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REVIEW

Changes in glycosylation of acute-phase proteins in health and disease: occurrence, regulation and function

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The pathophysiological variations in different glycoforms of acute-phase glycoproteins in serum most likely result from changes in the glycosylation process during their biosynthesis in the parenchymal cells of the liver. Biosynthesis in other cells or tissues may contribute, but in general appears to play a minor role. Inflammatory cytokines appear to regulate the process, but glycosylation changes are independent of protein synthesis. In addition, other humoral factors such as corticosteroids and growth factors are involved. The interplay of these factors is determined by the stage of the disease (*e.g.* rheumatoid arthritis), the physiological situation (*e.g.* pregnancy), or directly or indirectly by extraneous factors such as drugs (*e.g.* ethanol). Information about the functional implications of the changes is limited, but some reports suggest that for α_1-acid glycoprotein the changes might affect the operation of the immune system.

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Pathophysiological changes in the glycosylation of acute-phase proteins

Specific alterations in the glycosylation of acute-phase proteins (APP) occur in many pathophysiological states, *e.g.* acute and chronic inflammation, cancer, a variety of liver diseases and during pregnancy [see ref. 1 for recent review]. These alterations accompany changes in the serum concentration of APP, but are independent of their rate of synthesis and are found on both positive (*e.g.* α_1-acid glycoprotein (AGP), α_1-protease inhibitor (PI), haptoglobin (HG), α_1-antichymotrypsin (ACT) [1–9]) and negative APP (*e.g.* α_2-HS glycoprotein (α_2 HS), transferrin (TF) [6, 10]). As outlined below, the pattern of change in glycosylation is dependent on the particular state (*e.g.* acute inflammation or pregnancy) or the type of disease (*e.g.* rheumatoid arthritis (RA) or alcoholic liver cirrhosis), and to some extent on the APP studied.

APP contain one or more asparagine-linked (*N*-linked) carbohydrate structures, which may have from two to four branches (diantennary, trianten-

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nary and tetraantennary structures) arising from the $\alpha1\rightarrow3$- and $\alpha1\rightarrow6$-linked mannose (Man) residues of the core structure: Man$\alpha1\rightarrow$ 3(Man$\alpha1\rightarrow$ 6)Man$\beta1\rightarrow$4GlcNAcβ $1\rightarrow$4GlcNAc. For human APP these branches generally consist of Gal$\beta1\rightarrow$4GlcNAc units (lactosamine units) which can be further substituted with $\alpha2\rightarrow3$- and/or $\alpha2\rightarrow6$-linked sialic acid, $\alpha1\rightarrow2$- or $\alpha1\rightarrow3$-linked fucose, or other sugars in a number of different configurations [11, 16].

Variation in the structure of an oligosaccharide glycan has been previously referred to as microheterogeneity. Two types of inflammation- or disease-induced microheterogeneity have been distinguished for APP: major microheterogeneity, which reflects differences in the number of branches in the antennary structures [2, 4, 9, 17], and minor microheterogeneity, which is caused by variations in sialic acid, galactose and/or fucose content [3, 10, 18–21]. In addition, the multiple glycosylation sites on an APP mostly are occupied by different glycans. As a result, different glycoforms of an APP can be distinguished in serum and the proportions of these will change depending upon the condition or disease that is present. Both types of microheterogeneity can be studied in serum by using techniques employing lectins such as crossed immunoaffinoelectrophoresis or affinity chromatography [see 1, 22 for reviews].

Changes in the degree of branching

A reversible decrease in the degree of branching of APP glycans due to increased diantennary glycan content is called Type I major microheterogeneity. This has been demonstrated by increased reactivity of APP with concanavalin A (Con A) during acute-phase reactions after surgery [2, 20], in trauma after severe burning [5, 23, 24], and in animal models or isolated liver cells after treatment with dexamethasone [9, 25], phenobarbital [26] or galactosamine [27] and after laparotomy [25]. The detection of this type of change has been shown to be useful as an additional marker in the determination of intercurrent infections in chronic inflammatory diseases, like RA [28] and systemic lupus erythematosus [29].

