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Leptin, soluble interleukin-6 receptor, C-reactive protein and soluble vascular cell adhesion molecule-1 levels in human coronary atherosclerotic plaque

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Summary

The aim of the present study was to explore the relationship between tissue levels of leptin, soluble interleukin-6 receptor (sIL-6R), high-sensitive-C-reactive protein (hs-CRP) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in atherosclerotic plaques, and traditional risk factors. Coronary artery specimens were obtained from 35 consecutive patients (26 men and nine women) who underwent coronary artery bypass grafting procedure. The mean tissue levels of leptin, hs-CRP and sIL-6R were significantly higher in patients with diabetes mellitus than without diabetes mellitus. When patients were classified according to the smoking status, the mean tissue levels of leptin, hs-CRP and sIL-6R were significantly higher in current smokers than both former smokers and non-smokers. In addition, the mean tissue levels of leptin and sIL-6R were significantly higher in former smokers than non-smokers. There was a positive association between leptin and hs-CRP, sIL-6R and plasma glucose in all patients. Plasma HDL levels were associated negatively with atherosclerotic tissue levels of leptin. Tissue levels of sIL-6R were associated significantly in a positive manner with leptin, hs-CRP and plasma glucose, while tissue levels of hs-CRP were associated with both leptin and sIL-6R. In conclusion, it is attractive to speculate that hs-CRP, sIL-6R and leptin could act synergistically in course of local inflammatory activity and those molecules may not be just markers of inflammation and cardiovascular risk but are also likely to play a pathogenic role in atheromatous plaque. In addition, atherosclerotic tissue levels of CRP, sIL-6R and leptin were significantly higher in current smokers and patients with diabetes.

Keywords: atherosclerotic plaque, high-sensitive-C-reactive protein, interleukin-6 receptor, leptin, vascular cell adhesion molecule-1

Introduction

There is clear evidence today that the monocyte-derived macrophages, T lymphocytes and a large number of proinflammatory cytokines play a key role in all the phases of atherosclerosis [1,2]. In this context, the atherosclerotic plaque may be viewed as a chronic inflammatory nidus that is filled with immune cells that can orchestrate and effect inflammatory responses.

Leptin was thought originally to be an anti-obesity hormone, but it is also a crucial molecule for a number of diverse physiological processes, such as inflammation [3], immune function [4] and atherosclerosis [5]. Previous data indicate that hyperleptinaemia is involved in the pathogenesis of atherosclerosis [6,7].

Interleukin (IL)-6 plays a central role in various host defence mechanisms, such as the general immune response, acute phase reactions and haematopoiesis [8]. The biological activity of IL-6 is mediated via two membrane receptor proteins, a unique low-affinity binding receptor (IL-6R) and the high-affinity transducing β-subunit gp130. Although IL-6 may bind the IL-6 receptor (IL-6R) and elicit a biological response, it also induces the release of soluble IL-6 (sIL-6R). Recent evidence indicates that the pathophysiological effects of IL-6 may depend strongly on a soluble form of the receptor [9]. It has been demonstrated that IL-6 and sIL-6R are

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associated with the processes of inflammation and myocardial injury during the acute phase of acute myocardial infarction [10]. Furthermore, IL-6 gene transcripts are expressed in human atherosclerotic lesions [11,12].

Experimental studies *in vitro* have generated direct evidence that C-reactive protein (CRP) is involved in the pathogenesis of atherosclerosis [13,14]. CRP also appears to be produced locally in atherosclerotic plaques by resident macrophages and vascular smooth muscle cells and may be involved in several important steps in plaques genesis and progression [15]. Levels of CRP mRNA and protein are increased in atherosclerotic plaque obtained from autopsy samples [16]. In a recent report, Ishikawa *et al.* demonstrated that the gene for CRP was expressed in coronary plaque tissue obtained during the atherectomy procedure [17].

Vascular cell adhesion molecule-1 (VCAM-1) is an endothelial adhesion molecule of the Ig gene superfamily that has been considered as a biomarker of atherosclerosis [18]. VCAM-1 is involved primarily in the adhesion of mononuclear leucocytes to the endothelium, and is induced rapidly by the proinflammatory cytokines. O'Brien *et al.* have observed a striking association between the degree of macrophage accumulation and expression of VCAM-1 on neovasculature and on neoendothelial cells of atherosclerotic plaques [19]. VCAM-1 has been found to play a role in the initiation of atherosclerosis in mice [20].

