Acta Diabetol (1999) 36:67-72

ORIGINAL

S. Kado • T. Nagase • N. Nagata

Circulating levels of interleukin-6, its soluble receptor and interleukin-6/interleukin-6 receptor complexes in patients with type 2 diabetes mellitus

Received: 24 August 1998 / Accepted in revised form: 2 February 1999

Abstract We evaluated the relationship between plasma fibrinogen concentration and the serum levels of interleukin-6 (IL-6), its soluble receptor, and their complex in patients with type 2 diabetes mellitus. The study comprised 57 patients with type 2 diabetes and 15 normal healthy controls. Serum levels of IL-6, soluble IL-6 receptor (IL-6R), and circulating IL-6/IL-6R complex were determined by enzyme-linked immunosorbent assays. Correlations between the different study parameters and serum IL-6, IL-6R, or IL-6/IL-6R complex levels were determined by multiple linear regression analysis. Any association between the different study parameters and the serum levels of IL-6, IL-6R, or IL-6/IL-6R complex were determined by stepwise linear regression analysis. The serum IL-6 level in diabetic subjects was significantly higher than in normal healthy controls (3.48 ± 3.29 pg/ml vs 0.784 ± 0.90 pg/ml, mean ± SD, respectively, $P = 0.0001$). The specific optical density of the serum IL-6/IL-6R complex in diabetic patients was also significantly higher than in normal healthy controls, although there was no significant difference in the serum IL-6R level between diabetic patients and controls. The serum IL-6 concentration was correlated significantly with the HbA_{1C} level ($\beta = 0.58$, $P = 0.04$) by multiple regression analysis. Stepwise regression analysis revealed that the levels of serum IL-6 ($F = 8.251$), HbA_{1C} ($F = 7.108$), and serum urea nitrogen ($F = 5.603$) were associated with the plasma fibrino gen concentration. These results suggest that hyperglycaemia and increased levels of serum IL-6 can increase the plasma fibrinogen concentration, one of the known risk factors for atherosclerosis in patients with type 2 diabetes mellitus.

Key words Interleukin-6 • Interleukin-6 receptor • Fibrinogen • Diabetes mellitus

S. Kado (✉)
Department of Internal Medicine 5,
Tokyo Medical University, Chuo 3-20-1,
J-300-0395 Ami, Inashiki, Ibaraki, Japan

T. Nagase • N. Nagata
Department of Internal Medicine 3, National
Defense Medical College, Saitama, Japan

Introduction

Patients with diabetes mellitus have been reported to have increased levels of fibrinogen in their plasma and this is now recognized to be one of the cardiovascular risk factors in diabetes mellitus [1, 2]. The Prospective Cardiovascular Munster (PROCAM) study also showed that increased plasma fibrinogen is an independent risk factor for atherosclerosis [3]. Additionally, in the same study, raised fibrinogen was found to be associated with obesity, lack of exercise, serum triglycerides, serum cholesterol, male sex, and diabetes. It is important to recognize the existence of this putative hypercoagulability state in the overall care of diabetic patients since they have a high incidence of ischaemic heart disease and other vascular diseases.

Interleukin-6 (IL-6) is a multifunctional cytokine that induces a variety of biological responses in different target cells.

IL-6 has been shown to induce fever and the secretion of acute phase proteins such as fibrinogen from hepatocytes [4, 5]. Two membrane proteins, IL-6 receptor (IL-6) and gp130, are both necessary and sufficient for constituting a functional receptor complex. Recently, the presence of soluble IL-6R and IL-6/IL-6R complexes was reported in serum, and levels of the IL-6/IL-6R complex are increased in patients with systemic juvenile rheumatoid arthritis [6].

Therefore, in this study, we have investigated the levels of IL-6, its soluble receptor, and their complex in sera taken from patients with type 2 diabetes in order to clarify the influence of this cytokine system on the plasma fibrinogen concentrations.

