Tissue Factor Expression of Endothelial Cell in Response to Atherosclerotic Risk Factors

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ABSTRACT

Objective: To study the effect of serum from patients with atherosclerotic risk factors on the synthesis of endothelial tissue factor.
Methods: Serum from 30 diabetic patients, 30 hyperlipemic patients, 30 smokers and 30 normal serum were incubated with cultured endothelial cells from a human umbilical vein. The tissue factor of endothelial cells was measured using the assay that was developed in house after 24 hours incubation time.
Results: Smokers serum can significantly cause the increase in endothelial tissue factor. The mean level of tissue factor induced by smokers serum is 1.12 microunits/cell whereas the mean level of tissue factor induced by diabetic serum, hyperlipemic serum and normal serum is 0.4, 0.48 and 0.2 microunit/cell, respectively.
Conclusion: Smoking may increase the risk of thrombosis by increasing the tissue factor production of endothelial cells.

Keywords: Endothelial cell, tissue factor, risk factors

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Endothelial cells play an important role in the control of normal hemostasis.[1] Under physiologic conditions, endothelial cells provide the effective thromboresistant surface and help to maintain the fluidity and free flow of blood. In some pathological states, however, endothelial cells synthesize and express tissue factor (TF) which is the most potent initiator of blood coagulation leading to the thrombus formation.[2-4]

Aberrant TF expression triggers intravascular thrombosis associated with various diseases, such as atherosclerosis and cancer.[5,6] In atherosclerosis, TF is expressed on the surface of macrophage-derived foam cells within atherosclerotic plaques.[1] Recent studies have suggested that an additional source of TF, known as plasma TF or circulating TF may also contributes to thrombosis.[7,8] Importantly, several studies have shown that levels of circulating TF are increased in various diseases, such as atherosclerosis, diabetes, and sepsis.[9-11] Some studies have also shown a correlation between the levels of circulating TF and acute myocardial infarction.[12]

Thrombus formation is one of the pathophysiologic changes in atherosclerotic disorders.[13] However, the cause of thrombotic tendency in these patients is unknown. It is our postulation that modulation of TF expression of endothelial cells by atherosclerotic risk factors may be one cause responsible for thrombotic tendency in atherosclerotic disorders.

MATERIALS AND METHODS

Serum

Four groups of serum were used in the study. The first group consisted of 30 diabetic serum with blood sugar over 100 mg% without other abnormalities in blood chemistry. The second group was a group of 30 serum which had serum LDL over 160 mg%. The third group was serum from 30 active smokers with a history of smoking for more than 10 years and had normal blood chemistry. The fourth group was 30 healthy individuals which had normal blood chemistry. Each group contained 15 males and 15 females. All serum used in the study are leftover from specimens sent to the laboratory of the Department of Clinical Pathology and also from specimens previously collected for other unrelated research during the year 2005-2006. All of the specimens are not individually identifiable.

Serum was obtained from the clotted and fasting blood by centrifugation at 1,500 g for 10 minutes. Then the serum was sterilised using a filter with 0.2 µm pore size. The filtered serum was inactivated at 56 °C for 30

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minutes to destroy heat-labile complement proteins and then was stored at 80 °C until use. Hemolysed serum and serum with more than one abnormal chemistry test were excluded from the study.

Endothelial cell culture

Endothelial cells were isolated from a human umbilical vein according to the method of Jaffe.[14] The cells were cultured in a 35 mm tissue culture petridish with M-199 tissue culture media supplemented with 20% fetal calf serum, 5% human serum albumin, 10 ug/ml insulin, 10 ug/ml transferrin, 5 ng/ml acid fibroblast growth factor. Upon confluence, which was usually 1 week, cells were passed into a 96-well tissue culture plate for 48 hours. Then the endothelial cells were incubated with serum from the subjects which were 50% diluted in culture media. The incubation time was 24 hours and then the cells were assayed for tissue factor. Each serum was studied in duplicate and the mean tissue factor was reported. Endothelial cells incubated with endotoxin were used as a positive control. The number of endothelial cells cultured in each well was also counted in the counting chamber using a light microscope with the same technique as for white blood cell count in order to express the results of TF per endothelial cells.

