MONOKINES REGULATE GLYCOSYLATION OF ACUTE-PHASE PROTEINS

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After inflammatory stimuli, the rates of hepatic synthesis of several plasma proteins, the acute-phase proteins (APP), increase dramatically while some others, most notably albumin, decrease substantially (1). Among these APP are a number of glycoproteins that manifest microheterogeneity as a result of structural variations of their glycan chains. Increased plasma levels of APP are often accompanied by changes in the patterns of glycosylation of some of these proteins (2–6), readily detected as changes in relative affinity to lectins such as Con A. While monokines, products of activated mononuclear phagocytes, are known to play a major role in regulation of APP gene expression, leading to increased or decreased synthesis and secretion of plasma proteins (7–10), the mechanisms governing the alterations in glycosylation of APP are not known. It has not been firmly established whether these changes in glycosylation reflect differences occurring within hepatocytes or whether they reflect differential clearance of various glycosylated forms of APP from the circulation. If the former possibility is the case, it is unclear whether a common mediator induces both changes of APP synthesis and changes in their patterns of glycosylation, or whether separate mechanisms are responsible for these alterations. The lack of correlation between changes in concentration and pattern of glycosylation of α_1 acid glycoprotein in certain clinical states suggests that different mechanisms mediate these changes (5). To study the mechanisms governing both glycosylation and synthesis of APP in a single system, we used the human hepatoma cell line Hep 3B as a model.

Materials and Methods

Preparation of Conditioned Medium (CM). Human monocytes were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation followed by attachment to plastic culture dishes (100 mm) for 1.5 h in RPMI 1640 (Mediatech-Fisher Scientific Co., Springfield, NJ) with 20% FCS (Gibco Laboratories, Grand Island, NY). Adherent cells (1.5 × 10⁶/dish) were washed and incubated for 24 h in 5 ml of serum-free RPMI 1640 with dexamethasone (1 µM/ml), insulin (0.02 U/ml), and LPS (20 µg/ml). As expected, after incubation in the presence of dexamethasone (11), CM tested by the thymocyte proliferation assay (12) did not show IL-1 activity.

Induction of APP in the Hep 3B Cell Line. After subculture, cells were maintained in

RPMI 1640 with 10% FCS, dexamethasone (1 μM/ml), and insulin (0.02 U/ml) for 5 d and then for 24 h in serum-free RPMI 1640 supplemented with dexamethasone and insulin. At this point (time 0), Hep 3B cells were exposed to a number of inducers of acute phase protein changes: (a) CM prepared from LPS-activated human monocytes, (b) recombinant generated human IL-1β, a generous gift of Dr. C. A. Dinarello (Tufts University Medical School, Boston, MA), (c) recombinant generated human tumor necrosis factor (TNF), kindly provided by Dr. B. A. Beutler (University of Texas, Dallas, TX), (d) medium from the keratocarcinoma cell line, COLO-16, which contains hepatocyte-stimulating factor (HSF), and (e–g) COLO-16-derived purified HSF I, II, and III (8), a generous gift of Dr. H. Baumann (Roswell Park Medical Institute, Buffalo, NY). In other experiments, cells were exposed to tunicamycin (2 μg/ml). Cells were then incubated for an additional 24, 48, 72, or 96 h with replacement of medium every 24 h. Analyses were carried out in media collected over the final 24 h. Each experiment was run in triplicate.

Quantitation of APP Accumulated in Culture Media. Concentrations of albumin, α_1 proteinase inhibitor (αPI) and ceruloplasmin (Cp) secreted by Hep 3B cells into culture media were determined by quantitative electroimmunoassay (13) with monospecific antibodies, using a human serum calibrator kit (Atlantic Antibodies, Scarborough, ME) as a standard. Results were expressed as percentage of increase or decrease over control experiments.

Determination of αPI and Cp Microheterogeneity. Patterns of glycosylation were studied by means of agarose affinity electrophoresis (AFF-EP) with free Con A as a ligand (14). Briefly, in the first dimension of AFF-EP, 40 μM Con A (Sigma Chemical Co., St. Louis, MO) was incorporated into agarose, and culture medium was added to the application well. Electrophoresis was run for 1 h at 10 V/cm and the gel was transferred onto the second dimension plate. Two gels were cast, one containing anti-αPI or anti-Cp antibodies, and the second an intermediate gel containing 0.3 M α-methyl-D-mannoside (to dissolve Con A–glycoprotein complexes). Electrophoresis in the second dimension was run for 18 h at 2 V/cm. The gel was then washed, dried, and stained with Coomassie Brilliant Blue R250 (Sigma Chemical Co.). The area under the precipitate curves was determined by planimetry and the relative amounts of the different microheterogeneous forms were expressed as percentages of the total. A reactivity coefficient (RC) for each sample was calculated according to the formula: sum of Con A reactive variants/Con A nonreactive variant.

All statistical analyses were carried out by the Mann-Whitney test.