Subjects and methods

Subjects

Fifty-seven patients with type 2 diabetes (mean age 57.6 years, range 23-77; mean duration of diabetes 9.8 years, range 2-23) and 15 normal healthy controls (mean age 50.0 years, range 25-76) were studied. All of the subjects were non-smokers. Twenty-four diabetic patients had simple retinopathy, 14 patients had proliferative retinopathy while 19 had no retinopathy. Microalbuminuria was present in 12 patients, while 18 had overt proteinuria. Mild neuropathy was seen in 33 patients, while the other 24 patients had no neuropathy. Informed consent was obtained from all subjects according to the 1964 declaration of Helsinki. Blood and urine samples were taken early in the morning while the patients fasted for serum glucose, serum total cholesterol, serum triglycerides, serum high density lipoprotein (HDL)-cholesterol, serum urea nitrogen, serum creatinine, plasma fibrinogen, and HbA_{1C}. Urinary albumin and urinary creatinine levels were also determined.

Serum glucose was measured by the hexokinase method. Serum total cholesterol and triglycerides were determined by enzymatic calorimetric method (Determiner TC555 and TG555 diagnostic kits, Kyowa Medex, Tokyo, Japan). Serum HDL-cholesterol was determined on the supernate obtained following precipitation of plasma with heparin-manganese (Determiner-L HDL-C diagnostic kits, Kyowa Medex). Plasma fibrinogen was determined using an automated photo-optical coagulometer (CoagmasterII, Sankyo, Tokyo, Japan). HbA_{1C} was measured by HPLC method (Hi-AUTO A1C, KDK, Kyoto, Japan). Urinary albumin was determined by latex turbimetric immunoassays (Eiken alb, Eiken Chemicals, Tokyo, Japan). Baseline characteristics of the subjects are shown in Table 1.

Table 1 Baseline characteristics of the subjects

	Control subjects	Diabetic patients	*P*-value
n	15	57	
Age (years)	49.4±14.6	57.4±12.6	0.02
Duration of diabetes (years)	-	9.8±5.4	
Fasting serum glucose (mmol/L)	4.99±0.96	9.43±5.03	0.0001
HbA_{1C} (%)	5.4±0.8	8.7±2.0	0.001
Serum total cholesterol (mmol/L)	5.39±0.93	5.40±1.10	NS
Serum triglycerides (mmol/L)	1.37±0.87	1.75±0.92	NS
Serum HDL-cholesterol (mmol/L)	1.42±0.32	1.33±0.39	NS
Serum urea nitrogen (mmol/L)	4.33±0.88	6.03±2.66	NS
Serum creatine (μmol/L)	64.09±21.04	73.20±64.09	0.005
Plasma fibrinogen (μmol/L)	8.44±1.16	10.35±1.82	0.02
Urinary albumin-creatinine (mg/g creatinine)	8.5±6.7	225.9±461.6	0.001

Data are *n* or means ± SD
HDL, high-density lipoprotein; *NS*, not significant

Serum samples for IL-6, sIL-6R, and circulating IL-6/IL-6R complexes were stored at -80°C until enzyme-linked immunosorbent assays (ELISAs) were performed.

Determination of serum IL-6, IL-6R, and IL-6/IL-6R complex

Serum IL-6 and IL-6R levels were determined using commercially available ELISA kits (Cytoscreen human IL-6 immunoassay kit and Cytoscreen human sIL-6R immunoassay kit; BioSource International, Camarillo, Calif. USA). The limits of sensitivity of ELISAs for serum IL-6 and sIL-6R levels were 2 pg/ml and 0.5 ng/ml, respectively.

To measure levels of the IL-6/IL-6R complex, a 96-well dish coated with anti-IL-6R antibody (B-N12, BioSource International) was used. Serum (200 μl) and biotinylated antibody to IL-6 (50 μl; 8H12, BioSource International) were added to each well and incubated for 2 h at room temperature. After the incubation, the sera were discarded and the wells were washed three times with tris-buffered saline containing 0.02% Tween 20. Streptavidin-horseradish peroxidase conjugate was then added and incubated at room temperature for 20 min. The reaction was stopped by the addition of 100 μl of 2N H_2SO_4, and absorbance was measured at 450 nm.

To compare the levels of IL-6 present in the IL-6/IL-6R complex, the amount of IL-6 was extrapolated from a standard curve obtained by adding increasing concentrations of human recombinant IL-6 to reference serum from a healthy adult control subject and incubating for 45 min at 37°C.

