Alterations in Protein Glycosylation in PMA-differentiated U-937 Cells Exposed to Mineral Particles

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Carbohydrate moieties of cell glycoconjugates play a pivotal role in molecular recognition phenomena involved in the regulation of most biological systems and the changes observed in cell surface carbohydrates during cell activation or differentiation frequently modulate certain cell functions. Consequently, some aspects of macrophage response to particle exposure might conceivably result from alterations in glycosylation. Therefore, the effect of mineral particles on protein glycosylation was investigated in phorbol myristate acetate (PMA)-differentiated U-937. Jacalin, a lectin specific for O-glycosylated structures, showed a global increase in O-glycosylation in particle-treated cells. In contrast, no significant modifications were observed with concanavalin A, a lectin that recognizes certain N-glycosylated structures. The silicic acid-specific lectins Sambucus nigra agglutinin and Maackia amurensis agglutinin and the galactose-specific lectin Ricinus communis agglutinin revealed a complex pattern of alterations in glycoprotein glycosylation after crystalline silica or manganese dioxide treatments. Expression of sialyl Lewisα in glycosylated structure implicated in leukocyte trafficking, could not be detected in control or treated cells. This finding was consistent with the decrease in sialyl Lewisα expression observed during PMA-induced differentiation. In conclusion, various treatments used in this study induced quantitative as well as qualitative changes in protein glycosylation. Whether these changes are due to glycosidase release or to an alteration in glycosyltransferase expression remains to be determined. The potential functional implications of these changes are currently under investigation. — Environ Health Perspect 105(Suppl 5):1153–1156 (1997)

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Introduction

It is now widely accepted that the carbohydrate moieties of glycoproteins might act as ligands in biological recognition and for this reason are implicated in many biological processes such as cell–cell interaction, cell migration, and proliferation (1–4). This concept is well illustrated by the regulation of leukocyte trafficking, which relies on the specific interaction between membrane-bound lectins, selectins, and the oligosaccharidic structure sialic acid-α(2,3)-galactose-β(1,4)-(fucose-α(1,3))-N-acetylgalosamin (sialyl Lewisα) that allows leukocytes to roll along the blood vessels. This process, which results in activation and extravasation of inflammatory cells to the injured site, is of paramount importance in inflammation (4).

Several lines of evidence further support the biological importance of carbohydrates. For instance, alterations in the glycosylation of cell surface and/or soluble glycoconjugates were reported in many diseases (5,6). This is particularly evident in cancer, where these alterations are believed to be involved in the disturbance of cell adhesiveness, which leads to metastasis (7–10). Alternatively, carbohydrates and especially sialic acids are essential in self/nonself discrimination by the immune system (11). Consequently, uncontrolled alterations in cell surface glycosylation may ultimately lead to disease (11,12).

The interaction between inhaled particles and alveolar macrophage(s) (AM) is the first step in macrophage activation, which is recognized as the origin of later dysfunctions in pneumoconiosis (13). Many studies have demonstrated the diversity of macrophage responses to mineral particles and it is likely that other aspects of this response remain to be determined (13). In this context, there is evidence suggesting that carbohydrates might be important in macrophage biology. For instance, glycosylation patterns were altered in murine macrophages upon activation (14), and similar observations were made in AM from patients with interstitial lung diseases such as sarcoidosis and idiopathic pulmonary fibrosis (15).

Given the potential physiopathological interest of carbohydrates, this study was designed to determine whether alterations in protein glycosylation occur in macrophages following mineral particle exposure. The effect of particles on protein glycosylation was investigated in U-937 cells, a human myelomonocytic cell line that can be induced to differentiate into monocyte/macrophage by a phorbol ester (phorbol myristate acetate [PMA]) (16). Several reasons dictated the choice of this cell line. First, it was essential to use human cells because glycosylation is a species-specific parameter (2,3) that frequently makes studies on animal cells difficult to extrapolate to man. Second, although normal human AM would constitute a more realistic model, the use of such cells in experimental studies is limited by ethical considerations. Finally, PMA-differentiated U-937 cells were frequently used as a
macrophage model in immunological studies (16), and in our research they were an interesting surrogate for investigating AM responses to mineral particles (Trabelsi et al., in preparation).

