Modification of oligosaccharides by reactive oxygen species decreases sialyl lewis x-mediated cell adhesion

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Introduction

Reactive oxygen species (ROS) are produced during several pathological conditions such as in inflammation and reperfusion injury and damage various biological molecules because of its high and nonspecific reactivity (Halliwell and Gutteridge, 1999). As reported in earlier studies, ROS cause oxidative damage to proteins such as Cu,Zn-superoxide dismutase and collagen, and can also be a causal agent of lipid peroxidation in cell membranes (Farmer et al., 1943; Greenwald and Moy, 1979; Curran et al., 1984; Okawara et al., 1992; Halliwell and Gutteridge, 1999). It has been suggested that ROS are capable of degrading glycosaminoglycans, which are important components of extracellular matrices, thus leading to an impairment of proteoglycan functions (Moseley et al., 1995, 1997). It was also reported that ROS are capable of modifying glycosaminoglycan units of hyaluronic acid in vitro (Moseley et al., 1995, 1997; Grootveld et al., 1992; Ponta et al., 2003). The sialyl lewis x (sLex) epitope [NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc−] has been shown to be a specific oligosaccharide ligand for E-selectin (Phillips et al., 1990; Walz et al., 1990), a member of the selectin family that is expressed on activated endothelial cells. Interaction of the sLex epitope and E-selectin promotes the initial attachment and subsequent movement of leukocytes over vessel walls, firming their adhesion (Kasai, 1996; Vestweber and Blanks, 1999). The crystal structure of selectin and the sLex complex revealed that E-selectin directly recognizes the sLex epitope and that the sialic acid residue of the epitope is of particular

Modification of cell surface oligosaccharides by reactive oxygen species (ROS) and the biological effect of such modifications on cell adhesion were investigated. Treatment of HL60, a human promyelocyte leukemia cell line, with ROS, generated by a combination of hypoxanthine and xanthine oxidase (HX/XO), decreased the sialic acid content on the cell surface, as indicated by a flow cytometric analysis involving sialic acid-specific lectins, and a concomitant increase of free sialic acid was observed in the supernatant. A cell adhesion assay showed that the HX/XO treatment of HL60 cells decreases their capability of binding to human umbilical vein endothelial cells (HUVEC), probably because of an impairment of the interaction involving E-selectin, whereas the decrease in the binding was canceled by the addition of superoxide dismutase (SOD) and catalase. In fact, cell surface sialyl lewis x (sLeα), but not lewis x (Leα), was decreased by HX/XO treatment. Thus, it is more likely that the impaired interaction is based on diminished levels of the selectin ligand. Cleavage of sialic acid by ROS was further verified by the degradation of 4MU-Neu5Ac by HX/XO in the presence of hydrogen peroxide and iron ion. These results indicate that glycosidic linkage of sialic acid is a potential target for superoxide and other related ROS. It is well known that ROS cause cellular damages such as lipid peroxidation and protein oxidation, but, as suggested by the findings reported in the literature, ROS may also regulate cell adhesion via the structural alteration of sialylated oligosaccharides on the cell surface.

Key words: cell adhesion/reactive oxygen species/selectin/sialic acid/sialyl lewis x

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importance in this binding (Graves et al., 1994; Somers et al., 2000).

ROS facilitate the adhesion of leukocytes to endothelial cells via inducing the expression of adhesion molecules such as selectins and ICAM-1 (Patel et al., 1991; Palluy et al., 1992; Lo et al., 1993). For example, P-selectin, which is known to be present on activated platelets, is translocated from intracellular stores to the cell surface within minutes by ROS stimulation, and leukocytes adhesion then occurs very soon (Patel et al., 1991). A previous study found that treatment of human umbilical vein endothelial cells (HUVEC) with ROS for a few minutes facilitates the cellular adhesion of colon cancer cells and neutrophils without the de novo protein synthesis of adhesion molecules, such as P-selectin, E-selectin, and ICAM-1 (Suzuki et al., 1999).