Tissue factor assay

Tissue factor was measured both on the surface of the endothelial cell (surface TF) and in the cells (total TF) with the assay method previously described.[15] Surface TF was measured while the endothelial cells remained attached to the bottom of the culture dish. Total TF was determined in the cells that were disrupted by freezing at 70 °C and thawing at 37 °C for 3 times.

In brief, the assay was based on a two-stage amidolytic method. In the first stage, the cells were incubated with 150 µl of phosphate buffered saline with calcium and magnesium (PBS+), 10 µl of F IX concentrate and 20 µl of 0.025 M $CaCl_2$ for 10 minutes at 37 °C. The reaction in the first stage was stopped by 25 µl of 50 mM EDTA. In the second stage, 25 µl of the reaction mixture form the first stage was added to the wells containing 200 µl of tris-imidazole buffer and 25 µl of 2.5 mM S-2238. After 5 minutes of incubation at room temperature, the hydrolysis of the S-2238 was stopped by adding 50 µl of 50% acetic acid. The color produced from the reaction was determined by measuring the OD 405 nm with a Thermo Scientific GENESYS 20 spectrophotometer. The TF activity was then read from a standard curve constructed using standard tissue factor that was arbitrarily assigned to have an activity of 1 unit/ml. The TF of endothelial cells were reported as TF per cell derived from TF activity measured dividend by numbers of endothelial cell count.

Statistical analysis

The difference in tissue factor of endothelial cells stimulated by each group of serum was compared by one way analysis of variance. Significant difference was considered when $p < 0.05$.

RESULTS

1. Study serum

Each study group consisted of 30 serum. The mean blood sugar in the diabetic group was 202 ± 49 mg%. The mean LDL-cholesterol in the hyperlipemic group was 202 ± 33 mg%. For the smoker group, the mean duration of smoking was 18 years. The detailed of the serum study is demonstrated in Table 1.

TABLE 1. Mean and SD of blood sugar and LDL-cholesterol levels in each group of study serum and the results of surface and total TF in each group.

Group (n=30)	Blood sugar (mg%)	cholesterol LDL (mg%)	Surface TF (uU/cell)	Total TF (uU/cell)
Diabetic	202±49	108±16	0.08±0.05	0.4±0.3
Hyperlipemic	90±12	202±33	0.06±0.04	0.4±0.5
Smoker	93±7	108±14	0.02±0.02	1.1±0.8
Normal control	92±7	119±14	0.05±0.04	0.2±0.1

2. Effect of the study serum on the surface TF of endothelial cells

None of the study serum was able to stimulate the production of surface TF of endothelial cells. The mean surface TF produced in group 1 was 0.08 ± 0.05 microunit/cell (uU/cell), group 2 was 0.06 ± 0.04 uU/cell, group 3 was 0.02 ± 0.02 uU/cell and group 4 was 0.05 ± 0.04 uU/cell.

3. Effect of the study serum on the total TF of endothelial cells

Serum from the smokers (group 3) can induce the total TF production of the endothelial cells significantly compared to the serum from groups 1, 2 and 4 ($p< 0.0001$). The mean and standard deviation of total TF of endothelial cells induced by smokers serum was 1.12 ± 0.8 uU/cell which was significantly higher than that induced by serum from other groups ($p< 0.0001$). The mean and standard deviation of total TF induced from the serum of groups 1,2 and 4 was 0.4 ± 0.3, 0.48 ± 0.5, 0.2 ± 0.1 uU/cell respectively (Fig 1). There was no statistical difference of total TF between the normal group and the diabetic group and also between the normal group and the hyperlipemic group.

