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O- and *N*-glycosylation lead to different molecular mass forms of human monocyte interleukin-6

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The biosynthesis and secretion of human interleukin-6 (IL-6) was studied in monocyte cultures stimulated with endotoxin. After labeling with [^{35}S]methionine and immunoprecipitation with a specific antiserum one major (24 kDa) and four minor (27.5, 23.3, 22.5 and 21.8 kDa) molecular mass forms of IL-6 could be found in the cells and media. Incubation of monocyte media with sialidase and subsequently with endo-α-*N*-acetylgalactosaminidase, which cleaves Gal(β1-3)GalNAc from serine or threonine, led to the formation of only two forms of IL-6 with apparent molecular masses of 25 and 21.8 kDa. The latter had an electrophoretic mobility indistinguishable from that of ^{125}I-labeled recombinant human IL-6. The results suggest that human monocyte IL-6 carries *O*-glycosidically bound carbohydrates with a Gal(β1-3)GalNAc core to which only sialic acid is bound. Differences in *O*-glycosylation are the major cause for the molecular heterogeneity of IL-6. A small part of IL-6 (27.5 kDa form) is in addition *N*-glycosylated. Incubation of monocytes with tunicamycin and 1-deoxymynnojirimycin and treatment of IL-6 with endoglucosaminidase H suggested that the 27.5 kDa form of IL-6 carries at least one *N*-linked complex-type oligosaccharide chain.

Interleukin-6; Glycosylation, *N*-; Glycosylation, *O*-; (Human monocyte)

1. INTRODUCTION

IL-6 is a cytokine produced by various cell types, among others, monocytes, macrophages, fibroblasts and endothelial cells. Its synthesis can be induced by different stimuli, e.g. IL-1, TNF-α, PDGF and different viruses and bacterial products such as endotoxon [1-7]. IL-6 exerts a multitude of biological activities in the network of cytokines that is important in the regulation of the hematopoetic and immune systems (for a recent review see [8]). IL-6 is the major stimulus for the induction of acute-phase protein synthesis in the liver [9-11].

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Multiple forms of IL-6 have been described with molecular masses ranging between 20 and 30 kDa [12-16]. May et al. [14] found at least five forms of IL-6 in human fibroblasts. They demonstrated that IL-6 is both *O*- and *N*-glycosylated and in addition phosphorylated [15]. Similarly, Bauer et al. [16] described five molecular mass forms of IL-6 in human monocytes and confirmed the existence of *N*-glycosylated forms. Absence or presence of *N*-glycosylation, however, can only account for two different molecular mass forms of IL-6 and does not explain the observed molecular heterogeneity. We therefore analyzed the *O*-linked carbohydrate side chains of human monocyte IL-6 by digestion with sialidase and endo-α-*N*-acetylgalactosaminidase and found that differences in *O*-linked carbohydrate side chains are the major cause for the molecular heterogeneity of IL-6.

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2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{35}S]methionine (>1000 Ci/mmol) was purchased from Amersham-Buchler (Braunschweig, FRG). Ficoll-paque and protein A-Sepharose were from Pharmacia (Freiburg, FRG). 1-Deoxymannojirimycin, endoglucosaminidase H from *Streptomyces plicatus* and endo-α-*N*-acetylgalactosaminidase were from Boehringer (Mannheim, FRG). Sialidase from *Vibrio cholerae* was from Calbiochem (Giessen, FRG), tunicamycin from Sigma (München, FRG).

2.2. *Preparation of an antiserum against human IL-6*

A specific antiserum against human IL-6 was obtained in a rabbit by two successive subcutaneous injections of 100 μg of recombinant human IL-6 in Freund's complete adjuvant at a three week interval followed by a third injection four weeks later with 50 μg of IL-6 in Freund's incomplete adjuvant. The antiserum impaired the biological activity of IL-6 measured by the induction of fibrinogen mRNA in Fao cells [10].

2.3. *Iodination of recombinant human IL-6*

Iodination of recombinant human IL-6 was carried out as described [17].

2.4. *Human monocyte cultures, radioactive labeling, immunoprecipitation and electrophoresis*

Human monocytes were prepared from buffy coats as described by Andreesen et al. [18]. Mononuclear cells were separated from granulocytes and residual erythrocytes by centrifugation over Ficoll-paque. Monocytes were then separated from lymphocytes by adherence to plastic at 37°C in RPMI 1640 medium containing 10% fetal calf serum. The adherent cells were then incubated overnight with RPMI 1640 medium containing 5% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Monocytes (about 2×10^6 cells/dish) obtained after overnight incubation were radioactively labeled with [^{35}S]methionine (150 μCi/dish) for 3 h. When monocytes were incubated with tunicamycin (5 μg/ml) or 1-deoxymannojirimycin (4 mM), the respective drugs were added to the cells 1 h prior to the radioactive labeling and were kept in the cell media at the same concentrations during the labeling period. At the end of the incubation the media were separated from the cells. IL-6 was immunoprecipitated from cells and media as described [19]. The immunoprecipitated IL-6 was analyzed by SDS-polyacrylamide gel electrophoresis [20] and fluorography [21].