The findings presented in this report indicate that the accelerated cell adhesion is the result of the modification of glycoconjugates on the surface of endothelial cells by ROS. In fact, it has been demonstrated histochemically that ischemia reperfusion causes the disruption of the endothelial glycoconjugate (Ward and Donnelly, 1993; Czarnowska and Karwatowska, 1995; Beresewicz et al., 1998). Therefore, the possibility that ROS regulate cell adhesion as a result of the direct modification of glycoconjugates on the cell surface cannot be excluded. However, details of the modification of glycoconjugate structure by ROS are not currently well understood.

In this study, we reported on an investigation of the ROS-mediated modification of glycoconjugates and the biological consequence of such a modification. The data obtained here reveal that oxidative stress due to hypoxanthine and xanthine oxidase (HX/XO) led to cleavage of the sialic acid from glycoconjugates on the cell surface without the activation and/or induction of sialidase, thereby leading to an impairment in sLe^a-dependent cell adhesion. These findings suggest that ROS affect cell adhesion via the direct modification of cell surface sialylated glycoconjugates.

Results

The issue of how cell surface glycoconjugates are modified by ROS was investigated. It is known that the HX/XO system generates superoxide anion and H_2O_2 and is also capable of producing hydroxyl radical via the fenton reaction, providing trace amounts of transition metal ions are present. As judged by a cytochrome c reduction assay (Fridovich, 1970), superoxide production by HX/XO was comparable to the levels produced by isolated neutrophils stimulated with either PMA or formyl methionyl leucyl phenylalanine (fMLP): HX/XO, 5.8 nM/min; neutrophils (10^7/mL) stimulated by PMA, 9.1 nM/min; and neutrophils (10^5/mL) stimulated by fMLP, 3.5 nM/min. HL60 cells were exposed to ROS using HX/XO system as a radical source, and the resulting alteration in glycoconjugate structure was examined by flow cytometric analysis based on the binding specificity of several lectins. The lectins used, Maackia amurensis (MAM), Sambucus sieboldiana (SSA), Ricinus communis (RCA), and Canavalia ensiformis (ConA), preferentially bind to α2-3 linked sialic acid, α2-6 linked sialic acid, Galβ1–4GlcNAc, and α-D-mannose, respectively. As shown in Figure 1, the binding of SSA and MAM was decreased because of the loss of sialic acid residues by sialidase treatment, and a corresponding increase in RCA lectin binding was observed, indicating that the SSA and MAM lectins recognized the sialic acid residue and the RCA lectin recognized the galactose at the terminal position of oligosaccharides. The binding of ConA to the HL60 cells was not affected by the HX/XO treatment whereas the binding of MAM and SSA lectins were significantly less, compared to non-treated controls. Because the addition of superoxide dismutase (SOD) and catalase canceled the impairment of their binding (data not shown), the decrease in the reactivity of MAM and SSA lectins appeared to be based on damage directly caused by superoxide and its related ROS. On the other hand, the binding of the RCA lectin was increased in contrast to the sialic acid-specific lectin binding. This inverse correlation suggests that non-reducing terminal sialic acids are removed by ROS, which then allows β-Gal residues to be exposed. The binding of Lotus lectin, which binds to L-fucose, was not altered by ROS, indicating that fucose residues were not affected by HX/XO treatment (data not shown). As indicated by these results, the terminal sialic acid residues appear to be most susceptible to treatment with the HX/XO system, and thus it is suggested that the sialic acid residues are vulnerable target for damage by ROS.

To determine whether the loss of sialic acid residues from the non-reducing terminus is due to the release of sialic acid from the cell surface, we analysed the content of sialic acid in the supernatant of HL60 cells by high-performance liquid chromatography (HPLC) using a fluorescent labeling procedure. After the treatment of HL60 with HX/XO for