Coronary atherectomy specimens provide a unique source of atherosclerotic plaque tissues because they make it possible to correlate tissue concentrations of inflammatory markers with the clinical status of the patient. The aim of the present study was to explore the relationship between tissue levels of leptin, sIL-6R, hs-CRP and sVCAM-1 in atherosclerotic plaques, and traditional risk factors.

Materials and methods

Study participants

Coronary artery specimens were obtained from 35 consecutive patients (26 men and nine women) who underwent coronary artery bypass grafting procedure at Gülhane School of Medicine Hospital between 2003 and 2004. Excluded from the present study were individuals with severe inflammatory or infectious diseases, cancers, any systemic disease besides atherosclerosis, prior myocardial infarction within 30 days before surgery, immunological disease, familial hypercholesterolaemia, secondary hyperlipoproteinaemia, hepatic or renal disease, any medication other than aspirin ≤250 mg/day, a β-blocker, calcium antagonists, angiotensin-converting enzyme inhibitors, angiotensin-II receptor blockers, nitrates, oral anti-diabetics or statins. Informed written consent was obtained from each subject included in the study. The study was approved by the Ethical Committee of Gülhane School of Medicine.

Clinical history was assessed for diabetes mellitus, smoking, hypertension, hyperlipidaemia, prior acute myocardial infarction and whether any first-degree relatives had coronary heart disease (CHD) below the age of 55 years. We calculated body mass index (BMI) according to Quetelet's formula as the ratio of body weight to body height squared (kg/m^2). Blood pressure was calculated as the average of three measurements taken under standardized conditions in a supine position with a sphygmomanometer.

Diabetes was diagnosed in patients with dietary treatment or anti-diabetics or current fasting plasma glucose levels higher than 7·0 mmol/l. Hypertension was diagnosed according to World Health Organization (WHO) criteria (blood pressure ≥ 140/90 mmHg or current anti-hypertensive treatment). Hyperlipidaemia was defined if the total cholesterol level was > 5·2 mmol/l or the low-density lipoprotein (LDL) cholesterol level was > 3·4 mmol/l, or if a patient was taking a lipid-lowering drug. Cigarette smoking was classified as current smoker (smoking more than five cigarettes within the past 3 months), former smoker (> 3 months and <40 years) or non-smoker.

Tissue preparation

Thirty-five coronary atherosclerotic plaque specimens were derived from proximal lesions in the left anterior descending coronary artery ($n = 32$), right coronary artery ($n = 1$) and left circumflex artery ($n = 2$) during the elective coronary artery bypass grafting surgery. Immediately after the procedure, all extracted atherosclerotic plaques were frozen and stored at –80°C until tissue homogenization.

Frozen plaques (mean tissue weight, 0·020 g ± 0·001) were homogenized in ice-cold homogenization buffer (50 mM HEPES, 0·2% Triton X-100, 1 mM ethylenediamine tetraacetic acid (EDTA) and 0·1 mM phenylmethylsulphonyl fluoride (PMSF), pH 7·4) at 13 500 r.p.m. in Ultraturrax T25 (Janke & Kunkel, IKA® Labortechnik, Staufen, Germany) using a method described previously [21]. The homogenates were centrifuged at 2900 ***g*** for 15 min and the supernatants were stored at –80°C until analysis. Samples were coded to ensure anonymity and all analyses were performed in a blinded fashion. Before performing the assays, samples were brought to room temperature (18–25°C) and mixed gently. Tissue contents of leptin, soluble VCAM-1 (sVCAM-1), sIL-6R and high-sensitive-CRP (hs-CRP) in plaques were measured by enzyme-linked immunosorbent assay (ELISA) techniques using the ELX 800 (Bio-Tek Instruments Inc., Winooski, VT, USA) with commercially available kits, and normalized to the total protein content of the tissue homogenates.