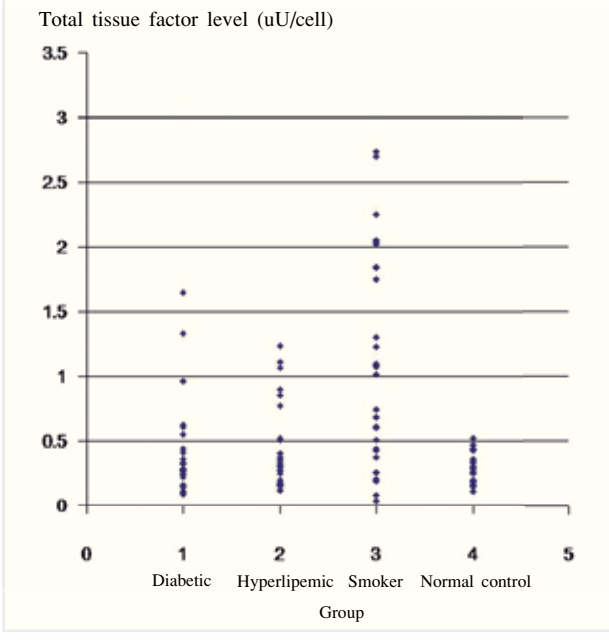

Fig 1. Total tissue factor levels of endothelial cells after incubation with different groups of study serum.

DISCUSSION

The method we used to detect the TF activity of endothelial cell was sensitive and was able to detect as low as 0.06 mU of TF. The sensitivity of the method was comparable to that from other studies.[16,17] In the study of the serum effect on the TF activity of endothelial cells, we found that the difference in cell numbers will give a significant difference in TF activity. Therefore, we reported the TF activity of endothelial cells per cell to solve this problem.

In our study, we did not find a significant effect of hyperglycemic serum and hyperlipemic serum on the endothelial tissue factor. Incubation of the endothelial cells with a high concentration of glucose leads to a severe delay in proliferation and in the adhesive and synthetic properties of the cells[18,19] and a change in the intracellular signaling pathway.[20] Data from Weis et al also showed that oxidized LDL is able to stimulate the endothelial cells to produce tissue factor.[21] It is possible that there are many different mechanisms in which glucose and lipids cause endothelial injury without interfering with the TF production.

Cigarette smoking is an important risk factor in the pathogenesis of atherosclerosis. Burke et al have demonstrated that sudden cardiac death in smokers was mainly associated with thrombosis regardless of the underlying plaque pathology and was not associated with the number of lipid-rich vulnerable plaques.[22] Possible mechanisms for this include leukocytes and platelet activation and damage to the endothelium.[23,24] Several studies have also suggested that significantly higher levels of circulating tissue factor are found in patients with acute myocardial infarction.[25] Potential sources of this circulating tissue factor include endothelial cells. However, data about the effect of smoking on tissue factor expression of the endothelial cells are limited.

When we studied the effect of smokers serum on the production of endothelial tissue factor, we found an evidence of an increase in tissue factor production that may lead to an acute thrombotic event in smokers. The mechanisms by which cigarette smoking contributes to the endothelial damage are not understood. Blann et al also found the injurious effect of smokers serum on the endothelial cells by demonstrating the increase in the von Willebrand factor which is one of the markers of endothelial cell injury.[26] The effect of smoking on the endothelail cell injury may be the result of nicotine which is known to injure the endothelial cells even at a low concentration.[27-30] However, in this study, we found that the levels of tissue factor produced were not correlated with the duration of smoking. It can be postulated that the free radicals produced during the smoking might be the cause of endothelial injury. In cigarette smokers, a substantial amount of free radicals are introduced exogenously and cigarette smoke contains more than 4,000 known and more than 100,000 unknown constituents.[31] These free radicals have a short effect and may be more harmful in the smokers who frequently smoke more than those who smoke for a long time. The mechanism by which smoking causing endothelial injury requires further study.

In conclusion, in 3 of the atherosclerotic risk factors we studied, we found that smoking cause endothelial cells to produce TF. However, the mechanism underlying tissue factor expression is still poorly understood.

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