A reversible increase in branching (Type II major microheterogeneity: decreased diantennary glycan content) has been shown for AGP in maternal serum during pregnancy [30–32]. Interestingly, in the fetal serum, AGP molecules express a Type I microheterogeneity which changes progressively during the development of the fetus to the normal pattern of the newborn [30, 33]. Increases in branching of APP have also been found in RA grade III and IV [34], during liver diseases like alcoholic liver cirrhosis [35] and in hepatitis [36, 37]. Both increases and decreases in branching have been described for a number of APP in cancer [1, 38–44].

Changes in terminal glycosylation

The majority of reported changes in terminal glycosylation of APP (also called minor microheterogeneity) are related to the degree and the type of substitution of the *N*-linked glycans with sialic acid and/or fucose [*e.g.* 1, 3, 10, 18–21, 39, 42, 44–46]. These changes differ according to the disease and the protein studied. In RA, sialylation is increased in TF [10], but decreased in AGP [46, 47], and unchanged in HG [21]. Consumption of large amounts of alcohol induces a decreased sialylation of TF [48]. Furthermore, decreased sialylation of AGP has been found in relation to benign liver diseases [35], whereas the sialylation of AGP and TF have been reported to be increased in cancer sera compared with healthy sera [42, 46, 49, 50]. In some studies, the type of glycan substitution with sialic acid was also investigated and these showed that increased expression of $\alpha2\rightarrow3$-linked sialic acid occurs for TF in cancer [42] and for AGP in liver cirrhosis [51]. When in the latter glycan a sialylated lactosamine unit is substituted with an $\alpha1\rightarrow3$-linked fucose residue, as suggested for TF in liver cancer [42], this will result in increased expression of the blood group antigen sialyl Le^x (SLEX; NeuAc$\alpha2\rightarrow$ 3-Gal$\alpha1\rightarrow$4(Fuc$\alpha1\rightarrow$3)GlcNAc-R).

Increased expression of SLEX in combination with an elevated fucosylation of AGP occurs during acute inflammation [20], in liver cirrhosis [51, 52] and was suggested to occur in RA [53]. Inflammatory conditions and tumor growth appear to affect also the fucosylation of other APP [3, 18, 19, 21, 42–45, 49, 53–55]. Most of these changes represent an increased occurrence of $\alpha1\rightarrow3$-linked fucose residues on the APP. Changes in fucosylation frequently coincide with changes in branching, but they are not necessarily determined by it [20, 45, 53, 55]. It must be kept in mind, however, that in most studies the changes described have been detected by indirect methods. Therefore, almost no structural data are available yet concerning which of the branches of a glycan are modified by the disease, or whether the type of branching or the glycosylation site at which it occurs on the APP can have influenced the fucosylation and sialylation.

Regulation of the changes in glycosylation in APP

Cytokine-induced alterations in the glycosylation of APP in the liver

The liver is known to be the major source of APP under normal physiological and inflammatory conditions, and the changes in serum concentrations of APP result mainly from the effects of inflammatory cytokines and other humoral factors on their biosynthesis by the liver [56, 57]. It is thought, therefore, that the accompanying changes in the proportions of different APP glycoforms originate from modifications in the post-translational glycosylation mechanisms in the liver, rather than from degradative processes in the circulation (see later). This latter conclusion is supported by the effects of liver damage on the various glycoforms of AGP and PI in serum [27, 35–37, 45]. Much of the experimental evidence has been obtained by *in vitro* studies of the effects of cytokines and glucocorticoids on the glycosylation of newly-synthesized APP in (primary) cultures of hepatocytes isolated from human [4, 6], rat [6, 25, 58] or mouse livers [59] and cultures of human hepatoma cell lines [6, 41, 55, 60, 61]. The validity of this approach for the study of inflammation-related events has been established by investigating the effects of cytokines and glucocorticoids on the regulatory mechanisms of APP gene expression [57, 62]. The hepatocytes and hepatoma cell lines appeared to be capable of synthesizing and secreting all the differently branched glycoforms of the APP studied as occurring in control and patient or animal sera.

