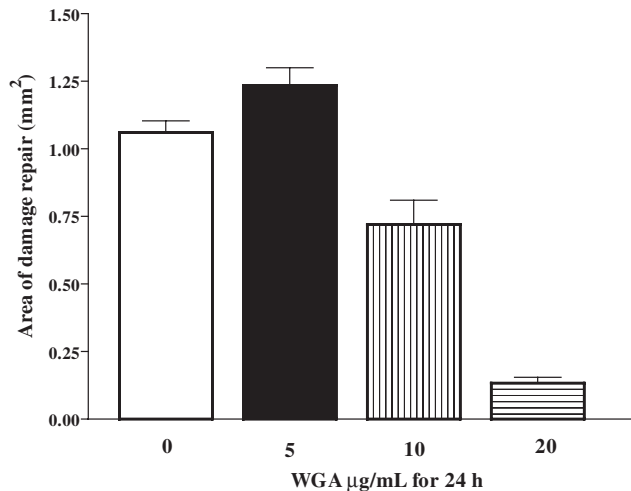


Fig. 2. Dose–response of H292 monolayer cultures to addition of the lectin WGA to the culture medium. H292 cells were grown to confluence and mechanically damaged by scoring with a pipette tip. WGA additions at varying concentrations were made to the culture medium and the areas of remaining damage were measured after 24 h. From these measurements, the area of damage repair was calculated. A marked reduction in damage repair was demonstrated at the 20 µg/mL level, which gradually decreased with reduced concentration of lectin. The results are the mean $\pm$ SEM for three experiments.

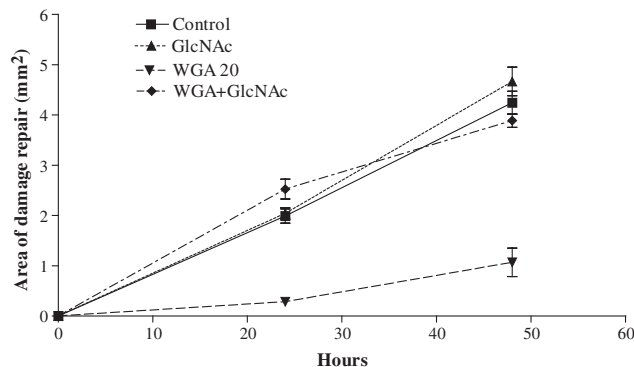


Fig. 3. Significant decrease in damage repair with addition of the lectin WGA. The cells were grown to confluence, mechanically damaged and either WGA alone, WGA pre-incubated with GlcNAc, GlcNAc alone or no addition was made, and the areas of damage repair determined after 24 and 48 h. The cultures of damaged H292 cells with WGA addition demonstrated a significant decrease in damage repair when compared with a control culture (no additions to the culture medium), addition of WGA pre-incubated with *N*-acetylglucosamine (GlcNAc) or addition of GlcNAc only. The results are the mean $\pm$ SEM for five experiments.

in all of the experiments the 48 h results were always consistent with the 24 h results and either showed complete recovery or a proportionate increase in repair.

When the lectin WGA was pre-incubated with its specific sugar GlcNAc and then added to the culture medium of damaged H292 cells, wound repair was no longer inhibited as with WGA alone and the culture repaired normally in a manner similar to the control culture and the culture with GlcNAc-only addition (Fig. 3).

Unpaired *t*-tests revealed a significant difference between the control cultures and the cultures with WGA additions ($P = 0.03$) and also compared with the cultures where WGA had been pre-incubated with GlcNAc prior to addition ($P = 0.008$). There was no significant difference between the control cultures, those cultures where the lectin had been pre-

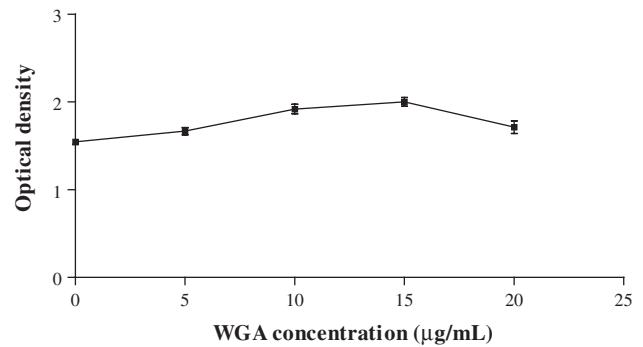


Fig. 4. A cell proliferation assay demonstrated no change in viability of H292 cells after incubation with WGA lectin for 24 h. An MTS cell proliferation assay was performed and the OD of the resulting medium measured at 492 nm. No significant difference was seen between those cultures with WGA addition and the control.