Statistical analysis

Data were analysed by the Mann-Whitney U test for unpaired samples. Correlation among the levels of serum IL-6, IL-6R, and IL-6/IL-6R complex were determined by simple linear regression analysis. Correlation between the different study parameters and serum IL-6, IL-6R, or IL-6/IL-6R complex was determined by multiple linear regression analysis. Any association between the different study parameters and the values for serum levels of IL-6, IL-6R, or IL-6/IL-6R complex was determined by stepwise linear regression analysis. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Serum levels of IL-6, IL-6R in diabetic patients

As shown in Fig. 1, serum IL-6 levels in the diabetic subjects were significantly higher than in normal healthy controls (3.48 ± 3.29 pg/ml vs 0.784 ± 0.90 pg/ml, mean ± SD, respectively, $P = 0.0001$). All of the values for control subjects except two were below 2 pg/ml, the limit of sensitivity.

In contrast to the results of the serum IL-6 study, there was no significant difference in the levels of serum IL-6R between the diabetic patients and controls (97.1 ± 23.9 vs 100.1 ± 23.4 ng/ml, Fig. 2). Simple regression analysis showed there to be a significant correlation ($r = 0.254$, $P = 0.057$) between serum IL-6 and serum IL-6R levels in diabetic patients.

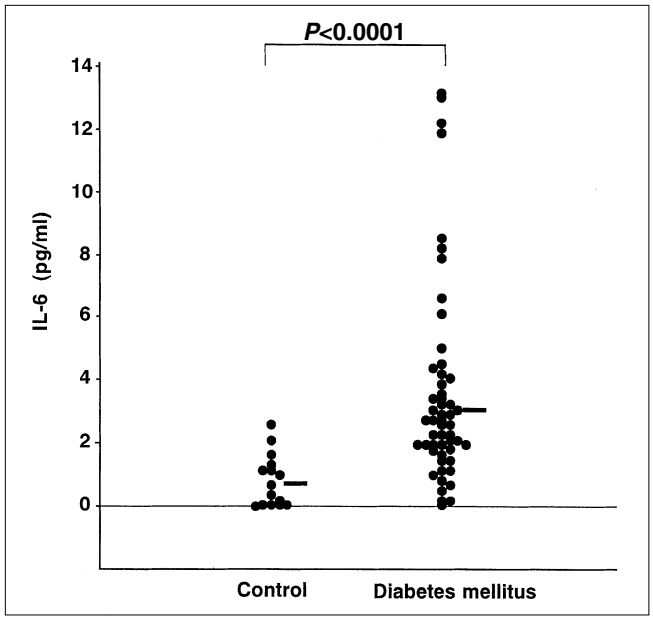

Fig. 1 Serum IL-6 concentrations in normal controls and diabetic patients. The median values for each group are shown by *horizontal bars*

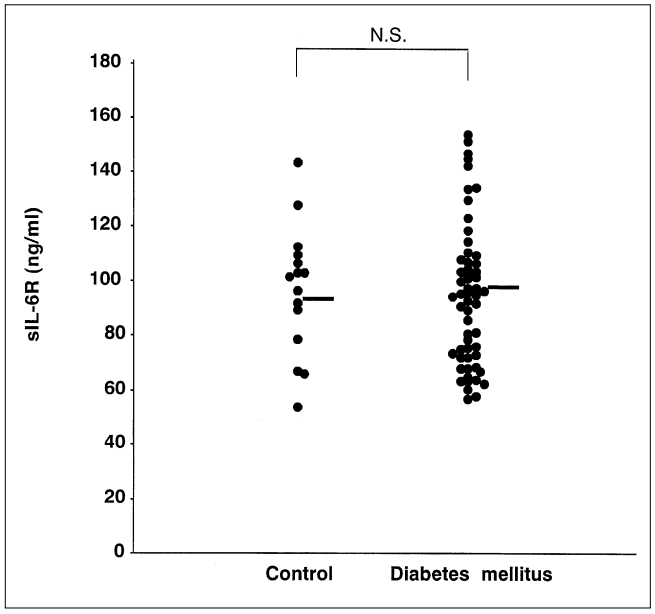

Fig. 2 Serum IL-6R concentrations in normal controls and diabetic patients. The median values for each group are shown by *horizontal bars*. *NS*, not significant