Results

Exposure of Hep 3B cells to varying amounts of CM and to supernatant obtained from the COLO 16 line resulted in dose-dependent reduction in accumulation of albumin and increase of accumulation of αPI and Cp (Table I). Incubation with IL-1, TNF, and HSF I caused reduction of albumin and increase of Cp accumulation, but had no effect on αPI. HSF II and HSF III did not significantly affect accumulation of any of the proteins studied. The changes described were most pronounced after 48 h of incubation (Table II).

AFF-EP revealed three microheterogeneous variants of αPI: one nonreactive and two reactive with Con A, and four variants of Cp: two nonreactive and two reactive with the lectin (Fig. 1). Incubation of Hep 3B cells with CM and COLO 16 caused increased accumulation of forms of αPI and Cp reactive with Con A compared to controls (p < 0.001) (Fig. 1 and Table I). These changes were dose (Fig. 2) and time (Table II) dependent, reaching a maximum at 72 h of culture. The effect was reversed within 24 h of removal of CM from the cell culture medium. None of the other purified or recombinant monokines affected reactivity of αPI or Cp with Con A. Addition of tunicamycin, known to block the

TABLE I

Accumulation of Alb, αPI, and Cp and Glycosylation Patterns of αPI and Cp Secreted into Culture Medium by Hep 3B Cells Exposed to Putative Inducers for 48 h

Inducer	n	Accumulation			Glycosylation pattern (RC)	
		Alb	αPI	Cp	PI	Cp
		%	%	%		
Control	12	100	100	100	1.67 ± 0.1*	0.34 ± 0.01
CM (10%)	12	29 ± 5‡	359 ± 18‡	1642 ± 120‡	3.55 ± 0.12‡	0.85 ± 0.02‡
IL-1 (1.25 ng/ml)	8	36 ± 4‡	105 ± 10	446 ± 52‡	1.7 ± 0.11	0.33 ± 0.02
TNF (250 ng/ml)	6	36 ± 3‡	103 ± 8	348 ± 32‡	1.65 ± 0.13	0.32 ± 0.01
COLO-16 (10%)	6	38 ± 2‡	154 ± 4‡	608 ± 65‡	3.0 ± 0.1‡	0.72 ± 0.03‡
HSF I (10%)	2	54 ± 1‡	98 ± 2	385 ± 26‡	1.68 ± 0.11	0.34 ± 0.01
HSF II (10%)	2	90 ± 3	99 ± 1	105 ± 8	1.67 ± 0.12	0.34 ± 0.01
HSF III (10%)	2	101 ± 2	101 ± 2	103 ± 10	1.66 ± 0.11	0.33 ± 0.02

* Mean ± SD.
‡ Differences statistically significant when compared with control ($p < 0.001$).

TABLE II

Time-dependent Changes in Accumulation over a 24-h Period and Reactivity with Con A of αPI Secreted by Hep 3B Exposed to 10% CM for Various Time Periods

Period of incubation	Accumulation	Glycosylation changes (RC)
h	%	
Control	100*	1.67 ± 0.1§
24	171 ± 26‡	2.95 ± 0.6
48	359 ± 18	3.55 ± 0.21
72	252 ± 13	4.77 ± 0.19
96	227 ± 22	3.92 ± 0.13

* αPI accumulation at each time point is expressed as percentage increase over the corresponding control.
‡ Mean ± SD.
§ Reactivity coefficient of αPI in control cultures, in the absence of added inducers, did not change significantly in any time period.

synthesis of N-glycosidically linked oligosaccharides, completely inhibited reactivity of both APP with the lectin (results not shown).

