Different capabilities of monocytes from patients with systemic lupus erythematosus and rheumatoid arthritis to induce glycosylation alterations of acute phase proteins in vitro

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Abstract

The effect of conditioned medium on the biosynthesis and glycosylation profile of acute phase proteins secreted by the human hepatoma cell line Hep G2 was studied. Conditioned medium was prepared from non-activated [CM-LPS(-)] and ex vivo lipopolysaccharide activated [CM-LPS(+)] monocytes from eight patients with active rheumatoid arthritis (RA), five patients with active systemic lupus erythematosus (SLE), and seven healthy subjects. The biosynthesis of albumin, α1-antichymotrypsin and α1-proteinase inhibitor and the profile of glycosylation of proteinase inhibitor were analysed. CM-LPS(-) from patients with SLE had a similar effect to CM-LPS(-) from healthy subjects. In contrast, CM-LPS(-) from patients with RA had the same effect as CM-LPS(+). The treatment of CM-LPS(-) with antibodies against interleukin 6 neutralised most of its ability to induce changes in the biosynthesis and glycosylation of acute phase proteins. Antibodies to interleukin 1 and tumour necrosis factor α had only a limited effect on the ability of CM-LPS(+) to induce changes of albumin and α1-antichymotrypsin syntheses, whereas they had no effect on the biosynthesis and glycosylation of proteinase inhibitor. These results indicate that: (a) monocytes isolated from patients with active SLE and active RA have different capabilities of inducing alterations of acute phase proteins in vitro; (b) ex vivo activation of monocytes from patients with SLE leads to the full induction of its capabilities to change acute phase proteins, whereas the activation of monocytes from patients with RA has no additive effect; and (c) interleukin 6 seems to be a major cytokine involved in the regulation of the glycosylation pattern of acute phase proteins.

Following tissue injury, infection, or various inflammatory processes, a number of normal homeostatic mechanisms are substantially changed. The characteristic pattern of these alterations is termed the acute phase response and includes changes in the concentration of a number of plasma proteins referred to as acute phase proteins. Quantitative changes in acute phase proteins are often accompanied by qualitative alterations of their carbohydrate moieties; this is referred to as microheterogeneity. Two types of microheterogeneity have been distinguished: major microheterogeneity, which reflects changes in the number of branches on heteroglycan antennary structures; and minor microheterogeneity which includes variations in the sialic acid or fucose content. Affinity electrophoresis with concanavalin A as a ligand has been widely used for the determination of major microheterogeneity of acute phase proteins in serum samples. In this system microheterogeneous forms with biantennary heteroglycans can be separated from forms having tri- and/or tetra-antennary units. For multiheteroglycan proteins the degree of reactivity with concanavalin A depends on the number of biantennary structures present on the molecule.

Two different types of changes in the glycosylation pattern of acute phase proteins in serum samples were found; type I, characterised by an increase in the relative amount of biantennary over more branched structures (increased concanavalin A reactivity), which is seen in patients with acute inflammatory processes; and type II, with a decrease in the relative amount of biantennary over more branched units (decreased concanavalin A reactivity), which is found in patients with chronic inflammatory disease. A decrease in reactivity with concanavalin A of a number of acute phase proteins, mostly marked for α1 acid glycoprotein, which was parallel to the increase of disease activity has been seen in serum samples from patients with RA. However, in serum samples from patients with systemic lupus erythematosus (SLE), the reactivity of α1 acid glycoprotein with concanavalin A was similar to that seen in serum samples from healthy subjects. Serum samples from patients with RA and SLE who had intercurrent infections showed a dramatic increase of reactivity between concanavalin A and α1 acid glycoprotein.

An in vitro system has been developed to study the mechanisms controlling the glycosylation changes of acute phase proteins. With this system we have shown that alterations in the glycosylation of acute phase proteins observed in serum samples result from changes occurring within hepatocytes and are mediated by cytokines such as interleukin 6, transforming growth factor β1, and, to some extent, tumour...
necrosis factor α. Moreover, the activity of interleukin 6 and transforming growth factor β  
may be modulated by interleukin 1 and tumour necrosis factor α. We have also found that  
the mechanisms regulating the glycosylation changes of acute phase proteins are independent of  
mechanisms regulating the gene expression of these proteins.

