附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※ 具動脈硬化危險因子病人治療前後單核細胞與內皮細胞接合之研究 ※

※ **Study on mononuclear cell adhesion to endothelial cells before and after treatment in patients with atherosclerosis risk factors** ※

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計畫類別：☑個別型計畫　☐整合型計畫

計畫編號：NSC 89-2314-B002-139

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

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- ☐國際合作研究計畫國外研究報告書一份

執行單位：臺大醫學院內科

中 華 民 國 90 年 2 月 1 日

目錄

關鍵詞：

動脈粥樣硬化，單核細胞，危險因子，細胞接合分子，內皮細胞

動脈粥樣硬化最初的變化是單核細胞與血管的內皮細胞接合。我們研究各種動脈硬化危險因子對單核細胞與培養之人類臍靜脈內皮細胞(HUVEC)接合之影響，以及改善這些危險因子後其改變情形。此外，細胞接合分子 VCAM-1 及 ICM-1 之角色亦加以研究。

單核細胞取之於具有各種危險因子的病人血液，將之與 HUVEC 作用，並計數附著於 HUVEC 之單核細胞數與對照組比較。研究的危險因子包括高血壓，糖尿病，高膽固醇，高三酸甘油酯，低密度脂蛋白膽固醇過高，高密度脂蛋白膽固醇過低等。病人接受治療後再作一次追蹤檢查。VCAM-1 及 ICM-1 之重要性則以阻斷之單株抗體來研究。

結果發現，具有危險因子病人其單核細胞與內皮細胞之接合有很明顯增加。與控制者相比較，高血壓者之細胞接合為 138.5±28.2%，糖尿病人為 142.6±25.2%，高膽固醇者為 140.1±29.7%，高三酸甘油酯者為 146.1±23.3%，高低密度脂蛋白膽固醇者為 136.7±26.9%，低高密度脂蛋白膽固醇者為 139.0±29.3%。而在治療後，即使危險因子未完全改善，亦有明顯降低細胞接合情形，自 150.6±22.3%減為 120.2±19.7%。在研究 VCAM-1 及 ICM-1 方面，發現各種危險因子病人，其細胞接合方面，ICM-1 佔有重要地位，而 VCAM-1 則只在高密度脂蛋白膽固醇過低病人佔有重要地位。但在糖尿病病人，則 VCAM-1 及 ICM-1 均不佔重要角色。

從以上研究結果，我們發現動脈硬化危險因子會促使單核細胞與內皮細胞之接合增加，而治療危險因子則能減少此種細胞接合。在動脈硬化危險因子病人的細胞接合，ICM-1 佔有重要地位。

Abstract

Key words: atherosclerosis, mononuclear cell, risk factor, cell adhesion molecule, endothelial cell

Atherosclerosis is initiated by adhesion of mononuclear cells (MC) to the vascular endothelial cells. We studied the effects of various atherosclerosis risk factors on the adhesion of MC to cultured human umbilical vein endothelial cells (HUVEC) and the changes after correction of these risk factors. The role of VCAM-1 and ICAM-1 was also investigated.

MC isolated from patients with various risk factors were incubated with HUVEC. MC adhered to HUVEC were then counted and compared with that obtained in normal control. The effects of treatment of risk factors were then studied at follow-up. The roles of VCAM-1 and ICAM-1 were studied by using blocking monocloncal antibodies.

The results showed that patients with risk factors had increased MC adhesion to HUVEC for all risk factors studied (hypertension, diabetes mellitus, hypercholesterolemia, hypertriglyceridemia, high low density lipoprotein cholesterol and low high density lipoprotein). Comparing with the control, the cell adhesions were 138.5±28.2% for hypertension, 142.6±25.2% for diabetes mellitus, 140.1±29.7% for hypercholesterolemia, 146.1±23.3% for hypertriglyceridemia, 146.1±23.3% for high low density lipoprotein cholesterol, and 139.0±29.3% for low high density lipoprotein cholesterol. After treatment, the cell adhesion was found significantly improved, decreased from 150.6±22.3% to 120.2±19.7% although the risk factors were not totally corrected.