Laboratory methods

Tissue concentrations of leptin were measured with a human leptin ELISA kit from Ray Biotech, Inc (Norcross, GA, USA). The minimum detectable dose of leptin was less than

70 pg/ml. The intra- and interassay coefficients of variation (CV) for leptin were < 10% and < 12%. sIL-6R and sVCAM-1 were determined with kits (human soluble IL-6R and human soluble VCAM-1) from BioSource International, Inc. (Camarillo, CA, USA). The minimum detectable dose of sIL-6R was < 8 pg/ml, while the intra- and interassay CV for sIL-6R were < 5% and < 5·6. The minimum detectable dose of sVCAM-1 was < 0·5 ng/ml, while the intra- and interassay CV for sVCAM-1 were < 4·6% and < 8·2. hs-CRP was measured with a human CRP kit from Generic Assays GmbH (Dahlewitz, Germany). The analytical sensitivity of the human CRP kit was determined at 0·2 mg/ml. The intra- and interassay CV for hs-CRP were < 5·7% and < 13·6%.

Blood samples for laboratory assays were obtained at approximately 08·00 h following overnight fasting before surgery. Serum was separated and frozen at –80°C until the time of the assay. Total plasma cholesterol, triglyceride, high-density lipoprotein (HDL)-cholesterol and glucose levels were measured by enzymatic colorimetric method with the Olympus Corp. AU 600 autoanalyser using reagents from Olympus Corp. (Hamburg, Germany). LDL-cholesterol was calculated by Friedewald's formula.

Table 1. Clinical and laboratory characteristics of study participants ($n = 35$).

Factor	Mean ± s.d.
Age, years	58·40 ± 9·85
Male, %	74·29
Body mass index, kg/m^2	23·86 ± 3·42
Systolic blood pressure, mmHg	135 ± 19
Diastolic blood pressure, mmHg	80 ± 10
Plasma concentrations	
Total cholesterol, mmol/l	4·50 ± 1·10
LDL cholesterol, mmol/l	2·83 ± 0·90
HDL cholesterol, mmol/l	1·00 ± 0·21
Triglycerides, mmol/l	1·45 ± 0·59
Fasting glucose, mmol/l	6·84 ± 0·16
*Tissue concentrations**	
Leptin, pg/ml	10 503·86 ± 11894·16
Soluble interleukin-6 receptor, pg/ml	5 542·58 ± 1 819·79
Soluble vascular adhesion molecule-1, ng/ml	84·18 ± 13·99
High-sensitive-C-reactive protein, mg/ml	2·40 ± 0·32

*Atherosclerotic plaque. LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Statistical methods

Statistical analyses were performed using SPSS version 11·5 (SPSS Inc., Chicago, IL, USA) software. Descriptives of the parameters were quoted as the mean ± s.d. and 95% confidence intervals (CI). Frequencies of categorical variables were calculated. Normality of the data was assessed using the Kolmogorov–Smirnov test. As they were not normally distributed, we applied logarithmic transformations to the leptin and sIL-6R data. Independent-samples *t*-test was used to compare two group values and analysis of variance (ANOVA) with Bonferroni as *post-hoc* tests for more than two groups. Parameters were adjusted for age, sex, BMI and statin use. Multiple stepwise linear regression analysis was used to investigate the relations among the parameters. *P*-values less than or equal to 0·05 were considered statistically significant.

Results

Clinical and laboratory characteristics of study participants are shown in Table 1. Hypercholesterolaemia was present in 10 (28·6%) patients, hypertension in 18 (51·4%), diabetes mellitus in 14 (40·0%), family history of CHD in 15 (42·9%) and prior myocardial infarction (MI) in 16 (45·7%). At the time of the study, the patients were being treated with aspirin ($n = 35$, 100%), beta-blockers ($n = 22$, 62·9%), angiotensin-converting enzyme inhibitors/angiotensin-II receptor blockers ($n = 18$, 51·4%), oral anti-diabetics ($n = 14$, 40·0%), nitrates ($n = 13$, 37·1%), calcium antagonists ($n = 13$, 37·1%) and statins ($n = 7$, 22·9%).

Comparisons of tissue concentrations of parameters between patients with and without diabetes mellitus are given in Table 2. The mean tissue levels of leptin, hs-CRP and sIL-6R were significantly higher in patients with diabetes mellitus than without diabetes mellitus. These differences in the variables between the presence and absence of diabetes mellitus remained significant after adjusting for age, sex, BMI and statin use. However, the mean tissue levels of sVCAM-1 were not found to be statistically significant between the groups according to diabetic state (data not shown). We did not observe any significant differences in any of the inflammatory parameters in relation to the type of

Table 2. Comparisons of tissue concentrations of parameters between patients with ($n = 14$) and without ($n = 21$) diabetes mellitus (age, sex, body mass index and statin use adjusted).