Determination of circulating IL-6/IL-6R complex levels

We constructed an ELISA based on the use of monoclonal antibodies to human soluble IL-6R for capture and monoclonal antibodies to human IL-6 for detection of the serum IL-6/IL-6R complex. The addition of human recombinant IL-6 to normal sera (serum IL-6R: 100.4 ng/ml) resulted in a dose-dependent increase in specific optical density (Fig. 3). These data indicated that our assay could detect the serum IL-6/IL-6R complex formed by the binding of exogenous IL-6 to its soluble receptor. The specific optical density of the serum IL-6/IL-6R complex in the patients with type 2 diabetes was significantly higher than that in normal healthy controls (Fig. 4).

We studied the correlation between the levels of serum IL-6/IL-6R complex, and serum IL-6 or IL-6R by simple regression analysis in the diabetic patients. There was a sig-

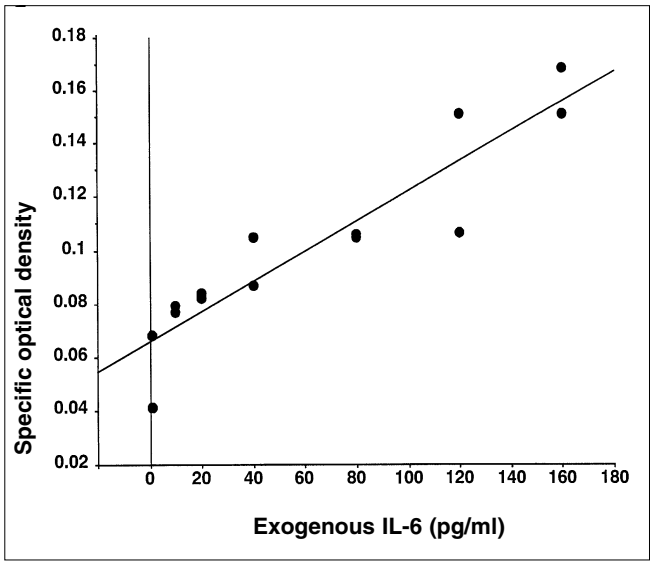

Fig. 3 Dose-response curve of the ELISA for the IL-6/IL-6R complex

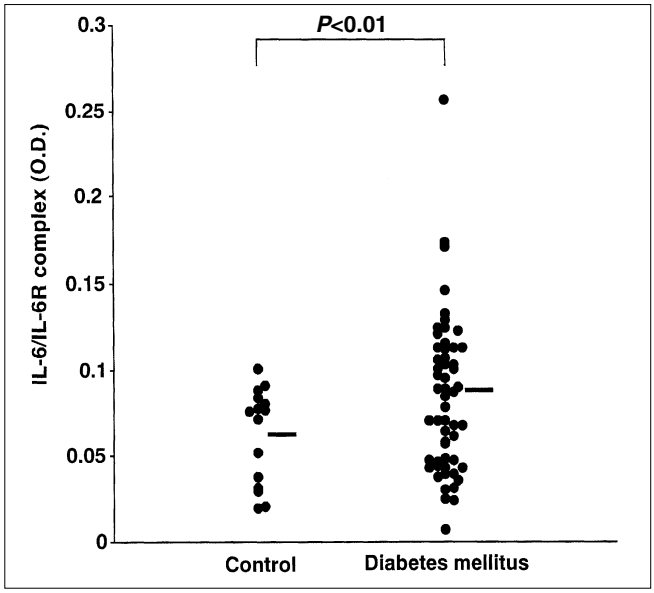

Fig. 4 Specific optical density of serum IL-6/IL-6R complex in normal controls and diabetic patients. The median values for each group are shown by *horizontal bars*

nificant correlation between serum levels of the IL-6/IL-6R complex and IL-6 ($r = 0.448$, $P = 0.005$), but there was no significant correlation between serum IL-6/IL-6R complex and serum IL-6R.