Studies on the significance of VCAM-1 and ICAM-1 in cell adhesion in various risk factors by using monoclonal antibody blocking revealed that ICAM-1 played more important role in cell adhesion in patients with risk factors. VCAM-1 was important for patients with low high density lipoprotein. Both VCAM-1 and ICAM-1 was not significantly involved in cell adhesion in diabetes mellitus.

From our results we concluded that various atherosclerosis risk factors could increase MC adhesion to HUVEC. The cell adhesion could be improved by correction of the risk factors. The cell adhesion molecule, ICAM-1, played more important role in the cell adhesion in patients with various risk factors.

Atherosclerosis is one of the most important causes of morbidity and mortality in the modern life. Coronary artery disease, cerebrovascular disease and various peripheral vascular diseases are mainly caused by atherosclerosis. In Taiwan, atherosclerosis-associated conditions, coronary artery disease and cerebrovascular accident, hold 4th and 2nd positions of the ten leading causes of mortality in past decades (1).

The initial event in the process of atherosclerosis is adhesion of circulating blood mononuclear cells, especially monocyte, to the endothelial lining of the vessel (2,3). The adhered cells then transmigrate across the endothelium to the subintimal layer where they replicate and, by engulfing lipids, transform into foam cells. The resulted fatty streak is the earliest pathological manifestation of atherosclerosis (4). Therefore, cell adhesion is the first step in the process of atherosclerosis (2,3). The studies about adhesion of mononuclear cells to endothelium is now one of the most active fields of research in the scope of atherosclerosis (5).

Endothelial dysfunction plays an important role in the pathogenesis of atherosclerosis (2,3,5). Many risk factors for the development of atherosclerosis have been identified in the past decades, among them are hypertension (6), dyslipidemia (7), smoking (8), diabetes mellitus (9), and obesity (10), etc. It has been reported that hypertension (11-13), hypercholesterolemia (14-18), hypertriglyceridemia (15,17,19), and diabetes mellitus (20-26) all enhanced mononuclear cell adhesion to endothelial cells in culture and/or vascular endothelium. Yet, in most of the reports, changes of the interaction between endothelial cells and mononuclear cells after correction of the abnormal conditions were not studied.

We, therefore, studied adhesion of mononuclear cell to the cultured human umbilical vein endothelial cells (HUVEC) in patients with various risk factors in Chinese patients. We further investigated the effects of treatment and correction of risk factors on the cell adhesion in these patients. Also, the role of adhesion molecules, VCAM-1 and ICAM-1, in the adhesion of cells to endothelial cells was studied.

Subjects and Methods

1. Culture of HUVEC

The HUVEC were obtained and cultured following the methods of Jeffe et al (27). The cells were cultured with M199 supplemented with 20% fetal bovine serum, 20 mM HEPES, 100 μg/ml endothelial cell growth substance (Collaborative Research, Bedford, MA), 5 U/ml heparin, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (28). Subculture was undertaken when confluence of the cell occurred, by applying 0.25% trypsin-EDTA (Gibco, Gaithersburg, MD) for 3 minutes. Cells of 3rd to 6th passage were used in experiments.

2. Study subjects

Normal controls and subjects with hypertension, hypercholesterolemia, hypertriglyceridemia, or combined hyperlipidemia, low high density lipoprotein cholesterol (HDL-C), and diabetes mellitus were collected for study. The patients were first studied before treatment. Then, after 3 months or longer of treatment with at least partial correction of the abnormal conditions, the subjects were studied again.

The criteria for the diagnosis of risk factors were: for hypertension, systolic blood pressure $\geq$160 mmHg and/or diastolic blood pressure $\geq$95 mmHg; for diabetes mellitus (DM), fasting blood sugar $\geq$140 mg/dl, and/or 2 h PC sugar $\geq$200 mmHg, and/or HbA1c $\geq$6.5%; for high cholesterol, $\geq$240 mg/dl; for high triglyceride, $\geq$240 mg/dl; for high low-density lipoprotein cholesterol (LDL-C), $\geq$130 mg/dl; and for low HDL-C, <40 mg/dl.