![Fig. 1. Lectin analysis of reactive oxygen species (ROS) treated HL60.](https://academic.oup.com/glycob/article-abstract/15/11/1094/578283)
5 min at 37°C, sialic acid in the supernatant was labeled with 1,2-diamino-4,5-methylenedioxylenebenzene (DMB), a fluorogenic reagent for alpha-keto acids, and the resulting fluorescent derivative was then assayed by reversed phase HPLC, with a fluorescence detection. It was confirmed that glycosidic linkage of sialic acid is not hydrolyzed under the conditions used for DMB labeling (data not shown). As shown in Figure 2, the analyses showed that the sialic acid content in the supernatant of ROS-treated HL60 cells was three times higher than that in a non-treated sample. This increase of sialic acid was partially inhibited by the addition of SOD, and completely abolished by the addition of SOD and catalase. These results indicated that ROS liberate sialic acid from the cell surface. Assuming that the amount of sialic acid that could be cleaved by sialidase is 100%, almost half of the total sialic acid on the cell surface was cleaved by ROS. In addition, even if sugar chains in the supernatant were hydrolyzed by hydrochloric acid, the amount of sialic acid available for conjugation to DMB was not increased. Because DMB reacts with α-keto acids, this labeling reagent produces fluorescent derivatives only with free sialic acid but not its glycosidically bound form. The increase in sialic acid levels in the supernatant by ROS appeared to result from the specific release of sialic acid but not to any nonspecific damage to sugar chains. In addition, because sialidase activity was not detected in HX/XO treatment (data not shown), it was unlikely that the release of sialic acid was due to the enzymatic hydrolysis. Therefore, these results further support the view that the glycosidic linkage of sialic acid is labile to attack by ROS, and it appears that the linkages of other sugars such as galactose and mannose are stable and not affected by ROS.

To examine the biological significance of ROS-mediated desialylation, we investigated the effect of ROS on cell adhesion, a process that involves sialic acid residues. E-selectin expression in HUVEC by IL-1β was confirmed by flow cytometric analysis using a specific antibody for E-selectin (data not shown), and it was observed that HL60 cells bind to the IL-1β-stimulated HUVEC, as shown in Figure 3A(b). This binding was completely blocked by the addition of either of anti-sLeα or anti-E-selectin antibodies [Figure 3A(f) and (g)], thus confirming that cellular binding entirely depends on protein–carbohydrate interactions. Treatment of HL60 cells with HX/XO impaired their binding to the IL-1β-stimulated HUVEC [Figure 3A(d)], as found for the sialidase treatment of HL60 cells [Figure 3A(h)]. However, the presence of SOD and catalase rescued the inhibitory effect of ROS [Figure 3A(e)]. As shown by these results, ROS actually interfered with cell adhesion most probably via damage to the oligosaccharide structures.

The issue of whether the ROS diminished sLeα epitope levels on the cell surface of HL60 cells was investigated. After treatment of HL60 cells with HX/XO, sLeα and its desialylated form, lewis x (Lex), were analyzed by flow cytometry using specific antibodies for sLeα and Lex. Sialidase treatment of HL60 cells resulted in the decrease and increase of the binding of both anti-sLeα and Lex antibodies to cell surface, respectively, indicating that the binding of these antibodies were affected by the presence of sialic acid at the terminus of oligosaccharides (Figure 4E and J). When HL60 cells were treated by HX/XO, the decrease in sLeα epitope levels was observed along with the corresponding increase in Lex (Figure 4A, B, F, and G). Consistent with the results of the cell adhesion assay, these structural alterations caused by ROS were almost completely abolished in the presence of SOD and catalase (Figure 4C, D, H, and I). Thus, ROS inhibit cell adhesion which is involved in the interaction of selectin and its ligand, sLeα, by converting the sialylated ligand into the asialo form.

To characterize the ROS-directed desialylation in more detail, a reaction with a model glycoside, 4MU-Neu5Ac, was analyzed. This substrate, as well as the corresponding galactoside, was incubated with a ROS generating system involving HX/XO, H2O2, and Fe2+. As shown in Figure 5A, the reaction with 4MU-Neu5Ac resulted in the release of the fluorescent aglycan, 4MU, indicating that the glycosidic linkage of sialic acid can also be cleaved in vitro. As revealed by the HPLC analysis, in addition, sialic acid was also detected as well as 4MU in the ROS treatment, whereas no by-products were detected (data not shown). On the other hand, the galactoside was not significantly cleaved, consistent with the observation that treatment of HL60 cells with ROS liberated only sialic acid residues at the nonreducing ends of the cell surface oligosaccharides with no further degraded. In addition, similar to the data from experiments using the cells, the addition of SOD and catalase