Multivariate analysis of serum IL-6, IL-6R, and IL-6/IL-6R complex levels in patients with type 2 diabetes

We analysed the correlation between serum levels of IL-6, IL-6R, or Il-6/IL-6R complex and various clinical parameters (age, duration of diabetes, HbA_{1C}, serum glucose, total cholesterol, triglycerides, HDL-cholesterol, urea nitrogen, creatinine, and urinary albumin-creatinine ratio) by multiple regression analysis in patients with diabetes (Table 2). In diabetic patients, serum IL-6 correlated significantly with HbA_{1C}, and serum IL-6R correlated significantly with serum creatinine and the urinary albumin-creatinine ratio. There was no correlation between the values of serum IL-6/IL-6R complex and these clinical parameters.

We analysed the determinants of plasma fibrinogen using various clinical parameters (age, duration of diabetes, HbA_{1C}, serum total cholesterol, triglyceride, HDL-cholesterol, urea nitrogen, creatinine, glucose, IL-6, IL-6R, IL-6/IL-6R complex, and urinary albumin-creatinine ratio) by stepwise regression analysis in patients with diabetes. In diabetic patients, serum IL-6, HbA_{1C}, and serum urea nitrogen were independently associated with plasma fibrinogen concentrations (Table 3).

Table 2 Multiple regression analysis between serum IL-6, IL-6R, or IL-6/IL-6R complex and other clinical parameters in patients with diabetes

	IL-6		IL-6R		IL-6/IL6R complex	
	β	*P*	β	*P*	β	*P*
Age	0.14	0.39	0.20	0.20	0.20	0.20
Duration of diabetes	-0.22	0.20	-0.24	0.13	-0.12	0.47
HbA_{1C}	0.58	0.04	0.18	0.47	0.10	0.71
Serum glucose	-0.09	0.69	0.03	0.87	0.27	0.23
Serum total cholesterol	-0.09	0.58	0.03	0.82	0.01	0.96
Serum triglycerides*	-0.04	0.85	0.09	0.60	0.14	0.46
Serum HDL-cholesterol	-0.05	0.76	-0.04	0.80	-0.19	0.25
Serum urea nitrogen	0.05	0.79	-0.20	0.26	0.19	0.32
Serum creatinine	0.12	0.53	0.42	0.02	-0.26	0.80
Urinary albumin-creatinine ratio*	0.08	0.20	0.44	0.01	0.14	0.46

β, standard regression coefficient
* Log-transformed values were used

Table 3 Factors associated with plasma fibrinogen in patients with diabetes

Dependent	Indipendent	Standard regression coefficient	*F*-value
Plasma fibrinogen	IL-6	0.354	8.251
	HbA_{1C}	0.326	7.108
	Serum urea nitrogen	0.264	5.603
		$R^2 = 0.414$ ($P < 0.0001$)	

F-value to enter was set at 4.0 at each step
R^2, multiple coefficient determination

Discussion

Interleukin-6 (IL-6) is a member of a class of pleiotropic cytokines involved in regulating homeostasis and the immune response. IL-6 has diverse biological activities, and has been proposed as a mediator in infectious diseases [7], and various inflammatory diseases [8], including autoimmune disorders [9].

In our study, the serum IL-6 concentration in patients with type 2 diabetes was increased when compared to that observed in control subjects. Our study also revealed that the serum IL-6 concentration correlated significantly with HbA_{1C} in diabetic patients. It can therefore be hypothesized that hyperglycaemia stimulates IL-6 production in hepatocytes, monocytes, or endothelial cells.

Morohoshi et al. [10] reported that IL-6 production in monocytes increases in conditions with high glucose media. They speculated that osmolarity and tumour necrosis factor partially affect IL-6 induction in monocytes in the hyperglycaemic state. Koj et al. [11] reported an increase in IL-6 production from monocytes in vitro in the presence of advanced glycation end-products. Yan et al. [12] also reported that intracellular oxidative stress generated by advanced glycation end-products results in the activation of the free radical-sensitive transcription factor NF-KB, one of the major transcription factors involved in the regulation of IL-6. From these facts, the formation of advanced glycation endproducts may, at least partially, contribute to the induction of IL-6 under hyperglycaemic conditions.