PMA-differentiated U-937 cells were treated with three reference particles: titanium dioxide (TiO$_2$), a harmless particle (17,18); manganese dioxide (MnO$_2$), which induces a strong inflammatory response (19,20); and crystalline silica (DQ$_{12}$), a potent fibrogenic particle (21,22). The results indicate that MnO$_2$ and DQ$_{12}$ induce specific patterns of change in protein glycosylation. The possible functional implications of these changes are discussed.

**Materials and Methods**

**Reagents**

Immobilon, 0.45 µM polystyrene particle-fluorescent blotting membrane, was purchased from Millipore (Bedford, MA). Hyperfilm enhanced chemical luminescence (ECL) and ECL Western blotting detection kits were purchased from Amersham Labs (Little Chalfont, UK). Biotinylated lectins, including *Arachis hypogaea* agglutinin (peanut agglutinin (PNA)), *Ariocarpus integrifolius* agglutinin (Jacalin), *Canavalia ensiformis* agglutinin (concanavalin A (ConA)), *Bandeiraea simplicifolia* agglutinin (Griffonia simplicifolia agglutinin (GS I-B4)), *Maackia amurensis* agglutinin (MaaA), *Ricinus communis* agglutinin I (RCA), *Sambucus nigra* agglutinin (SNA), and *Ulex europeaus* agglutinin I (UEA), were purchased from Vector Laboratories (Burlingame, CA). Murine monoclonal IgM (clone: KM93) specific to sialyl Lewis$^a$ was purchased from Valbiotech (Paris, France). Specific gold-labeled reagents and silver enhancement kits were obtained from Janssen Life Sciences (Piscataway, NJ). Electrophoresis and Western blotting apparatus (mini Protein II cell, mini transblot cell) and reagents were from Bio-Rad Laboratories (Richmond, CA). RPMI 1640 medium, glutamine, penicillin, and streptomycin were obtained from Gibco BRL (Cergy-Pontoise, France). All other reagents were of analytical grade.

**Cell Culture**

U-937 cells (a myelomonocytic cell line derived from a patient with histiocytic lymphoma) were maintained as a suspension culture at 37°C in a humidified chamber in the presence of 5% CO$_2$. They were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Routinely, they were seeded at 1 x 10$^5$ cells/ml and readjusted at this concentration with fresh complete medium three times a week. In the experiments described herein, U-937 was differentiated with 0.162 µM PMA over 4 days, as previously described by Öberg et al. (16).

**Mineral Particles**

In this study we used three reference particles: DQ$_{12}$, with a diameter of 2.2 µm and specific area of 3 m$^2$/g; MnO$_2$, with a diameter of 3.7 µm and specific area of 59 m$^2$/g; and TiO$_2$, with a diameter of 0.33 µm and specific area of 7.5 m$^2$/g. Particle-specific area was determined as previously described (23). Particles were heated for 2 hr at 200°C to inactivate bacterial endotoxins, then sonicated for 5 min (50 kHz, 20 W) just before use.

**Cell Treatments**

After 4 days of PMA differentiation, U-937 cell lines were exposed to particles at 50 µg/cm$^2$ over 48 hr. When sialyl Lewis$^a$ expression was investigated during U-937 differentiation, cells were harvested 1, 2, 3, or 4 days after addition of PMA.