![Fig. 2. Sialic acid analysis of the supernatant of reactive oxygen species (ROS) treated HL60. HL60 were incubated with hypoxanthine and xanthine oxidase (HX/XO) at 37°C for 5 min in the presence or absence of superoxide dismutase (SOD) and catalase. In the sialidase treatment, HL60 cells were incubated with 0.1 U/mL sialidase at 37°C for 30 min. The sialic acid in supernatant was labeled with 1,2-diamino-4,5-methylenedioxylenebenzene (DMB), and measured by reversed phase high-performance liquid chromatography (HPLC). For hydrolysis assay, the supernatant was treated with hydrochloric acid described in experimental procedures. (mean ± SD, n = 4).](https://academic.oup.com/glycob/article-abstract/15/11/1094/578283/1096)
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protected the 4MU-Neu5Ac from cleavage (Figure 5B). Because this substrate was not cleaved solely by either of HX/XO, H₂O₂, or Fe²⁺ but by a combination of these reagents, it appeared that another, such as hydroxyl radical, produced by cooperative interactions of HX/XO, H₂O₂, or Fe²⁺ is primarily required for the glycosidic linkage to be attacked. These results suggest that ROS is capable of directly eliminating non-reducing terminal sialic acid residues without inducing sialidases in cells.

Discussion

The effect of ROS on the cell surface oligosaccharides was investigated. The findings clearly show that non-reducing
Many reports have indicated that ROS increase cell adhesion, as mediated by the interaction of selectins and their ligands, may be regulated differentially in a manner that is dependent on ROS levels. When the level of ROS is relatively low, the adhesion would be facilitated by the induction of adhesion molecules. In contrast, if ROS levels are unusually increased, the loss of sialic acid from the oligosaccharides required for adhesion could suppress cell adhesion. Under these conditions, the relatively rapid modification of oligosaccharides could play a more significant role, for example, in the regulation of the excessive accumulation of neutrophils in various inflammatory states. Because L- and P-selectin-mediated cell adhesion in which sialic acid in the sLe^x epitope is involved can also be impaired by ROS, such an oxidative damage of sLe^x epitope could affect other types of cell–cell interactions.

It has been reported that ascorbic acid oxidation causes the decarboxylation of sialic acid in glycoproteins (Ericson and Pratt, 1987). The cleavage of sialic acid by ROS, as found in this study, did not involve decarboxylation because DMB reacts with the sialic acid whose carboxyl group is intact. Thus, it is unlikely that the mechanism of sialic acid cleavage by HX/XO is similar to the ascorbic oxidation. However, the carboxyl group of sialic acid appears to be the most susceptible target of oxidative damage in the structure of the sugar. A synthetic sialo-type substrate was cleaved by ROS, whereas a galactoside substrate was essentially not damaged. In addition, as shown by flow cytometric analyses using lectins, significant changes in cell surface oligosaccharides were not observed except for alterations based on elimination of sialic acids. Therefore, it seems most likely that a carboxyl group is required for a sugar to be damaged by ROS, and thus the ROS would primarily attack the carboxyl group of the sugar, subsequently leading to the cleavage of the glycosidic linkage. It is likely that the radicals generated by HX/XO transfer one electron to carbohydrate group of sialic acid, and the resulting sugar-radical in which an unpaired electron is donated by the carboxyl group may then attack C-2 of the sialic acid, consequently leading to cleavage of the glycosidic linkage. On the other hand, Hawkins and Davies (1998) examined the degradation of hyaluronic acid by HOCl/OCl^- and suggested that homolysis of the chloramide species forms a nitrogencentered radical which leads to a formation of a carbon-centered radical on the N-acetyl group. In the light of this report, it is also possible that ROS generated by HX/XO form the sugar-radicals on the N-acetyl group. Subsequent modifications of functional groups such as hydroxyl one in sialic acid, which is known to import in the interaction with selectin, may abrogate the binding of HL60 cells. A significant fraction of the sLe^x epitope could be immunoreactive toward the antibody used but not functional in the selectin-binding. In the HX/XO treatment, loss of the binding was drastic compared to that of the antibody staining in contrast to the case of sialidase treatment (Figures 3 and 4). However, the radical species responsible for the cleavage of the glycosidic
linkage as well as a detailed mechanism remain to be investigated.