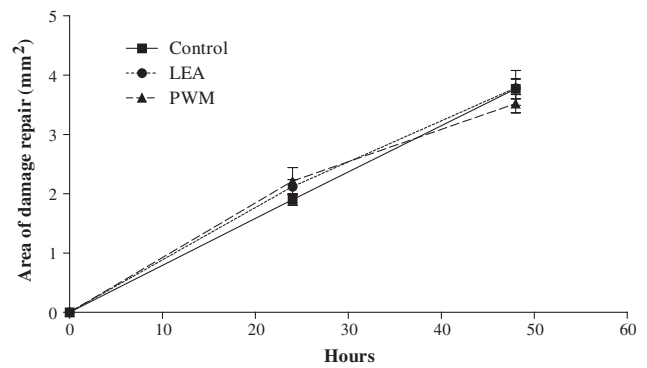


Fig. 5. Lectins with similar carbohydrate specificities to WGA showed no effect on the damage repair of H292 monolayer cultures after damage. H292 cells were grown to confluence, mechanically damaged and LEA, PWM or no lectin addition was made. The areas of remaining damage were measured after 24 and 48 h, and the areas of repair were calculated. There was little difference between the control, LEA addition and PWM addition. The results are the mean $\pm$ SEM for four experiments.

incubated with GlcNAc prior to addition and the cultures that had received additions of GlcNAc alone. The propidium iodide viability studies showed that all cultures were at least 95% viable after 24 h and at least 90% viable after 48 h. The proliferation assay after 24 h incubation with WGA showed no significant difference to the control culture (Fig. 4).

Other lectin additions Two other lectins, LEA and PWM, with similar carbohydrate specificities to WGA (GlcNAc) (for details, see Table 1) were added to the cultures, but no differences were observed in damage recovery when compared with the control (Fig. 5). In all cases, extensive recovery was achieved within 48 h. Unpaired *t* tests showed no significant difference between these cultures.

Figure 6 demonstrates the effects of adding WGA at 20 µg/mL to a damaged culture, followed by additions of the inhibitory sugar at varying time intervals. The damage repair achieved after 24 h is shown. Those cultures where GlcNAc was added 1, 2, 3 and 5 h after WGA addition demonstrated no significant differences to the control culture, although a slight downward trend in repair was observed. Addition of GlcNAc after 7 h showed an approximate repair rate of 66% of that achieved in the control. In all cases, complete repair was achieved eventually.

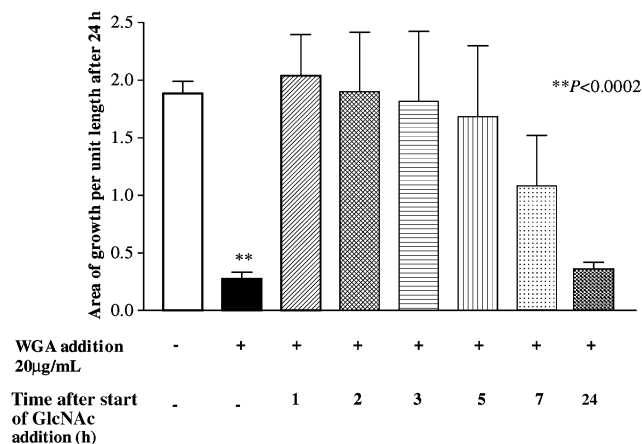


Fig. 6. The inhibition of damage repair after WGA addition to H292 cells recovered after GlcNAc addition. Cells were grown to confluence, mechanically damaged and WGA additions (20 µg/mL) were made to all except the control at time 0. Subsequent GlcNAc additions were made at different time intervals and the areas of remaining damage were measured after 24 h. After calculation of the areas of repair, the results showed that the inhibition was reversible if GlcNAc was added to the culture medium subsequent to the WGA addition. The results are the mean $\pm$ SEM for three experiments.