	Diabetes mellitus (–)		Diabetes mellitus (+)			
Parameters	mean ± s.e.	95% CI	mean ± s.e.	95% CI	*t*	*P*
Leptin (pg/ml)*	3·43 ± 0·07	3·28–3·57	4·19 ± 0·09	4·02–4·38	28·019	< 0·001
sIL-6R (pg/ml)*	3·65 ± 0·02	3·61–3·70	3·83 ± 0·03	3·77–3·89	21·298	< 0·001
hs-CRP (mg/ml)	2·25 ± 0·06	2·13–2·37	2·64 ± 0·07	2·49–2·79	17·610	< 0·001

*Logarithmic transformed values sIL-6R, soluble interleukin-6 receptor; hs-CRP, high-sensitive-C-reactive protein.

Table 3. Comparisons of tissue concentrations of parameters according to cigarette smoking status* (age, sex, body mass index and statin use adjusted).

Cigarette smoking status ($n = 35$)	Parameters: Leptin** (pg/ml)	sIL-6R** (pg/ml)	hs-CRP (mg/ml)
Current smoker	4·37 ± 0·17[a]	3·88 ± 0·08[d]	2·98 ± 0·86[g]
Former smoker	3·72 ± 0·30[b]	3·73 ± 0·04[e]	2·33 ± 0·04[h]
Non-smoker	3·24 ± 0·11[c]	3·60 ± 0·08[f]	2·22 ± 0·03[i]

*ANOVA with Bonferroni test. **Logarithmic transformed values. sIL-6R, soluble interleukin-6 receptor; hs-CRP, high-sensitive-C-reactive protein. $P < 0{\cdot}001$ for [a] *versus* [b], [b] *versus* [c], [a] *versus* [c], [d] *versus* [f], [e] *versus* [f], [d] *versus* [e], [g] *versus* [h]; $P = 0{\cdot}001$ for [g] *versus* [i]; $P = 0{\cdot}012$ for [g] *versus* [h].

medication, hypercholesterolaemia, hypertension, family history of CHD and prior MI (data not shown).

When patients were classified according to smoking status, the mean tissue levels of leptin, hs-CRP and sIL-6R were significantly higher in current smokers than both former smokers and non-smokers (Table 3). In addition, the mean tissue levels of leptin and sIL-6R were significantly higher in former smokers than non-smokers. These differences remained significant after adjusting for age, sex, BMI and statin use. However, the mean tissue levels of sVCAM-1 were not found to be statistically significant between the groups according to smoking status (data not shown).

Regression analysis results for leptin, hs-CRP and sIL-6R are given in Table 4. There was a positive association between leptin and hs-CRP, sIL-6R and plasma glucose in all patients. Plasma HDL levels were associated negatively with atherosclerotic tissue levels of leptin. Tissue levels of sIL-6R were associated significantly positively with leptin, hs-CRP and plasma glucose, while tissue levels of hs-CRP were associated with both leptin and sIL-6R.

Discussion

In the present study, tissue levels of leptin were associated positively with hs-CRP and sIL-6R in atherosclerotic plaques. Our results are in line with previous reports showing that leptin may regulate immune responses, and stimulation of cultured human endothelial cells with leptin may lead to enhanced proinflammatory activity [22–24]. Leptin, either directly or indirectly through the immune system, may alter CRP levels. First, similar to previous reports [23,25], there was a positive association between leptin and both hs-CRP and sIL-6R. Secondly, the long form of the leptin receptor (Ob-R) resembles the gp120 family of cytokine receptors, which includes the IL-6 receptor [26]. Moreover, Ob-R has been shown to have the signalling capabilities of IL-6-type cytokine receptors [27]. Ultimately, the relationship between leptin, IL-6 and CRP in the plaques appears to be more complex. Leptin can stimulate a proinflammatory

Table 4. Regression analysis results.