Two different types of change in the major microheterogeneity of APP were observed for human hepatocytes and the human hepatoma cell lines Hep-3B and Hep-G2 cells after treatment with cytokines (*e.g.* interleukin (IL)-1, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, TNF-β) and/or glucocorticoids. For human hepatocytes and Hep-3B cells, the changes resembled the Type I changes observed in sera of patients with acute inflammatory states. This occurred with both positive (AGP, PI, HG, ACT) and negative (α_2HS) APP, and was accompanied by increased synthesis of the positive [4, 6, 41, 63] and decreased synthesis of the negative APP [6]. For Hep-G2 cells, the opposite effect was observed for secreted AGP, PI, HG and ACT [41, 63], *i.e.* the secreted APP displayed the Type II changes observed in sera of patients with some chronic inflammatory states. This was also accompanied by increased synthesis of the APP [7, 28, 34]. The nature of the minor microheterogeneity of APP has not yet been thoroughly studied. However, isolated human hepatocytes have been found to secrete the fucosylated glycoforms of AGP that are found in normal human serum (T. W. Graaf and W. van Dijk, unpublished results), and increased fucosylation has been observed in Hep-G2 cells for secreted ACT and PI (W. van Dijk and A. Mackiewicz, unpublished results) and TF [55].

These results strongly support the theory that changes in the relative proportions of APP glycoforms are generated by variations in the post-translational glycosylation process in the parenchymal cells of the liver, and that cytokines and glucocorticoids are involved in inducing these changes. Surprisingly, all cytokines had a similar effect on the synthesis of APP, although the magnitude of the effect was dependent on the APP studied. The different effects of cytokines on the glycosylation suggest an uncoupling in the regulation of secretion and glycosylation. The existence of such uncoupling was also found for the glycosylation and secretion of rat AGP [6]. It can be concluded, therefore, that the signal transduction pathways for the regulation of the changes in glycosylation of APP are at least partly independent of those governing protein synthesis.

It seems unlikely that the cytokine-induced changes can be explained by the effects on expression of different genetic variants. Previous studies have shown that there are minor changes in the relative proportions of the products of the three AGP genes during acute inflammation, and these cannot account for the major alterations in the diantennary glycan content of AGP [64]. This has been further confirmed in a transgenic mouse model, where multiple glycoforms of human AGP were found in the sera of mice in which only one human AGP gene was expressed [65]. It cannot be excluded, however, that increased protein production could have an effect on the APP microheterogeneity, as was suggested from studies with isolated hepatocytes from transgenic mice expressing the rat AGP gene [59].

The effects of cytokines on glycosylation of APP depends on the particular cytokine and combinations of different cytokines can result in synergistic or antagonistic effects. The cytokines can be divided according to their effects into different classes: (i) those inducing Type I and Type II alterations (IL-6), (ii) those inducing Type I alterations (TGF-β), (iii) those inducing Type II alterations (LIF, TNF-α, IFN-γ), and (iv) those which

did not exert direct effects, but which could modulate the activity of other cytokines (IL-1) [reviewed in 66]. The various alterations in glycosylation found *in vivo* are most likely controlled by the cooperation of various cytokines and other factors, like glucocorticoids, rather than by a single factor. This is supported by *in vitro* studies showing that the magnitude and the type of glycosylation alteration were dependent on the composition and the amount of interacting cytokines, and that these effects could be further modulated by the treatment with the synthetic glucocorticoid dexamethasone [reviewed in 66]. The mechanisms by which cytokines induce these modifications in glycosylation of APP are unknown, but changes have been noted in components of the post-translational biosynthetic pathways in the liver during inflammation [67–70].

Contribution of extrahepatic cells

The *in vitro* studies with hepatocytes and hepatoma cell lines have clearly shown that these cells can produce all the glycosylation variants present in normal and patient sera. So it seems likely that in most situations the liver is the major source. Contributions from extrahepatic cells cannot be totally excluded since a few groups have reported on the secretion of APP by various blood cells. For instance leukocytes and alveolar macrophages are able to synthesize PI [71–73], and AGP can be secreted by human lymphocytes, granulocytes and monocytes [74, 75]. Since inflammation is associated with proliferation of leukocytes, part of the observed changes in glycosylation and synthesis of APP in inflammation could originate from these cells [75].