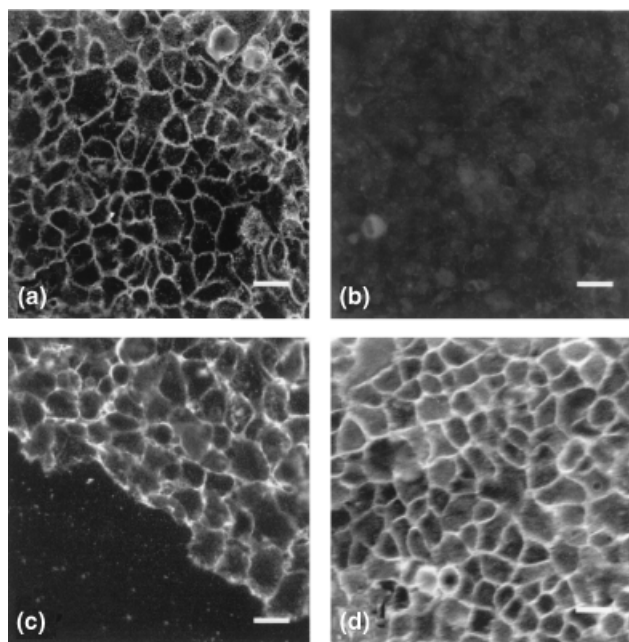


Fig. 7. WGA binds to the cell membranes of H292 monolayer cultures. H292 cells are grown to confluence and incubated with a WGA-FITC conjugate (a) and the pattern of fluorescent staining demonstrated that the WGA had bound to the surfaces of the cells. A monolayer of H292 cells incubated with a WGA-FITC conjugate pre-incubated with GlcNAc showed no visible membrane labelling (b). An H292 monolayer incubated with WGA-FITC after mechanical damage and 24 h culture (c) showed similar results to undamaged cells (a). An H292 monolayer incubated with an LEA-FITC conjugate showed a pattern of fluorescent staining similar to that seen after incubation with WGA-FITC (a). Bars = 25 µm.

Lectin binding Binding of the lectin WGA to H292 cell membranes was clearly demonstrated after the cells were incubated with WGA-FITC (Fig. 7a). This occurred at all the time points throughout the culture. This binding was not observed if the lectin was pre-incubated with GlcNAc before

application to the cells (Fig. 7b). Following damage to the H292 cells and addition of WGA-FITC after 24 h, the cultures showed a similar pattern of fluorescent staining (Fig. 7c) to undamaged cultures (Fig. 7a). The lectin LEA also bound predominantly to the surfaces of the cells (Fig. 7d), whereas the lectin PWM demonstrated no binding to the cells (indistinguishable from Fig. 7b, not shown).

Discussion

In this study we have shown that the binding of the lectin WGA to GlcNAc residues on the surface of H292 cells significantly retards the normal repair processes in this respiratory epithelial cell line. We have also demonstrated that this behaviour is specific to the WGA lectin and does not occur with two other GlcNAc-specific lectins (LEA and PWM). Furthermore, we have also shown that this effect is reversible for up to 7 h, providing additional confirmation that the inhibitory effect of WGA was brought about by cell surface binding. Taken together, these data provide strong evidence that carbohydrates are involved in the repair of damaged epithelium via surface interactions and that our experimental model provides a good method of probing carbohydrate moieties on the surfaces of cells to establish their role in fundamental processes such as damage repair.

These results are in agreement with work published by White et al. [16], where the authors demonstrated that addition of the lectin WGA to repairing cultures of a respiratory epithelial cell line resulted in inhibition of wound repair. However while their results were obtained from cultures treated with EGF which stimulated wound repair, our results reflect changes in baseline repair. We have shown that the addition of WGA at 20 µg/mL to the cultures resulted in a repair rate reduced to only 20% of that achieved by the control. In contrast, using WGA additions at a higher concentration of 30 µg/mL, in the presence of EGF, White et al. [16] showed a reduction in repair to about 75% of that achieved by the control using the 1HAEo⁻ cell line. White et al. [16] also demonstrated the presence of dystroglycan (both α and β sub-units) in their repairing cell-line cultures. α -Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to β -dystroglycan, which is a transmembrane protein, and dystroglycan is known to bind to laminin. They were able to show that WGA bound to some of the cells at the edge of damaged areas and suggested that WGA was binding to Dystroglycan. They suggested that WGA competed with dystroglycan resulting in inhibition of wound repair. The results with our cell line H292 are somewhat different. We have demonstrated consistent membrane labelling of all cells in the damaged cultures by WGA with no preference for the damaged edges. This labelling persists unchanged over the 48 h period of culture. We postulate that by binding to carbohydrate moieties on the cell surfaces, WGA is inhibiting wound repair in the cell line H292.

In order to repair damage, epithelial cells must be able to migrate, proliferate and finally differentiate. All these processes involve complex interactions between cells, between cells and matrix, and between cells and soluble mediators.