The monocyte/macrophage plays a central  
part in the mediation of acute phase response  
events. Cytokines released by this cell are  
involved in the regulation of gene expression  
and alterations of the glycosylation of the acute  
phase proteins in the liver. These studies  
were performed to appraise the capabilities of  
monocytes isolated from patients with active  
SLE and RA to affect the glycosylation profile of  
acute phase proteins in vitro. We evaluated  
both the spontaneous abilities and the abilities  
of ex vivo activated monocytes. To characterise  
the cytokines involved in these processes,  
we neutralised the inducing activity of cytokines  
secreted by monocytes using specific antibodies.

Patients and methods

Patients

Heparinised blood was obtained from five  
patients with active SLE (grade III of activity),  
eight patients with active RA (grade IV of  
activity) and seven healthy subjects. SLE and  
classic RA were diagnosed based on the  
American Rheumatism Association criteria.  
The activity of SLE was assessed according to  
the Rothfield and Pace scale, whereas the  
activity of RA was assessed according to Mallya  
and Mace.

Preparation of conditioned medium from  
activated and non-activated human  
peripheral blood monocytes

Mononuclear cells were isolated from 50 ml of  
heparinised peripheral blood collected from  
patients with SLE, RA, and healthy subjects,  
using Ficoll-uropolinum gradient centrifugation.  
The cell population obtained was suspended  
in Eagle’s minimal essential medium (Biomed,  
Poland) (3 × 10⁶ cells/ml) and supplemented  
with 20% fetal calf serum (Gibco Laboratories)  
and antibiotics. A 10 ml volume of the prepared  
suspension was placed into plastic Petri dishes  
(100 mm) and the cells were incubated for one  
and a half hours. Adherent cells, 80–90% of  
which were positive for non-specific esterase  
staining, were washed and incubated for  
a further 24 hours in 5 ml of serum free  
Eagle’s minimal essential medium containing  
antibiotics. This medium was referred to as  
CM-LPS(−). For the preparation of  
CM-LPS(+), 20 µg/ml of lipopolysaccharide  
from Escherichia coli 055:B5 (Sigma Chemical)  
was added to the medium.

Neutralisation of the activity of  
conditioned medium with antibodies  
directed against specific cytokines

In two independent experiments, CM-LPS(+)  
prepared from monocytes isolated from two  
different healthy subjects was incubated with  
specific polyclonal antibodies directed against  
cytokines. Antibodies to interleukin 6 (a gift  
of Dr P Seghal, Rockefeller University, New  
York, NY, USA), interleukin 1α (Genzyme,  
Boston, MA, USA), interleukin 1β (a gift of  
Dr C A Dinarello, Tufts University Medical  
School, Boston, MA, USA), tumour necrosis  
factor α (a gift of Dr J Mathison, Scripps  
Clinic Foundation La Jolla, CA, USA) were  
added separately and together to CM-LPS(+)  
and incubated for two hours at 37°C. The  
amounts of antibodies used were based on  
titration results. The minimum amount of each  
antibody generating the maximum neutralisation  
of conditioned medium to inhibit albumin  
synthesis was selected. As a control, preimmune  
rabbit serum was incubated with CM-LPS(+)  
in corresponding volumes.

Induction of changes in acute phase  
proteins in the human hepatoma cell line  
Hep G2

After subculture, cells were maintained in plastic  
Petri dishes (35 mm) in Eagle’s minimal essential  
medium with 10% fetal calf serum for five days  
and then for 24 hours in serum free minimal  
essential medium supplemented with dexamethasone (1 µmol/l) and insulin (0.02 U/ml).  
The Hep G2 cells were then exposed to a 10% solution of CM-LPS(−) and CM-LPS (+)  
prepared from healthy subjects and patients  
with SLE and RA, in addition to 20% CM- 
LPS(+) from healthy subjects treated with  
antibodies against cytokines. In control cultures  
cells were exposed to minimal essential medium  
alone. The cells were then incubated for an  
additional 72 hours with the replacement of  
medium (containing conditioned medium)  
every 24 hours. Analyses were carried out in  
media collected during the final 24 hours. Each  
experiment was run in duplicate or triplicate.

Quantitation of acute phase proteins  
accumulated in collected culture media

Concentrations of albumin, α₁-antichymotrypsin,  
and α₁-protease inhibitor secreted into the  
culture media by Hep G2 cells were determined  
by quantitative electroimmunoassay with  
monospecific antibodies, using a human serum  
calibrator kit (Atlantic Antibodies) as a standard.  
The results were expressed as a percentage  
increase or decrease over control experiments.  
We have previously shown that the increased  
accumulation of proteinase inhibitor in Hep 3B  
cell medium was parallel to the increase of  
newly synthesised 35S-methionine labelled  
proteinase inhibitor and the increase in its  
intracellular mRNA concentration. Thus, the  
changed accumulation of proteins in the medium  
reflects, in general, changes in the rate of their  
synthesis by the hepatoma cell lines.
Glycosylation alterations of acute phase proteins in SLE

...phoresis with concanavalin A (50 μmol/l; Sigma Chemical). 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 for each sample was calculated according to the relation: 

sum of concanavalin A reactive variants/ concanavalin A non-reactive variant.