Inflammation-induced changes in the glycan biosynthesis

The biosynthesis of glycans is accomplished by a multistep enzymatic process occurring on the endoplasmic reticulum and in the Golgi complex, and involves a series of highly specific glycosyltransferases and glycosidases [76–78]. Branching of *N*-linked glycans is initated at an early stage of the glycan formation in the *cis*-Golgi system by the addition of *N*-acetylglucosamine (GlcNAc) residues to the inner core structure. At least four different GlcNAc transferases (GNTases) are involved in the formation of the various branched glycans. Control of the relative activities of these enzymes is one of the mechanisms regulating the branching process [76–78]. GNTase IV and V catalyze the formation of tri- and tetraantennary glycans and therefore changes in the activities of these enzymes can be expected to occur under inflammatory conditions. Although detailed studies of GNTases have not been performed with liver cells, treatment of human myeloma cells with IL-6 stimulated the activities of GNTases IV and V, and was accompanied by increased expression of tri- and tetraantennary glycans on the membrane-bound glycoproteins [79].

Changes in sialylation and fucosylation are determined by the activities and specificities of the sialyl (STase) and fucosyltransferase (FTase) [78, 80], and it appears that the action of α6-STase (sialic acidα2 $\rightarrow$ 6Gal-) is mutually exclusive of the actions of α3-STase (sialic acidα2 $\rightarrow$ 3Gal-) and α3-FTase (Fucα1 $\rightarrow$ 3GlcNAc-). In addition, the branch specificities of these glycosyltransferases are non-identical, which results in differences in the terminal glycosylation of di-, tri- and tetraantennary glycans [81]. It can be expected that with increased branching of the acceptor glycan there will be a shift from branch termination by an α6-linked sialic acid residue to termination by an α3-linked sialic acid residue, which in combination with the action of the α3-FTase will yield the SLEX antigen [80]. Changes in the absolute and relative activities of the enzymes will determine the number of glycans that will be terminated in this way. So, it is possible that the high expression of SLEX structures on AGP during inflammation [20, 53] is caused by an increase in the activity of α3-FTase. Elevated serum levels of α3-FTase have been found in RA [S Thompson and GA Turner, unpublished observations].

There could be additional or alternative mechanisms. α6-STase in the Golgi system could be mislocated or prematurely released during the hepatic acute-phase response [68–70], which in turn could increase the incorporation of sialic acid and fucose residues by α3-STase and α3-FTase, respectively. Cytokines and dexamethasone have been shown to regulate the expression and secretion of α6-STase in rat and human hepatocytes [69, 82], and may also up- or down-regulate the expression of other glycosyltransferases.

Changes in other components involved in the biosynthetic mechanisms could contribute to the regulation of the changes in glycosylation of APP. It has been reported that inflammation induces variations in the availability of nucleotide-sugars (the substrates for the glycosyltransferases) [83–85], as well as the availability of dolichol and dolichol-phosphate (the precursors of the dolichol-linked oligosaccharide precursor for *N*-linked gly-

cans) [86]. Furthermore, an altered rate of intracellular transport or an altered route of transit of APP has been suggested to exist during inflammation [87–89].

Role of serum glycosyltransferases

The possible involvement of serum glycosyltransferases in changes in APP glycosylation can only be considered for those enzymes involved in the terminal addition of sugars, *i.e.* sialylation, fucosylation or galactosylation. Modifications, by serum enzymes, in the type of branching cannot occur because these are determined at an early stage of the biosynthetic process [77]. Galactosyl-, sialyl and fucosyltransferases are known to occur in sera, and changes in the levels have been described under various pathological and physiological circumstances [68–70, 90–97]. Large direct effects on the glycosylation in serum, however, seem unlikely, because serum glycoproteins are turning over rapidly and the appropriate nucleotide-sugar substrates are only present at very low levels in serum. Nevertheless, it cannot be excluded that at sites where cellular damage has occurred these compounds have been released and that they could be locally used by the respective serum glycosyltransferases. It seems possible that some glycosyltransferases are present in serum in high amounts as acute-phase reactants, as the liver can be induced to secrete α6-STase by IL-6 and dexamethasone [69, 70].