It is possible that the cleavage of sialic acid by ROS cause impaired functions of glycoproteins and sugar chains because the sialylation of many types of glycoconjugates is often associated with various biological events (Kodama et al., 1993; Pilatte et al., 1993; Schwarzkopf et al., 2002). As reported, for example, oxidative damage decreases the sialic acid content of erythropoietin, a highly glycosylated protein and, as a result, degradation of the protein follows (Uchida et al., 1997). Chappey et al. (1998) showed that the low density lipoprotein (LDL) sialic acid content in patients with advanced coronary artery disease was lower than in healthy subjects. Tanaka et al. (1997) reported that the content of sialic acid on LDL was decreased steadily by oxidation. Furthermore, it has been shown that serum sialic acid concentrations are elevated in cases of inflammation and cancer, under which conditions oxidative stress tends to arise and/or to be enhanced (Stefenelli et al., 1985; Painbeni et al., 1997; Goswami et al., 2003). Thus, it is reasonably certain that the loss of sialic acid residues by ROS can be a causal factor for dysfunction in cells, tissues and organs. The this study might provide a possible mechanism for the cleavage of sialic acid, and, in addition, explain how ROS is associated with pathogenesis and the progression of diseases such as atherosclerosis and cancer from the point of view of glycobiology.

Materials and methods

Chemicals

XO was purchased from Roche Diagnostics (Tokyo, Japan). 3′,6′-(Di(O-acetyl)-2′,7′-bis[N,N-bis(carboxymethyl)aminoethyl]fluorescein, tetraacetoxyethyl ester (calcine-AM) was purchased from Dojindo (Tokyo, Japan). Fluorescein isothiocyanate (FITC) conjugated lectins, SSA, MAM, RCA, ConA, and anti Le<sup>e</sup> monoclonal antibody were purchased from Seikagaku Corporation (Tokyo, Japan). Sialidase detection kit was purchased from Takara Biochemicals (Shiga, Japan). Sialidase from Arthrobacter ureafaciens, 4-methylumbelliferyl-N-acetylneuraminic acid (4MU-Neu5Ac) and 4-methylumbelliferyl-β-D-galactoside (4MU-Gal) were purchased from Nakalai tesque (Kyoto, Japan). Anti sLe<sup>e</sup> monoclonal antibody was purchased from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti human CD62E (E-selectin) neutralizing antibody was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). FITC-conjugated goat anti mouse Ig was purchased from Dako (Kyoto, Japan). The other reagents were of the highest grade available.

Cell culture

HL 60 cells were cultured on 100-mm Petri dishes in RPMI1640 medium containing 10% fetal calf serum (FCS). HUVEC were isolated by dispase treatment from umbilical vein cord as described previously (Suzuki et al., 1993). HUVEC were grown on 24-well flat bottom microplates in MCDB131 medium containing 10% FCS, 10 ng/mL recombinant human basic fibroblast growth factor, 1 µg/mL hydrocortisone, 18 µM/L heparin, and 0.25 µg/mL amphotericin B. These cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

ROS treatment of HL60

HL60 were washed twice with phosphate-buffered saline (PBS; pH 7.4) to remove the FCS-containing medium. HL60 (2 × 10⁵ cells) were treated with 2 mM HX and 50 µM XO in PBS at 37°C for 5 min. The ROS generation was terminated by the addition of 100 µg/mL superoxide dismutase and 100 µg/mL catalase (SOD/CAT) or FCS-containing RPMI1640 medium. ROS-treated HL60 (ROS-HL60) were used immediately in under experiments.