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

Results

EFFECT OF CONDITIONED MEDIUM ON SYNTHESIS OF ALBUMIN, α1-ANTICHYMOTRYPSIN AND PROTEINASE INHIBITOR BY THE HEP G2 CELL LINE

Table 1 shows changes in the synthesis of the studied proteins by Hep G2 cells exposed to various conditioned preparations. CM-LPS(−) and CM-LPS(+) obtained from healthy subjects reduced the synthesis of albumin to the same degree. Similarly, there was no significant difference between the effect on albumin production of CM-LPS(−) and CM-LPS(+) obtained from patients with RA and SLE. The total reduction of albumin synthesis by conditioned medium from patients with RA was more pronounced, whereas the reduction by conditioned medium from patients with SLE was less pronounced than the reduction caused by conditioned medium from healthy subjects. CM-LPS(+) obtained from healthy subjects or patients with SLE had stronger effects than the corresponding CM-LPS(−) on the increase of α1-antichymotrypsin and proteinase inhibitor synthesis. In contrast, CM-LPS(−) from patients with RA increased the synthesis of α1 antichymotrypsin and protease inhibitor to the same high degree as CM-LPS(+).

EFFECT OF CONDITIONED MEDIUM ON THE MICROHETERGENEITY OF PROTEINASE INHIBITOR SYNTHESISED BY THE HEP G2 CELL LINE

Affinity electrophoresis with concanavalin A as a ligand revealed three microheterogeneous forms of proteinase inhibitor (fig): variant 0, not reactive with concanavalin A; variant 1, weakly reactive with concanavalin A; and variant 2, reactive with concanavalin A. The figure shows the changes in the proteinase inhibitor glycosylation pattern caused by various conditioned medium preparations. Table 2 gives the reactivity of concanavalin A with proteinase inhibitor expressed as a reactivity coefficient. All conditioned medium preparations affected the reactivity of proteinase inhibitor with concanavalin A compared with controls. CM-LPS(+) obtained from healthy subjects and patients with SLE had a stronger effect on the reactivity of proteinase inhibitor with concanavalin A than CM-LPS(−) prepared from the same cells. The degree of alteration caused by CM-LPS(−) from patients with SLE was the same as that caused by the corresponding conditioned medium from healthy subjects. In contrast, CM-LPS(−) obtained from patients with RA affected the reactivity of proteinase inhibitor with concanavalin A to the same degree as CM-LPS(+), which was similar to that of CM-LPS(+) from patients with SLE and healthy subjects. The inducing capabilities for the synthesis and glycosylation of acute phase proteins of non-activated monocytes from healthy subjects [CM-LPS(−)], which served here as controls, may be due to the partial activation of these cells by adherence to the plastic and/or to possible endotoxin contamination in the laboratory.

Table 1 Albumin (Alb), α1-antichymotrypsin (ACT), and α1-proteinase inhibitor (PI) synthesised by Hep G2 cells exposed to conditioned medium obtained from healthy subjects and patients with rheumatoid arthritis and systemic lupus erythematosus. Control value was 100 in all instances; results expressed as mean (SD) percentage of control.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Protein</th>
<th>CM-LPS(−)</th>
<th>CM-LPS(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects (n=7)</td>
<td>Alb</td>
<td>40 (5)*</td>
<td>41 (10)*</td>
</tr>
<tr>
<td></td>
<td>ACT</td>
<td>555 (35)*</td>
<td>880 (50)*</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>140 (10)*</td>
<td>210 (25)*</td>
</tr>
<tr>
<td>Patients with RA (n=8)</td>
<td>Alb</td>
<td>35 (5)*</td>
<td>38 (5)*</td>
</tr>
<tr>
<td></td>
<td>ACT</td>
<td>910 (35)*</td>
<td>940 (50)*</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>205 (25)*</td>
<td>230 (20)*</td>
</tr>
<tr>
<td>Patients with SLE (n=5)</td>
<td>Alb</td>
<td>54 (2)*</td>
<td>50 (3)*</td>
</tr>
<tr>
<td></td>
<td>ACT</td>
<td>670 (30)*</td>
<td>800 (20)*</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>110 (15)</td>
<td>190 (20)*</td>
</tr>
</tbody>
</table>

*Significant difference compared with control.
†Significant difference compared with CM-LPS(−).