2.5. *Glycosidase treatment*

For sialidase treatment, monocyte media were dialyzed exhaustively against 50 mM sodium acetate buffer, pH 5.5, 1.0 mM $CaCl_2$ and subsequently incubated with sialidase from *Vibrio cholerae* (200 mU/ml medium) at 37°C for 16 h. The sialidase-treated monocyte media were further dialyzed against 20 mM sodium phosphate buffer, pH 6.5, and incubated with 25 mU of endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* at 37°C for 16 h.

For endoglucosaminidase H treatment, the IL-6-IgG complexes eluted from protein A-Sepharose were dialyzed exhaustively against 50 mM sodium phosphate buffer, pH 6.0, containing 0.01 mg/ml SDS and incubated in a total volume of 0.1 ml with 5 mU of endoglucosaminidase H at 37°C for 16 h.

3. RESULTS AND DISCUSSION

Human monocyte cultures (2×10^6 cells) were labeled with [^{35}S]methionine for 3 h to study the biosynthesis and secretion of IL-6. In monocyte cultures stimulated with endotoxin (100 ng/ml medium) different molecular mass forms of IL-6 could be found in cells and media (fig.1). Since the most of newly synthesized IL-6 had been secreted during the labeling period only faint bands which had the same electrophoretic mobilities as secreted IL-6 (fig.1, lanes 4,7) could be found in the cells (fig.1, lanes 3,5). The predominant form of human IL-6 had an apparent molecular mass of 24 kDa. This is in accordance with the findings of May et al. [14,15] and Bauer et al. [16]. Besides this 24 kDa form a higher molecular mass form (27.5 kDa) and three low molecular mass forms (21.8 kDa, 22.5 kDa and 23.3 kDa) could be detected. The 21.8 kDa and 22.5 kDa forms were only faint bands. The synthesis of all these different molecular mass forms of IL-6 was less when monocytes were cultured in endotoxin-free media (fig.1, lanes 1,2).

To exclude the possibility that the different molecular mass forms of IL-6 were due to contaminations appearing during immunoprecipitation competition experiments were carried out. When immunoprecipitations were done in the presence of 10 μg of unlabeled recombinant human IL-6 the intensity of all the bands representing IL-6 (27.5, 24, 23.3, 22.5 and 21.8 kDa) was strongly decreased, while the intensity of some higher molecular mass contaminating bands (especially found in cells) remained unchanged (fig.1, lanes 6,8). The faint band (arrow) above the 27.5 kDa form of IL-6 was not strongly suppressed when the immunoprecipitation was carried out in the presence of unlabeled IL-6, suggesting that this band represents a contamination rather than a form of IL-6.

Our pattern of human monocyte IL-6 obtained by SDS-polyacrylamide gel electrophoresis is similar, but not identical to that described by May et al. [14]. They found in fibroblasts a lower molecular mass doublet (23 and 25 kDa), and a higher molecular mass triplet (28, 29 and 30 kDa).

When *N*-glycosylation of IL-6 was inhibited by incubation of monocytes with tunicamycin, the 27.5 kDa form of IL-6 disappeared, whereas the

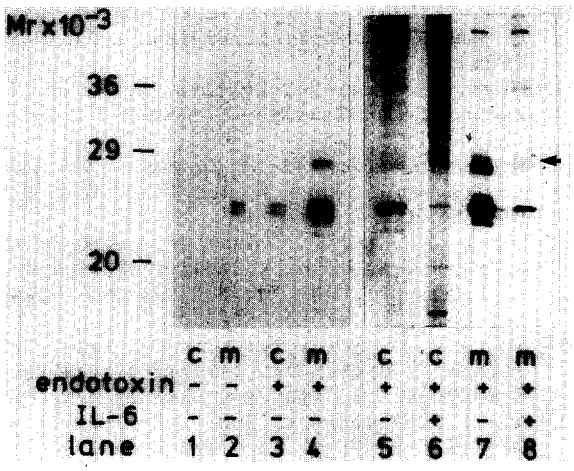

Fig.1. Biosynthesis and secretion of IL-6 by human monocytes. Human monocyte cultures were incubated without (lanes 1,2) or with (lanes 3–8) 100 ng/ml of endotoxin for 16 h and subsequently labeled with [^{35}S]methionine (150 μCi/2 × 10^6 cells) for 3 h. IL-6 was immunoprecipitated from the cells (c) (lanes 1,3,5,6) and media (m) (lanes 2,4,7,8) in either the absence (lanes 1–5,7) or presence (lanes 6,8) of 10 μg of unlabeled recombinant human IL-6.