Some of these pathways are partially understood, for example, changes in cell membrane carbohydrate antigens play an important role in carcinogenesis and metastasis [17]. The glycoconjugates sialyl Lewis A and sialyl Lewis X antigens are commonly expressed on human cancer cells and are ligands for the cell adhesion molecule E-selectin expressed on vascular endothelial cells [18]. They are specifically involved in the binding of the cancer cells to the endothelium, demonstrating the importance of carbohydrate expression as an indicator of metastasis [18].

The cell line H292 is tumour derived and may have an altered glycosylation profile compared with normal cells. However, we suggest that the carbohydrate interactions demonstrated in this study relate to fundamental interactions, which are involved in many *in vivo* situations either alone or as part of more complex processes. There are numerous examples of such situations – one such example is the requirement of cell surface carbohydrates for leukocyte binding to endothelium at sites of inflammation. Selectins bind specifically to sialylated or fucosylated glycans on leucocyte surface glycoproteins, tethering the leucocyte to the endothelium [19], and are an essential prerequisite for the subsequent cell crawling and extra-vasation.

These investigations and others demonstrate the important role that carbohydrate interactions play in a variety of normal and disease states. Our results indicate that specific carbohydrates are required for one or more epithelial repair processes. They could operate via a number of possible mechanisms. Firstly, cell membranes contain many transmembrane molecules, and any physical hindrance would affect the efficiency of cell-to-cell interactions. This is seen by the presence of a large carbohydrate complex – polysialic acid (PSA) – on the cell surface of a neuronal cell line, which increased the space between neighbouring cells [20], thereby sterically hindering the interaction of other surface molecules, an effect that was sensitive to enzymatic removal of PSA.

Two other lectins (LEA and PWM) with a similar nominal carbohydrate specificity to WGA [21, 22] showed no effect on the rate of damage repair, although LEA was shown to bind to the cell membranes. These findings (i) suggest that simple surface carbohydrate bulk may not be the key factor in this instance, (ii) demonstrate subtle differences in specific binding between lectins with similar affinities and (iii) indicate that these minor differences may well be more important than is currently appreciated. While all three lectins can be blocked with high concentrations of GlcNAc, at the physiological level their binding characteristics show linkage-specific preferences. WGA shows a preference for *N*-acetylchitotriose [9], while LEA has a greater affinity for three or more α -(1,4)-linked GlcNAc repeats. The PWM used in this study was a mixture of five or more isolectins exhibiting variable reactivity with GlcNAc oligomers [9]. These fine differences in binding characteristics can provide detailed information as to the precise composition and position of the carbohydrate concerned.

Secondly, if either the degree or the type of glycosylation of transmembrane proteins are modified, then the response of particular cellular interactions can be altered. A good example of this is the transmembrane cell adhesion molecule CD44, which is a major receptor for hyaluronan [23] and participates in cell–cell interaction and cell migration. CD44

exists in a number of alternatively spliced isoforms with varying degrees of glycosylation. Skelton et al. [24] have shown that the binding of CD44 to hyaluronan can be affected by changes in *N*-linked glycosylation. A further glycoconjugate-related effect has been shown by Bennett et al. [25], who demonstrated that some isoforms of CD44 are modified to contain heparan sulphate and will bind heparin-binding growth factors such as basic-fibroblast growth factor [25].

Our results complement a number of potential associations between carbohydrates and different diseases that have already been noted. *Pseudomonas aeruginosa* may attach to fucose residues on respiratory epithelium [10] and, furthermore, a link has been demonstrated between altered glycosylation patterns and the increased binding of this bacterium [26]. It has been postulated that human milk glycoconjugates and oligosaccharides may provide a major source of non-immunological protection for infants against several bacterial infections such as *Escherichia coli*, *Campylobacter jejuni* and *Streptococcus pneumoniae* [27], and that in addition these complex carbohydrates exert anti-pathogenic effects on, for example, Shigatoxin, Cholera toxin and labile toxin of *E. coli* [27]. It therefore would seem that glycosylation patterns and alterations to these patterns are important in diseases involving epithelial injury and subsequent repair responses. Our model system allows an insight into specific carbohydrate involvement in the particular area of epithelial repair, and future work should determine the precise mechanisms involved in altered cellular behaviour. This information can then be used to aid in the production of more effective therapies for diseases such as asthma and, in addition, may also be applicable to a number of other clinical conditions where altered glycosylation occurs.

Acknowledgements

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