Table 2 Reactivity of concanavalin A with proteinase inhibitor secreted by Hep G2 cells exposed to conditioned medium obtained from healthy subjects, and patients with rheumatoid arthritis and systemic lupus erythematosus.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>CM-LPS(−)</th>
<th>CM-LPS(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>7</td>
<td>2·0 (0·2)*</td>
<td>1·0 (0·3)*</td>
</tr>
<tr>
<td>Patients with RA</td>
<td>8</td>
<td>1·2 (0·2)*†</td>
<td>1·0 (0·1)*†</td>
</tr>
<tr>
<td>Patients with SLE</td>
<td>5</td>
<td>2·1 (0·2)*†</td>
<td>1·2 (0·1)*†</td>
</tr>
</tbody>
</table>

*p<0·001 compared with control.
†p<0·001 compared with CM-LPS(−) of healthy subjects.

Affinity electrophoresis with concanavalin A of proteinase inhibitor synthesised by the Hep G2 cell line. (A) Control experiment, (B) incubation with CM-LPS(−) from a healthy subject, and (C) incubation with CM-LPS(+) from a healthy subject.
EFFECT OF CONDITIONED MEDIUM NEUTRALISED BY ANTIBODIES ON THE SYNTHESIS AND GLYCOSYLATION OF THE STUDIED PROTEINS

Table 3 summarises the results obtained. Incubation of conditioned medium with antibodies to interleukin 6, interleukin 1, or tumour necrosis factor α caused partial neutralisation of its ability to inhibit albumin synthesis. The neutralising capabilities of all the antibodies were similar. The combination of all antibodies had an additive effect, but did not completely abolish the ability of the conditioned medium to inhibit albumin synthesis. All three antibodies had an inhibitory effect on the ability of the conditioned medium to increase α1 antichymotrypsin synthesis. Antibodies to interleukin 6 had the most pronounced neutralising activity, whereas the effects of antibodies to interleukin 1α and 1β and tumour necrosis factor α were marginal. The combination of three of these three antibodies had an additive effect, but did not completely neutralise the ability of the conditioned medium to increase α1 antichymotrypsin synthesis. Only the antibody to interleukin 6 was able to partially inhibit the effect of the conditioned medium on proteinase inhibitor synthesis and its reactivity with concanavalin A. Antibodies to interleukin 1α and β used separately or in combination with antibodies to interleukin 6 had an effect on proteinase inhibitor production and reactivity to concanavalin A. Antibodies to tumour necrosis factor α did not neutralise the capabilities of the conditioned medium to induce proteinase inhibitor synthesis. It is possible that antibodies to tumour necrosis factor α had a slight effect on the reactivity between proteinase inhibitor and concanavalin A; however, more data are required to confirm this observation.

Discussion

There are three major findings of these studies. (a) Monocytes isolated from patients with active SLE and active RA have different capabilities of inducing alterations of acute phase proteins in vitro. (b) Ex vivo activation of SLE monocytes leads to the secretion by these cells of cytokines altering glycosylation of acute phase proteins. In contrast, in vitro activation of monocytes from patients with RA does not change their capabilities of inducing alterations of glycosylation in acute phase proteins in Hep G2 cells. (c) Interleukin 6 seems to be a major cytokine secreted by monocytes involved in the regulation of the glycosylation pattern of acute phase proteins. These studies have also shown (by indirect measures) that during active SLE there is an impaired spontaneous generation of interleukin 6, interleukin 1, and tumour necrosis factor α by peripheral blood monocytes, and during active RA this activity is enhanced.

We have shown13,14 in vitro that two different human hepatoma cells lines, Hep 3B and Hep G2, show various patterns of alterations of the glycosylation of acute phase proteins on induction with a crude cytokine preparation. The alterations resemble those seen in serum samples from patients with SLE and RA. Using these two lines as a model, we have shown that a number of cytokines in various specific combinations may affect the pattern of glycosylation of proteinase inhibitor, a model positive acute phase protein. Interleukin 6 seems to induce two types of alterations, type I which is seen in patients with acute inflammatory processes and type II which is found in serum samples from patients with chronic inflammatory disease. Transforming growth factor β induces only type I alterations, whereas tumour necrosis factor α has only a slight effect on type II changes. Interleukin 1 used alone has no effect on any of the alterations. However, when interleukin 1 was added to interleukin 6, it increased its effect on type I but not on type II changes. In contrast, tumour necrosis factor α added to interleukin 6 potentiated its effect on type II, but not on type I alterations. The addition of either interleukin 1 or tumour necrosis factor α to transforming growth factor β inhibited the capabilities for inducing type I alterations. The combination of interleukin 6 with transforming growth factor β had an additive effect on inducing type I and the opposite effect on inducing type II glycosylation changes. These data, taken together with the results of changes in the biosynthesis of acute phase proteins by cytokines, strongly suggest that specific subsets of cytokines are responsible for mediating type I and type II alterations in the glycosylation of acute phase proteins in addition to the biosynthesis of these proteins.