IL-6R is cleaved and the 50-55 kDa soluble protein is released into the circulation [13, 14]. A recombinant soluble truncated form of the IL-6R can bind to IL-6, and interact with membrane gp130 and transduce the biological signal in target cells [15]. The soluble IL-6R binds the ligand IL-6 with an affinity that is comparable to that observed with the membrane-associated receptor [16]. Moreover, IL-6 bound to the soluble IL-6R generated by shedding elicits an IL-6 specific signal on cells which express gp130 on the cell surface [17]. For example, IL-6/IL-6R complex up-regulates the expression of acute-phase proteins in hepatic cells, and

augments the human chorionic gonadotropin in trophoblasts [18, 19]. May et al. [20] reported that soluble IL-6R 'chaperones' IL-6 in an active form and prevents its degradation. Although we did not find a significant correlation between clinical parameters and the elevated circulating levels of the IL-6/IL-6R complex, there is a possibility that these circulating complexes affect areas such as bone metabolism in diabetes.

Elevated plasma fibrinogen concentration is known to be an independent risk factor for atherosclerosis. Plasma fibrinogen is increased in diabetes, vascular disease, smoking, and in many physical and metabolic stresses. The factors which regulate plasma fibrinogen concentration are poorly understood. Thyroid hormone, glucagon and acute insulin deficiency are involved in the increase of fibrinogen concentration [21-23]. IL-4, IL-10 and IL-13 down regulate the biosynthesis of fibrinogen in vitro [24].

Recombinant IL-6 stimulates the synthesis of a wide spectrum of acute phase proteins from human hepatocytes including fibrinogen. In contrast, recombinant IL-1β and recombinant tumour necrosis factor-α exhibit suppressive effects on fibrinogen production [25]. Ceriello et al. [26] reported that the production of fibrinogen shows a direct correlation with hyperglycaemia in both diabetic patients and normal subjects in whom a hyperglycaemic state had been artificially produced. In our study, the plasma fibrinogen concentration was associated with serum IL-6 levels, but not with serum IL-6R or IL-6/IL-6R complex levels. Taken together, these data indicate that the elevated plasma fibrinogen levels in diabetes may, at least partially, be due to increased IL-6 secretion induced by hyperglycaemia.

In conclusion, we found that serum levels of IL-6 and the IL-6/IL-6R complex are elevated in diabetic patients and that plasma fibrinogen shows a significant association with serum IL-6 levels in diabetic patients. Given that serum IL-6 correlates with HbA_{1C} by multiple regression analysis, we postulated that IL-6 induced by the hyperglycaemic state is one of the mechanisms for the progression of atherosclerosis in patients with diabetes.

Acknowledgements We thank the staff of endocrinological section of the Department of Internal Medicine 3, National Defense Medical College for their technical assistance and sending us the patients' samples for this work.