3. Isolation of serum and mononuclear cells

Fasting venous blood from patients or control subjects was collected in sterile tubes containing 7.5% EDTA (CBC tube). To isolate mononuclear cells from the patients and the controls, the method of Menon et al. were used (21). The blood was layered over an equal volume of Histopaque 1077 (Sigma), and then centrifuged at 400x g for 30' at room temperature. Mononuclear cells are collected from the interphase, resuspended in Tris buffer (TBS, 20 mM Tris, 0.15 M NaCl, pH 7.4). After sedimentation by centrifugation at 250x g for 10', the cells are washed and resuspended in TBS buffer and counted for cell concentration. Exactly 400 K mononuclear cells in 0.25 ml of medium were applied to each well of the 6-well plates covered with confluent HUVEC (see below). For each sample 2 wells were studied (in duplicate).

4. Adhesion study

The method of Berliner et al. (29) were used. HUVEC were cultured in 6-well culture plates. At confluence, HUVEC monolayers were rinsed with serum-free medium for 3 times. Mononuclear cells from the patients or normal controls, $4x10^5$ cells in 250 µl in DMEM containing 1% heat-inactivated serum, were added to each well, and then kept in incubator for 1 h. The nonadherent mononuclear cells were rinsed off and the wells fixed with 1% glutaraldehyde. The attached cells were counted in each of 10 microscopic fields and then averaged. The results of adhesion study was compared to the parallel running study on the blood of a single control subject, the chief investigator of this study (CSL), who had normal laboratory data through the study period. The laboratory data of CSL were presented in Table 1.

5. Detection of the activation of VCAM-1 and ICAM-1 on HUVEC

To investigate the roles of VCAM-1 and ICAM-1 in cell adhesion in various conditions with coronary risk factors, the activities of VCAM-1 and ICAM-1

were studied using the method of Bochner et al. (30). Monoclonal antibodies against human VCAM-1 or human ICAM-1 were added to the wells of HUVEC monolayers at the same time the mononuclear cells were added. After 1 h the adhesion assay was performed as described above (29). Suppression of mononuclear cell adhesion to the HUVEC monolayers by monoclonal antibodies against VCAM-1 or ICAM-1 suggested the activation of VCAM-1 and ICAM-1 on HUVEC.

Results

1. Study subjects

A total of 300 subjects were studied. Among them, 91 subjects received follow-up studies, counting a total of 391 measurements. Of the 300 subjects, 159 were male and 141 female. The mean age of the study subjects was 63.9±10.9 years (range 28-100). The case numbers for individual risk factors were shown in Table 2. The data about the number of risk factors for the study subjects were presented in Table 3. The study subjects might have more than one risk factor. There were 53 subjects without any risk factors, some were changed from previous condition with some risk factors. Most of the study subjects were with 1 or 2 risk factors (90 and 99 subjects, respectively). Multiple risk factors were present in significant numbers of cases (3 risk factors in 36 subjects, 4 risk factors in 17 subjects and 5 risk factors in 5 subjects).

2. Adhesion study

The cell adhesion of the study subjects was expressed as percentage of the parallel running data of the control (CSL). The cell adhesion data for various conditions including that for risk factors were shown in Table 4. When comparing the cell adhesion data of all the risk factors to that of subjects without risk factor or to subjects with high HDL-C, the differences were all highly significant ($p<0.001$). Subjects with high TG levels also showed higher cell adhesion than subjects with high LDL-C ($p<0.025$).

Of the study subjects 91 cases (male 54 and female 37) received repeated studies. Of them, 7 subjects showed improvement in 3 risk factors, 22 subjects in 2 risk factors, 34 subjects in 1 risk factor, and 7 cases showed no improvement or worsening for 1 risk factor. The cell adhesion was 150.6±22.3% before treatment which became 120.2±19.7 at follow-up after treatment ($p<0.001$).

3. Detection of the activation of VCAM-1 and ICAM-1 on HUVEC

A total of 34 patients with various coronary risk factors were studied for the roles of VCAM-1 and ICAM-1 in mononuclear cell adhesion to the cultured HUVEC. All 34 cases were studied with anti-VCAM-1 monoclonal antibody, but only 30 cases were studied with anti-ICAM-1 monoclonal antibody. The risk factors of these patients were complicated. The numbers of patients with

various risk factors were: hypertension 10 cases, DM 8 cases, high cholesterol 8 cases, high triglyceride 7 cases, high LDL-C 16 cases and low HDL-C 16 cases. Suppression of mononuclear cell adhesion to cultured HUVEC was semiquantitatively measured by arbitrary assigned scores (0-4 score). The results were shown in Table 5. It showed that VCAM-1 played less important role in the adhesion of mononuclear cells to the cultured HUVEC for the risk factors studied, except for low HDL-C, in which VCAM-1 seemed worked as an important factor. On the other hand, ICAM-1 was found playing important role in the adhesion, except for DM, in which both ICAM-1 and VCAM-1 seemed having no important contribution.