In serum samples from patients with SLE with various degrees of disease activity, there are very limited or often no changes in the concentration of acute phase proteins.7,8 Alterations in the glycosylation of acute phase proteins in serum samples are not found in patients with active SLE, whereas patients with SLE with intercurrent infections have a significant increase in the number of acute phase proteins.9 Changes in the glycosylation of acute phase proteins (type I) are almost always found in these
patients. In contrast, in the course of RA, the whole spectrum of (type II) alterations of acute phase proteins is seen, and the observed changes correlate with the degree of disease activity. 

Intercurrent infections do not have an additive effect on the concentration of acute phase proteins in serum, but have an opposite effect on the alterations (type I) in the glycosylation of acute phase proteins to that which the exacerbation of the disease evokes.

These results indicate that differences in the pattern of glycosylation of acute phase proteins observed in patients with active SLE and active RA may be associated with different secretions of the cytokines involved in these processes. This finding suggests that activity of RA, but not SLE, is connected with activation of monocytes. Moreover, ex vivo activation by lipopolysaccharides of monocytes from patients with SLE corrected their impaired abilities to induce glycosylation alterations of acute phase proteins, indicating that these cells are capable of responding to exogenous inflammatory stimuli. This observation could explain the basis of altered glycosylation of acute phase proteins seen in patients with SLE with intercurrent infections; studies of monocytes isolated from such patients would directly support this hypothesis.

Monocytes secrete a number of cytokines known to be involved in mediating acute phase protein changes. These include interleukin 6, interleukin 1α and β, tumour necrosis factor α, and transforming growth factor β. The monocyte is not, however, the only cell which secretes cytokines mediating these events. Many other cell types can serve as sources of these cytokines, namely platelets, which are source of transforming growth factor β, or fibroblasts which secrete interleukin 6. However, fibroblasts, for instance, require interleukin 1 or tumour necrosis factor α for the induction of interleukin 6 production. Thus, there is a link between the functional capabilities of monocytes and the activation of other cells, indicating that the defective function of monocytes in patients with SLE may lead to the impaired function of other cells involved in the acute phase response. Accordingly, for a final evaluation, the measurement of various cytokines in serum samples from patients with SLE would be useful. Houssiau et al reported raised levels of interleukin 6 in serum samples from patients with RA, which correlated with the increased concentrations of a number of acute phase proteins in serum. Deng et al observed the same phenomenon in serum samples from patients with endotoxaemia.

In these studies using antibodies against various cytokines, we were not able completely to neutralise the effect of conditioned medium on the induction of changes in the glycosylation of acute phase proteins. It is possible that other cytokines secreted either by monocytes or other adherent cells, which might be present in monocyte preparations, are involved in these processes. At the time of these studies, no neutralising antibodies against transforming growth factor β were commercially available. However, the effect of transforming growth factor β in Hep G2 cells is opposite to the effect of interleukin 6, thus the neutralisation of transforming growth factor β should lead to the increased effect of interleukin 6. In fact, the human hepatoma cell lines Hep G2 and Hep 3B should be used to gain a better understanding of the effect of conditioned medium on various types of glycosylation changes. However, owing to the limited number of cells obtained from patients, especially patients with SLE, the application of both lines in these studies was impossible.

The so-called defective function of monocytes from patients with SLE has been reported, including reduced phagocytic activity, decreased hexose monophosphate shunt and glycolytic activity, and the defective production of interleukin 1. In contrast, the increased generation of interleukin 1 by monocytes from patients with active RA was found. Franchimont et al have shown that peripheral blood mononuclear cells from patients with RA did not spontaneously release tumour necrosis factor α. In our studies we confirmed (by indirect measurements) earlier results of impaired and enhanced generation of interleukin 1 by monocytes from patients with SLE and RA, respectively, and explored the similar effects for interleukin 6 and tumour necrosis factor α.


22 Rothfield N F, Page N. Relation of positive LE-cell prepara-