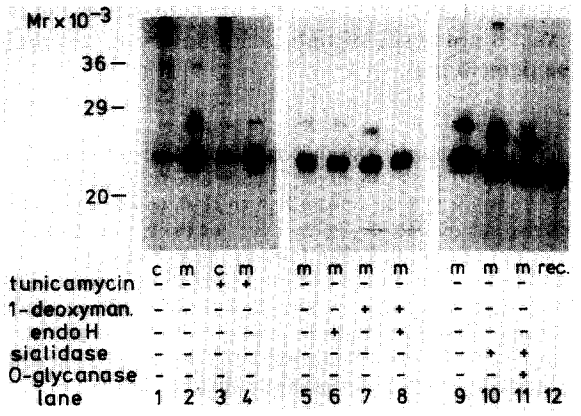

Fig.2. Deglycosylation of human IL-6. IL-6 was immunoprecipitated from the cells (lanes 1,3) or media (lanes 2,4) of untreated (lanes 1,2) or tunicamycin-treated (lanes 3,4) monocyte cultures. To analyze *N*-linked carbohydrates IL-6 was immunoprecipitated from untreated (lanes 5,6) or 1-deoxymannojirimycin-treated (lanes 7,8) monocyte cultures and incubated without (lanes 5,7) or with (lanes 6,8) endoglucosaminidase H. To analyze *O*-linked carbohydrates IL-6 was immunoprecipitated from untreated monocyte media (lane 9) or from media incubated with sialidase (lane 10) and from media incubated with sialidase and subsequently with endo-α-*N*-acetylgalactosaminidase (lane 11). For comparison, lane 12 shows ^{125}I-labeled recombinant human IL-6.

other molecular mass forms remained unchanged (fig.2, lanes 2,4). This indicates that only the 27.5 kDa form of IL-6 is *N*-glycosylated. The *N*-glycosylated form of IL-6 could not be deglycosylated by endoglucosaminidase H (fig.2, lanes 5,6). When monocytes were incubated with 1-deoxymannojirimycin, which impairs the synthesis of complex-type oligosaccharides and leads to the accumulation of high mannose-type precursor oligosaccharides [22], the *N*-glycosylated form of IL-6 had a smaller apparent molecular mass and could be deglycosylated by endoglucosaminidase H (fig.2, lanes 7,8). This indicates that the 27.5 kDa form of IL-6 carries at least one *N*-asparagine bound complex-type carbohydrate side chain. Since we found only one *N*-glycosylated form of IL-6, there is obviously less heterogeneity in the *N*-glycosylated forms of IL-6 in our monocyte system than in the fibroblasts used by others, who obtained a triplet of *N*-glycosylated IL-6 bands [14].

The primary structure of human IL-6 has two potential *N*-glycosylation sites [23]. Since incubation of monocytes with low concentrations of tunicamycin and incomplete endoglucosaminidase H treatment of monocyte media treated with 1-deoxymannojirimycin did not yield an IL-6 of a molecular mass between 27.5 and 24 kDa (not shown), IL-6 apparently carries only one *N*-linked oligosaccharide chain.

Incubation of monocyte media with sialidase led to increased electrophoretic mobilities of the 27.5, 24 and 23.3 kDa forms of IL-6 (fig.2, lane 10). Additional incubation of the sialidase-treated monocyte media with endo-α-*N*-acetylgalactosaminidase, which cleaves Gal(β1–3)GalNAc from serine or threonine, led to the formation of only two forms of IL-6 with apparent molecular masses of 25 kDa (cleavage product of the 27.5 kDa form) and 21.8 kDa (fig.2, lane 11). The electrophoretic mobility of the latter was indistinguishable from that of ^{125}I-labeled recombinant human IL-6 (fig.2, lane 12).

The glycosidase digestion experiments clearly indicate that the molecular heterogeneity of human monocyte IL-6 is mainly due to differences in *O*-glycosylation. The results allow the conclusion that human IL-6 carries *O*-glycosidically bound carbohydrates with a Gal(β1–3)GalNAc core to which only sialic acid is bound. The number of *O*-linked

carbohydrate side chains per IL-6 molecule and the exact binding site of sialic acid, however, remain to be determined.

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