Discussion

In this study we established the methods of measuring adhesion of mononuclear cells from patients with various coronary risk factors to cultured human endothelial cells derived from umbilical vein. It is well accepted that the adhesion of mononuclear cells, especially monocytes, as the first step of atherosclerosis (2,3). Using this method, the propensity of cell adhesion may suggest the tendency of atherosclerosis. Decreasing cell adhesion by interventions might be interpreted as having salute effects by reducing the development of atherosclerosis. This hypothesis needs further testing to prove.

In our study we found that all risk factors, including hypertension, diabetes mellitus, hypercholesterolemia, hypertriglyceridemia and low HDL-C, enhance the mononuclear cell adhesion to cultured HUVEC (Table 4). High proportion of our patients had multiple risk factors, as shown in Tables 2 and 3. This increases the difficulty for data analysis. In our study subjects, 91 cases had follow-up studies. After interventions, the risk factors were not totally improved or corrected in the patients. Yet, the adhesion of mononuclear cells was much improved.

One unique point worth to mention in this study is that the control was a single subject who had normal biological data which kept stable through the study period (Table 1). Although this may have some problem for the only person as the control, yet, this may assure the consistency of the control.

Another interesting finding was the studies on the roles of the cell adhesion molecules, mainly VCAM-1 and ICAM-1. In our studies, we found that ICAM-1 played more important role for cell adhesion in most of the risk factors. VCAM-1 was only became important in patients with low HDL-C. Also interesting is that in DM, both VCAM-1 and ICAM-1 seemed to play no significant role in the adhesion of mononuclear cells to endothelial cells. This finding needs further study on patients with isolated risk factors.

In conclusion, patients with various coronary risk factors had increased tendency of mononuclear cell adhesion to cultured human endothelial cells, suggesting the propensity of atherosclerosis. The cell adhesion promoted by risk factors may be mediated more by ICAM-1, while VCAM-1 playing less importance.

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Table 1. The laboratory data of the control subject*

	19/10/'99	28/2/'00	13/7/'00
Sugar	109	98	104
Cholesterol	165	185	191
Triglyceride	94	120	103
LDL-C	103	116	121.5
HDL-C	43	45	49

* The control subject was the chief investigator of this study.
* Blood samples were taken after more than 12 hour fasting.
All data are in the unit of mg/dl.
LDL-C= low density lipoprotein cholesterol;
HDH-C=high density lipoprotein cholesterol

Table 2. Presence of risk factors in the study subjects

Risk factor	Case Number
Hypertension	70
Diabetes mellitus	115
High cholesterol	95
High triglyceride	70
High LDL-C	185
Low HDL-C	141
High HDL-C	86
No risk factor	53

Note: Study subjects might have more than one risk factor (see Text)
LDL-C= low density lipoprotein cholesterol;
HDH-C=high density lipoprotein cholesterol

Table 3. Number of risk factors in the subjects studied

No. of risk factors	Case Number
0	53
1	90
2	99
3	36
4	17
5	5

Table 4. Adhesion data for the study subjects

Risk factor	Case No.	Adhesion (%)
No risk factor	53	116.6±25.2
Hypertension	70	138.5±28.2
Diabetes mellitus	115	142.6±25.2
High cholesterol	95	140.1±29.7
High triglyceride	70	146.1±23.3
High LDL-C	185	136.7±26.9
Low HDL-C	141	139.0±29.3
High HDL-C	86	124.7±27.1

Table 5. Suppression of mononuclear cells to cultured HUVEC in patients with various risk factors

Risk factor	Case No.	V-CAM MoAb	ICAM MoAb
Hypertension	10	1.1	1.7
Diabetes mellitus	8	1.0	1.2
High cholesterol	8	1.1	1.5
High triglyceride	7	1.1	1.8
High LDL-C	16	1.0	1.5
Low HDL-C	16	1.6	1.5