**Western Blotting**

After cells were extensively washed with phosphate-buffered saline (PBS), they were homogenized at 40 x 10$^6$ cells/ml in PBS supplemented with 1% Nonidet P40, 10 mM EDTA, and 25 µM of the serum proteinase inhibitor P-nitrophenyl-p'-guanido benzoxoate (Sigma, l'Isle d'Abeau, France). Proteins (10 µg for each sample) were first separated on 10% polyacrylamide continuous minigel gels according to the method of Laemmli (24), then electrophoretically transferred onto Immobilon membrane according to the method of Towbin et al. (25). Prestained molecular weight markers (Bio-Rad) were used to assess transfer efficiency and determine the size of blotted proteins. After its transfer, the Immobilon membrane was blocked overnight at room temperature in PBS supplemented with 2% polyvinylpyrrolidone (PVP) (wt/vol) and 0.1 mM CaCl$_2$ (PVP buffer), then incubated with various biotinylated lectins at 10 µg/ml in PVP buffer. The specificities of the lectins used in this study are described elsewhere (26–28). After their extensive wash, we incubated the membranes with peroxidase-conjugated streptavidin. After their wash, the lectin-labeled proteins were visualized on a Hyperfilm-ECL (Amersham) with an ECL Western blotting detection kit (Amersham) as recommended by the manufacturer. When the monoclal antibody anti-sialyl Lewis$^a$ was used as a carbohydrate-specific reagent, the Immobilon membrane was blocked overnight at room temperature with PVP buffer, then incubated with the monoclonal antibody at 0.5 µg/ml for 1 hr at room temperature. After the membrane was washed with PVP buffer, it was incubated with gold-labeled rabbit antibodies to mouse IgM for 2 hr at 37°C, washed again, and developed with the IntenseBL silver enhancement kit as described by the manufacturer (Janssen Life Sciences).

**Protein Assay**

Protein concentrations were estimated by a simplified Lowry's method (Bio-Rad) that was specially designed for detergent-containing samples. Bovine serum albumin was used as standard.

**Results**

Western blotting using lectins or carbohydrate-specific antibodies is an easy and convenient way to analyze glycoprotein glycosylation (29). This technique was then used to investigate the possible alterations in PMA-differentiated U-937 cell line glycoproteins following phagocytosis of three reference particles: DQ$_{12}$, MnO$_2$, and TiO$_2$. Special attention has been paid to sialic acid and fucose (Fuc) residues, which are located on the nonreducing termini of the saccharide chain, and to galactose (Gal) residues, which can be unmasked after enzymatic desialylation. The levels of O- and N-glycans were also evaluated.

**N- and O-Glycosylation**

Two major types of glycans are carried by glycoproteins: the first is attached through an asparagine side chain (N-glycans or N-glycosylation), and the second through a threonine or serine side chain (O-glycans or O-glycosylation). ConA, which binds certain mannolysed structures exclusively found in N-glycans, and Jacalin, which binds the disaccharide galactose-β(1,3)N-acetylgalactosamine; (Gal-β(1,3)GalNac) found exclusively in O-glycans, represent interesting tools to investigate N- and O-glycosylations (29).

As shown in Figure 1, more bands are observed with ConA than with Jacalin, which confirms that N-glycosylation is
more frequent than O-glycosylation in mammalian cells. No significant changes were observed with ConA after particle treatments. The labeling profile and band intensity were similar in treated and control cells, with one exception (Figure 1A). Only one band could be visualized at about 50 kDa in MnO2-treated cells, whereas a doublet was observed at this level in all other samples. This band corresponded to the larger component of the doublet and its intensity was slightly higher.

More dramatic changes were observed in O-glycosylation, as assessed by Jacalin labeling (Figure 1B). Electrophoretic profiles were similar in all analyzed samples. However, labeling intensity was increased in the three treatments. This increase was slight but significant in TiO2, moderate in MnO2, and more pronounced in DQ12-treated cells.