Effects of circulating glycosidases and the selective removal of different glycoforms

Glycosidases can be released into the circulation by inflamed tissues [82, 98] and by cancers [98, 99], and they could affect the glycosylation of APP by removing terminal carbohydrate residues such as sialic acid, fucose or galactose. Increased amounts of circulating undersialylated glycoproteins have been detected in liver disease [100–102]; however, this could be caused by the abnormality in the hepatic asialoglycoprotein receptor which has been found in these situations [102, 103].

Selective removal of different glycoforms by branch-specific tissue lectins may also contribute to the changes in glycosylation of APP observed in serum. This is suggested by a study in which different glycoforms of human AGP were injected into normal and acute-phase-response activated rats [104]. Activation of the acute-phase response in the rat resulted in a somewhat decreased half-life for the glycoform with only tri- and tetraantennary glycans (12 vs 14 h for the normal rat) and a slightly increased half-life for the glycoform in which one of the glycans was replaced by a diantennary glycan (16 vs 13 h for normal rats). The authors concluded, however, that the small changes observed in the elimination of the various glycoforms were not sufficient to explain the large changes in glycosylation of AGP observed in the acute-phase response.

Physiological functions

Inflammation-induced changes in the glycosylation of APP have been found in human, rat and mouse serum [see, *e.g.* 1, 25, 59] and therefore appear to be an essential phenomenon in the general inflammatory reaction. These changes occur on several glycoproteins at approximately the same time, leading to a completely different carbohydrate phenotype for the majority of the plasma proteins. This particularly happens during acute inflammatory reactions when the serum albumin levels are lowered to counterbalance the large increases in the positive acute-phase glycoproteins. Dependent of the stage of inflammation, the plasma glycoproteins will express predominantly certain glycan chains, *e.g.* diantennary glycans in the early phase of acute inflammation or highly fucosylated tri- or tetraantennary glycans towards the end of that condition [20] or in liver cirrhosis [51].

Glycan structures play a crucial role in a number of biological processes [105, 106], of which the selectin-mediated interaction between leukocytes and endothelial cells in homing and inflammatory processes are currently the most fascinating [107, 108]. Changes in the levels of the various glycoforms of APP, therefore, can be expected to be of importance in the function of these molecules in the inflammatory reaction. This aspect has not yet been thoroughly studied.

Most studies of the physiological role of the glycosylation of APP have been concentrated on AGP, most probably because the exact function of this major APP is not known and because AGP is the major carrier of tri- and tetraantennary glycans in normal human serum [109]. Several *in vitro* studies [110–113] have shown that AGP can modulate the immune system, *e.g.* (i) inhibition of T-cell proliferation [114, 115] and (ii) induction of an IL-1 inhibitor in macrophages [116]. These effects are concentration dependent, reach optima in the physiological range of concentrations and in many cases involve the carbohydrate portion of the molecule.

In other studies, the rheological properties of AGP have been shown to be dependent on its diantennary glycan content [117]. This might explain why in acute-phase-induced rats the distribution volume of human AGP is lower for the glycoforms containing only tri- and tetraantennary glycans than for the glycoforms containing also diantennary glycans [104].

Changes in the sialylation and fucosylation of APP can result in the increased expression of SLEX structures—structures that have been shown to be involved in the selectin-mediated interaction of leukocytes and endothelial cells in homing and inflammatory processes [108]. It has been postulated that the inflammation-induced changes in the expression of SLEX on AGP, and on other APP, may have an inhibitory effect on the selectin-mediated influx of leukocytes into inflamed areas [20, 53, 118] and may represent a humoral feedback response of the hepatic acute-phase reaction to dampen down the cellular inflammatory reaction.

Concluding remarks

This minireview has shown that change in the glycosylation of APP in health and disease is a complex and fascinating field with many unresolved questions for future studies. Of particular importance is the need to clarify the function(s) of these carbohydrate changes and to understand in more detail the mechanisms by which they are regulated.

Recent technical developments have resulted in easier methods to purify APP [44], analyze carbohydrate structure in small amounts of material [3, 44, 119] and isolate various glycoforms [120]. These new procedures, coupled with our increasing knowledge of glycosyltranferase genes, will enable us to test the various ideas suggested in this review and lead to a better understanding of the processes involved.

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