Lectin analysis

ROS-HL60 were labeled with FITC-conjugated SSA, MAM, RCA, or ConA lectin diluted to 1:500 with PBS at 4°C for 60 min. In the sialidase treatment, HL60 were incubated with 0.1 U/mL sialidase at 37°C for 30 min before the addition of lectins. After removing residual unbound lectin by washing with PBS three times, fluorescence was analyzed using flow cytometry (FACScan, Beckton Dickinson, Franklin Lakes, NJ).

Sialic acid analysis

After treatment of HL60 with HX/XO or 0.1 U/mL sialidase, the supernatants were collected, lyophilized, and dissolved in 50 µL of water. The fluorescence labeling of sialic acid using DMB was performed according to manufacturer’s instruction (Takara).

Fifty µL of sample was incubated with 200 µL of fluorescent reagent at 50°C for 2.5 h. For the hydrolysis assay, samples were incubated with 2.6 µL of 1 N HCl at 80°C for 60 min before the addition of the fluorescent reagent. The reaction was stopped by cooling the solution to 0°C. Sialic acid was analyzed by reversed phase HPLC. A 50-µL aliquot was applied to an HPLC system (600E, Waters, Milford, MA) equipped with a PALPAK Type R column (4.6 mm × 250 mm, Takara). Elution was performed at room temperature using an eluent composed of acetonitrile (92%)–methanol (7%) at a flow rate 0.8 mL/min. Fluorescence was monitored with excitation and emission wavelengths of 373 and 448 nm, respectively. Sialic acid concentrations were determined by determining the amount of DMB group using a known amount of sialic-acid–DMB complex as a standard.

Cell adhesion assay

A monolayer cell adhesion assay was performed as described previously (Takada et al., 1993). HL60 cells were washed with RPMI1640 medium and resuspended with 3 mL of the same medium. For the fluorescence labeling of HL60, 3 µL of calcine-AM was added and the solution was then incubated at room temperature for 15 min in dark. After washing with PBS, the HL60 cells were treated with HX/XO in the presence or absence of SOD/CAT. In the ligand antagonist assay, fluorescence labeled HL 60 were incubated with a neutralizing antibody for sLe<sup>e</sup> or E-selectin at room temperature for 30 min. In the sialidase treatment,
HL60 cells were incubated with 0.1 U/mL sialidase at 37°C for 30 min. HL60 cells were washed once with RPMI1640 medium and resuspended in the same medium. HUVEC were incubated with 1 ng/mL IL-1β at 37°C for 4 h before the cell adhesion experiment of HL60. After the washing of HUVEC with RPMI1640 medium twice, HL60 were added at a concentration of 1 x 10^5 in 500 µL to HUVEC and incubated at room temperature with rotation. After incubation for 20 min, HUVEC were washed gently with PBS, phosphate-buffered saline; RCA, Ricinus communis; ROS, reactive oxygen species; sLex, sialyl Lewis x; SOD, superoxide dismutase; SSA, Sambucus sieboldiana; XO, xanthine oxidase.

**References**


Farmer, H., Koch, H.P., and Sutton, D.A. (1943) The course of autoxidation of radicals generated during the hydroxyl radical-induced degradation of low density lipoprotein; Le x, lewis x; MAM, Maackia amurensis; PBS, phosphate-buffered saline; RCA, Ricinus communis; ROS, reactive oxygen species; sLex, sialyl Lewis x; SOD, superoxide dismutase; SSA, Sambucus sieboldiana; XO, xanthine oxidase.

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**Abbreviations**

4MU-Gal, 4-methylumbelliferyl-galactoside; 4MU-Neu5Ac, 4-methylumbelliferyl-N-acetylneuraminic acid; ConA, Canavalia ensiformis; DMB, 1,2-diamino-4,5-methylenedioxybenzene; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; HX, hypoxanthine; LDL, low density lipoprotein; Le x, lewis x; MAM, Maackia amurensis; PBS, phosphate-buffered saline; RCA, Ricinus communis; ROS, reactive oxygen species; sLex, sialyl Lewis x; SOD, superoxide dismutase; SSA, Sambucus sieboldiana; XO, xanthine oxidase.

**References**