**Sialic Acid Expression**

Sialic acids occur as terminal sugars in α(2,3) and α(2,6) linkages to Gal. Therefore, two lectins able to discriminate between these two linkages, which recognize sialic acid in α(2,6) and α(2,3), respectively, were used in this study (SNA and MAA) (27). In the typical experiment depicted in Figure 2A, the electrophoretic profiles obtained by MAA were similar in control and treated cells. However, the intensity of the major band observed at about 150 kDa decreased significantly in DQ12- and MnO2-treated cells. The electrophoretic mobility of two other bands at 85 and 110 kDa appeared slightly increased in DQ12- and MnO2-treated cells. With SNA, electrophoretic profiles and labeling intensity were almost identical in control cells, TiO2- and DQ12-treated cells (Figure 2B). In contrast, significant changes were noted after MnO2 treatment. One band around 35 kDa increased in intensity and two bands at about 20 and 25 kDa disappeared almost completely.

**Fucose Expression**

The presence of fucosylated structures was investigated by using Fuc-specific lectin UEA I. Weak labeling was observed with this lectin and no significant differences were noted between treated and untreated cells (data not shown).

**Terminal Galactose Expression**

Expression of terminal Gal was investigated by using three lectins: PNA, RCA, and GS I-B4. With RCA, which recognizes galactose-β(1,3)N-acetylglucosamine [Gal-β(1,3)GlcNac] and Gal-β(1,4)GlcNac structures found in N-glycans, an increase in labeling intensity was observed in particle-treated cells for a band at about 60 kDa. Overall, the labeling was increased in DQ12-treated cells and decreased in MnO2-treated cells; this was more pronounced for the bands found in the range of 140 to 200 kDa (Figure 3A).

With GS I-B4, which binds Gal residues in α(1,3) and α(1,4), the only significant changes were observed in DQ12-treated cells (Figure 3B). A slight decrease in intensity was observed for a band at 70 kDa, while two other bands at 46 and 60 kDa were barely visible. No significant differences in terminal Gal-β(1,3)GlcNac-type expression were noted between treated and untreated cells, as assessed by PNA labeling (Figure 3C). It should be noted that PNA is specific for the same disaccharide Gal-β(1,3)GlcNac as Jacalin, but unlike Jacalin, it does not bind the sialylated form of this disaccharide (28).

**Sialyl Lewisx Expression**

Expression of sialyl Lewisx, a carbohydrate structure that is the common counter ligand of selectins, has been investigated by using a monoclonal antibody. We found no evidence of sialyl Lewisx expression in treated or untreated cells (not shown). Because U-937 is a cell line known to express this structure (30), we hypothesized that it might have been lost during PMA-induced differentiation. Protein extracts were prepared from U-937 cells after various times of differentiation and analyzed. After 24 hr differentiation, only the larger band was visible and its intensity faded gradually during differentiation (Figure 4). These observations are consistent with the absence of labeling initially observed in particle-treated and control cells, as cell extracts were prepared 6 days.
Discussion

In this study we present the first evidence that quantitative as well as qualitative changes may affect the carbohydrate moity of cell glycoproteins following exposure to mineral particles. The observed alterations are probably specific, as each particle induced a typical pattern of changes. TiO₂ (17,18) showed only a moderate effect on protein glycosylation. In contrast, MnO₂ (19,20) and DQ12 (21,22) induced more significant alterations. One of the more prominent changes observed following treatment with these two particles was an increase in O-glycosylated structure expression detected by Jacalin. More subtle changes were noted with other lectins. A slight decrease was observed in the level of sialic acid linked in α(2,3) in both DQ12- and MnO₂-treated cells, whereas the level of sialic acid in α(2,6) linkage was diminished in MnO₂-treated cells only. Likewise, the level of galactosylated structures recognized by RCA was significantly increased in DQ12-treated cells and decreased in MnO₂-treated cells. The pH maintained in the cultures for the control and the various treated cells ranged from 6.85 to 6.95 (data not shown), which reflects the pH of the alveolar lining fluid (31). Moreover, these fairly similar pH values indicate that this parameter probably was not involved in the particle-specific profiles of glycosylation alterations.