References

1. Kannel WB, D'Agostino RB, Wilson PWF, Belanger AJ, Gagnon DR (1990) Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. Am Heart J 120:672-676
2. Ernst E (1990) Plasma fibrinogen – an independent cardiovascular risk factor. J Int Med 227:365-372
3. Heinrich J, Balleisen F, Schulte H, Assmann G, van de Loo J (1994) Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. Arterioscler Thromb 14:54-79
4. Gauldie J, Richards C, Hornish D, Lansdorp P, Baumann H (1987) Interleukin B2/B-cell stimulatory factor type 2 shares identity with monocyte-derived stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA 84:7251-7255
5. Andus T, Geiger T, Hirano T, Northoff H, Ganter U, Bauer J, Kishimoto T, Heinrich PC (1987) Recombinant human B-cell stimulating factor 2 (BSF-2/IFNβ2) regulates β-fibrinogen and albumin mRNA levels in Fao-9 cells. FEBS Lett 221:18-22
6. De Benedetti F, Massa M, Pignatti P, Albani S, Novick D, Martini A (1994) Serum soluble interleukin 6 (IL-6) receptor and IL-6/soluble IL-6 receptor complex in systemic juvenile rheumatoid arthritis. J Clin Invest 93:2114-2119
7. Waage A, Brandtzaeg P, Halstensen A, Kierulf P, Espevik T (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 1, interleukin 6, and fatal outcome. J Exp Med 169:333-338
8. Guerne PA, Zuraw BL, Vaughan JG, Carson DA, Lotz M (1988) Synovium as a source of IL-6 in vitro: contribution to local and systemic manifestations of arthritis. J Clin Invest 83:585-592
9. Linker-Israeri M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR (1991) Elevated levels of endogeneous IL-6 in systemic lupus erythematosus. A putative role for pathogenesis. J Immunol 147:117-123
10. Morohoshi M, Fujisawa K, Uchimura I, Numano F (1996) Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. Diabetes 45:954-959
11. Koj A, Guzdek A, Potempa J, Korzus E, Travis J (1994) Origin of circulating acute phase cytokines: modified proteins may trigger IL-6 production by macrophages. Preliminary report. J Physiol Pharmacol 45:69-80
12. Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D (1994) Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding protein. J Biol Chem 269:9889-9897
13. Novick D, Engelmann H, Leitner O, Rubinstein M (1989) Soluble cytokine receptors are present in normal human urine. J Exp Med 170:1409-1404
14. Honda M, Yamamoto S, Cheng M, Yasukawa K, Suzuki H, Saito T, Osugi Y, Tokunaga T, Kishimoto T (1992) Human soluble IL-6 receptor: its detection and enhanced release by HIV infection. J Immunol 148:2175-2180
15. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda K, Hirano T, Kishimoto T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer. Cell 58:573-581
16. Stoyan T, Michaelis U, Schooltink H, Van Dam M, Rudolph R, Heinrich P, Rose-John S (1993) Recombinant soluble human interleukin-6 receptor. Expression in *Escherichia coli*, renaturation and purification. Eur J Biochem 216:239-245
17. Rose-John S, Ehlers M, Grötzinger J, Müllberg J (1995) The soluble interleukin-6 receptor. Ann NY Acad Sci 762:207-211
18. Mackiewicz A, Schooltink H, Heinrich PC, Rose-John S (1992) Complex of soluble IL-6 receptor/IL-6 up-regulates acute-phase protein. Proc Natl Acad Sci USA 149:2021-2027

19. Matsuzaki N, Neki R, Sawai K, Shimoya K, Okada T, Sakata M, Saji F, Koishihara Y, Ida N (1995) Soluble interleukin-6 (IL-6) receptor in the sera of pregnant women forms a complex with IL-6 and augments human chorionic gonadotropin production by normal human trophoblasts through binding to the IL-6 signal transducer. J Clin Endocrinol Metab 80:2912-2917
20. May LT, Patel K, Garcia D, Ndubuisi MI, Ferrone S, Mittleman A, Mackiewicz A, Sehgal PB (1994) Sustained high levels of circulating chaperoned interleukin-6 after active specific cancer immunotherapy. Blood 84:1887-1895
21. Niessen RWLM, Pfaffendorf BA, Sturk A, Lamping RJ, Schaap MCL, Hack CE, Peters M (1995) The influence of insulin, β-estradiol, dexamethasone and thyroid hormone on the secretion of coagulant proteins by HepG2 cells. Thromb Haemost 74:686-692
22. Tessari P, Iori E, Vettore M, Zanetti M, Kiwanuka E, Davanzo G, Barazzoni R (1997) Evidence for acute stimulation of fibrinogen production by glucagon in humans. Diabetes 46:1368-1371
23. De Feo P, Gaisano MG, Haymond MW (1991) Differential effects of insulin deficiency on albumin and fibrinogen synthesis in humans. J Clin Invest 88:833-840
24. Vasse M, Paysant J, Soria J, Collet JP, Vannier JP, Soria C (1996) Regulation of fibrinogen biosynthesis by cytokines, consequences on the vascular risk. Haemostasis 26[Suppl 4]:331-339
25. Castell JV, Gomez-Lechon MJ, David M, Hirano T, Kishimoto T, Heinrich, PC (1988) Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett 232:347-350
26. Ceriello A, Giugliano D, Quatraro A, Russo P, Marchi E, Torella R (1989) Hyperglycemia may determine fibrinopeptide A plasma level increase in humans. Metabolism 38:1162-1163