Parameters	Leptin *B*	Leptin *t*	Leptin *P*	sIL-6R *B*	sIL-6R *t*	sIL-6R *P*	hs-CRP *B*	hs-CRP *t*	hs-CRP *P*	Plasma glucose *B*	Plasma glucose *t*	Plasma glucose *P*	Plasma HDL *B*	Plasma HDL *t*	Plasma HDL *P*	*F*	P	Adj. R^2
Leptin				0·616	5·115	< 0·001	0·478	3·798	0·001	0·088	2·366	0·024	− 0·183	3·079	0·004	1741·224	< 0·001	0·995
IL-6R	0·334	2·144	0·04				0·474	3·755	0·001	0·108	2·747	0·01				1577·336	< 0·001	0·994
hs-CRP	0·782	15·773	< 0·001	0·222	4·472	< 0·001										2613·411	< 0·001	0·993

HDL, high-density lipoprotein; sIL-6R, soluble interleukin-6 receptor; hs-CRP, high-sensitive-C-reactive protein.

response, but increased proinflammatory cytokines can also stimulate leptin production. Higher levels of leptin may affect the function and trafficking of inflammatory cells in the atherosclerotic plaques by modulating the production of these cytokines.

Leptin is associated negatively with HDL cholesterol in atherosclerotic plaques. Similar to our results, an inverse correlation between plasma leptin and HDL cholesterol was also observed in some human studies [28,29].

In the current study, the finding of a strong positive association between tissue levels of sIL-6R and hs-CRP was not surprising. Classically, synthesis of CRP in the liver is mainly under the control of IL-6 [30]. Previous studies have demonstrated that CRP is a physiological regulator of sIL-6R shedding in human neutrophils and increases markedly the formation of the sIL-6R/IL-6 complex [31]. Data from a previous experimental study suggest that CRP may also function to increase IL-6 secretion from endothelial cells [32]. Haddy *et al.* showed that there was a strong correlation between IL-6 and CRP [33]. Therefore, it may be speculated that IL-6 is not only a potent hepatic stimulus for CRP but also associated with increased tissue levels in atherosclerotic plaque.

Cigarette smoking is a well-established cardiovascular risk factor that is also associated with systemic inflammation [34]. Our data suggest that progressively elevated atherosclerotic tissue levels of leptin, hs-CRP and sIL-6R, but not sVCAM-1, were related significantly to smoking status. Furthermore, these findings support previous observations that significantly higher levels of hs-CRP and IL-6 were reported in current smokers than in non-smokers [35] and smoking influences circulating concentrations of both leptin and CRP [36,37].

In our study, atherosclerotic tissue levels of leptin, hs-CRP and sIL-6R were significantly higher in patients with diabetes mellitus than without diabetes mellitus. In addition, tissue levels of leptin are associated positively with plasma glucose. Consistent with present observations, elevated leptin levels, along with other proinflammatory cytokines, have been associated with an increased risk for type II diabetes [38,39]. Recent evidence suggests that CRP, like leptin, correlates with insulin resistance independently of BMI [40]. It has also been demonstrated that plasma leptin levels are associated with coronary atherosclerosis in type II diabetes [41]. Kado *et al.* reported that serum levels of IL-6 and the IL-6/IL-6R complex are elevated in diabetic patients [42]. Consequently, our results confirm that leptin, along with other inflammatory markers such as hs-CRP and sIL-6R, may contribute to the progression of atherosclerosis in diabetes mellitus.

Several limitations of the present study deserve mention. The sample size was too small compared to other studies, and the design was too simple. The detection of atherosclerotic tissue levels of other inflammatory markers, which are relevant to the atherosclerotic process, such as tumour necrosis factor-alpha, IL-1beta or chemokines, would have been more convincing. In addition, measurement of tissue IL-6 would have strengthened the study.

In conclusion, it is attractive to speculate that hs-CRP, sIL-6R and leptin could act synergistically in the course of local inflammatory activity and those molecules may not be just markers of inflammation and cardiovascular risk but are also likely to play a pathogenic role in atheromatous plaque. In addition, atherosclerotic tissue levels of CRP, sIL-6R and leptin were significantly higher in current smokers and patients with diabetes. In the current study there was no association between tissue levels of variables and blood lipids. However, based on these observations it is not possible to claim that circulating lipids, which are well known to influence both the initiation and progression of CHD, do not play a role in the pathogenesis of atherosclerosis.

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