It is interesting that the major cellular alterations were observed following exposure to MnO₂ and DQ12, which might be related to the damage caused to the cells by these particles, which are significantly more toxic than TiO₂ on PMA-differentiated U-937 (Trabelsi et al., in preparation). However, this assumption should be tempered by the fact that these particles induced opposite effects on the level of galactosylated structures recognized by RCA, whereas they induced similar cytotoxicity. Alternatively, phagocytosis of mineral particles is the first step of complex processes that lead to macrophage activation. Because the form of activation likely is dependent on the type of particles, the different effects of MnO₂ and DQ12 on protein glycosylation might be related to the fact that they trigger distinct activation pathways.

Similar but less pronounced results were obtained when the cells were exposed to particles at 25 instead 50 µg/cm² or over 24 instead of 48 hr (data not shown). However, the fact that a 48-hr incubation seems optimum to detect glycosylation alterations does not rule out the possibility that significant changes may occur during the early steps of particle phagocytosis. Further experiments are needed to clarify this point. Similarly, although the doses of particles used in this study were in the range of those frequently used in in vitro experiments, the pathophysiological relevance of the data reported in this paper remains to be fully ascertained, as such high doses of particles are unlikely to be encountered in vivo. Along this line, alterations in sialic acid expression similar to those observed here with DQ12 were found in AM from individuals occupationally exposed to silica (Matrat et al., in preparation).

Several mechanisms can account for these glycosylation changes. For instance, differences in glycosylation are frequently attributable to differences in the level of expression of specific glycosyltransferases (2,32). Therefore, the increased level of O-glycosylated structures detected by Jacalin in particle-treated cells might be due to the upregulation of the N-acetyl galactosaminyltransferase and the galactosyltransferase involved in the synthesis of O-linked glycan core structures. Mineral particles may also interfere with other as aspects of glycoprotein glycansynthesis. Oligosaccharide chains are assembled while the newly synthesized proteins are moving through a specific pathway from endoplasmic reticulum to the trans-Golgi network. Every compartment along this pathway is equipped with a specific set of enzymes to carry out the required reactions (33). Therefore, any disturbance of this membrane flow may generate alterations in protein glycosylation. Such disturbances are likely to be encountered in cells treated with high doses of particles, as decreases in organelle motion resulting from possible effects on the cytoskeleton were observed in AM in which the volume occupied by ingested particles...
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exceeded 7 to 8% of normal cell volume (34). Alternatively, as a given oligosaccharide can be carried by different glycoproteins (4), some of the changes observed for certain structures may be attributable to changes in the level of expression of glycoprotein scaffolds that display these structures. Finally, differentiated U-937 cells readily phagocytize the mineral particles used in this study (Trabelsi et al., in preparation), and phagocytosis is frequently associated with an important release of lysosomal glycosidases (35) that are able to degrade oligosaccharides. Therefore, the involvement of these glycosidases cannot be excluded when a band disappears or when its intensity decreases following treatment.

The functional significance of particle-induced alterations in glycoprotein glycans remains to be determined, but the reduced sialylation observed following DiQ2 and MnO2 treatment might be of importance. This observation is consistent with the concept that mineral particles activate macrophages; reduction in glycoprotein sialylation is known to occur during the priming step of macrophage activation (14). However, sialic acid is an important element in cell reactions with and perception by its external environment (11). Consequently, any qualitative or quantitative changes in cell sialylation may modulate or alter certain cell functions (11). For instance, interactions of macrophages with various subsets of T lymphocytes are influenced by the level of macrophage cell surface sialylation (11). Likewise, particle-induced changes in terminal Gal expression observed in this study are also of interest. Indeed, these galactosylated structures are likely ligands for mammalian membrane-bound and soluble Gal-specific lectins (11,36) and for naturally occurring anti-Gal antibodies (11,12).

In conclusion, we show that mineral particles induce specific patterns of changes in the carbohydrate moiety of glycoproteins. Given the biological implications of carbohydrates, these changes may be critical in modulating macrophage functions. Identification of the proteins affected by these changes, as well as elucidation of the mechanisms that cause them, might provide interesting clues in our understanding of macrophage response to mineral particles.

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