Discussion

Several mechanisms could be responsible for the effect of CM on patterns of glycosylation of αPI and Cp. Human αPI and Cp both consist of one polypeptide chain containing three oligosaccharides attached to three separate asparagine residues by N-glycosyl linkages. Both of these APP exhibit multiple microheterogeneous forms in serum, differing in the composition of their bi- and triantennary heteroglycan structures (15–17). Con A binds unsubstituted hydroxyl groups at carbons 3, 4, or 6 of α-linked 2-O-substituted mannose residues, and at least two interacting molecules are required for this binding (18). Only biantennary structures of αPI and Cp react with the lectin, while triantennary structures do not. Thus, the alterations in glycosylation patterns observed in the present studies may reflect a relative increase of bi- over triantennary structures attached to the polypeptide chain. It is possible that various glycosylating enzymes could be activated or depressed by constituents of CM (19). There could, for
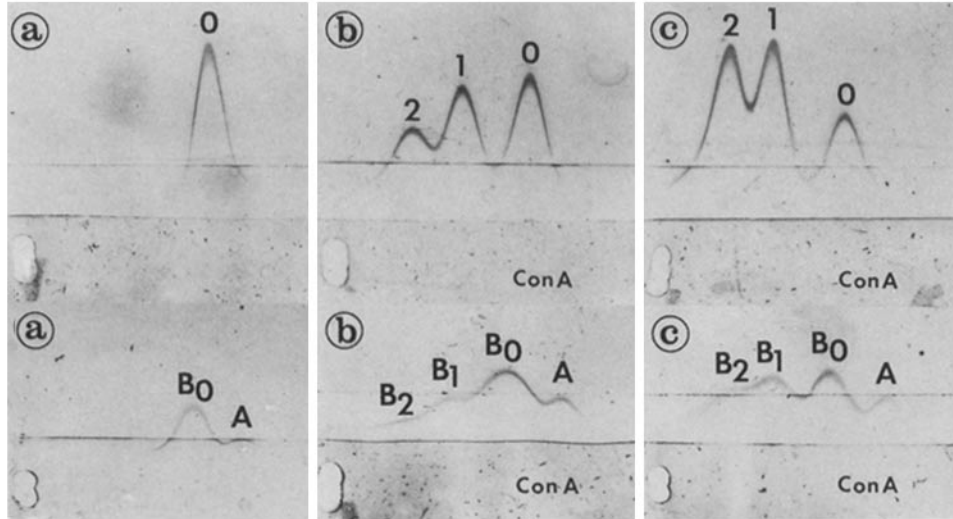
FIGURE 1. Affinity electrophoresis (AFF-EP) of αPI (*top*) and Cp (*bottom*) synthesized and secreted by Hep 3B cells. (*a*) Supernatant from Hep 3B cells maintained in culture medium alone for 48 h subjected to electrophoresis in the absence of Con A in the first dimension. (*b*) The same sample as in *a*, except that electrophoresis was carried out in the presence of Con A; (*top*) variant 0, nonreactive with Con A (migrating to the same distance as αPI in *a*); variant 1, weakly reactive with Con A; and variant 2, strongly reactive with Con A. (*Bottom*) Variants A and B0, nonreactive with Con A; variant B1, weakly reactive with Con A; and variant B2, strongly reactive with Con A. (*c*) Medium from Hep 3B cells incubated with 10% CM for 48 h.
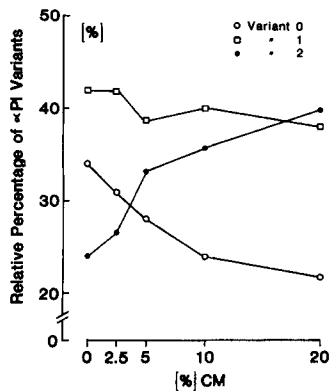

FIGURE 2. Dose-dependent changes of relative amounts of three αPI variants secreted by Hep 3B exposed to CM for 48 h. Variant 0, nonreactive with Con A; variant 1, weakly reactive with Con A; variant 2, strongly reactive with Con A.

example, be a decrease in synthesis or activity of the β-N-acetylglucosaminyl transferase that converts the biantennary to triantennary oligosaccharides. A second possibility is that other glycosylating enzymes that use the same substrates are activated. Finally, it is possible that different kinetics of intracellular transport or different routing may occur in hepatocytes during the acute phase response, with resulting alteration in glycosylation. It has been suggested (3) that increased rate of protein synthesis and lack of parallel activation of certain transferases may result in increase of attached biantennary structures. This possibility seems to be least likely, as increased synthesis of Cp induced by IL-1, TNF, and HSF I

in these studies was not accompanied by changes in its glycosylation. In addition, we observed a time-dependent, diminishing response to induction by CM while the effect on glycosylation pattern of αPI was not affected by time (Table II).

These studies demonstrate that changes of APP glycosylation seen in human sera during various inflammatory states can be explained by alterations occurring within hepatocytes, and that these changes are regulated by one or more monokines. Different mechanisms appear to regulate synthesis and glycosylation of APP, since changes in rates of synthesis of Cp caused by IL-1, TNF, and HSF I were not accompanied by alterations in glycosylation of this protein and the time required for maximal induction and maximal reactivity with Con A of αPI varied substantially.

Summary

The acute-phase response to inflammatory stimuli, characterized by increased synthesis of acute-phase proteins (APP), is often accompanied by changes in the glycosylation patterns of some of these proteins. While expression of APP genes in hepatocytes is regulated by monokines, mechanisms governing changes in glycosylation are not known. Exposure of human hepatoma cell line Hep 3B to conditioned medium from LPS-activated human monocytes and to medium from the keratocarcinoma cell line COLO-16 led to increased synthesis of α_1 proteinase–inhibitor and ceruloplasmin and to alterations of their glycosylation patterns similar to those seen in human serum in various inflammatory states. IL-1, tumor necrosis factor, and hepatocyte stimulating factor I increased synthesis of ceruloplasmin without alterations in the pattern of its glycosylation. These findings demonstrate that altered glycosylation seen in plasma in some inflammatory states can be explained by the effects of monokines on glycosylation in hepatocytes and that gene expression and glycosylation of some APP during the acute-phase response may be regulated by different mechanisms.

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