The effects of CRP and PlGF expression on plaque stability in human carotid atherosclerosis

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Objective: This study aimed to investigate the effects of C-reactive protein (CRP) or placenta growth factor (PIGF) on atherosclerotic plaque stability. **Methods:** Fifty-five patients were recruited from among individuals who underwent carotid endarterectomy (CEA) in the Vascular Surgery department of the Fifth Hospital of Zhengzhou University from January 2008 to June 2009. The patients were divided into symptomatic and asymptomatic groups. Symptomatic patients were stratified according to the transient ischemic attack (TIA) frequency within six months: level I (1-2), level II (3-5), or level III (6 or more). CRP and PlGF expression were assayed and analyzed to determine whether they were associated with plaque stability. **Results:** No significant differences were found in CRP expression between the two groups, but PlGF expression in asymptomatic patients was lower in symptomatic patients. PlGF expression in asymptomatic patients was found to be positively related with TIA frequency, suggesting that lowering the PlGF level may represent an effective strategy to stabilize atherosclerotic plaques. [Life Science Journal 2010;7(3):58-63]. (ISSN: 1097-8135).

Keywords: carotid atherosclerosis, unstable plaque, placenta growth factor, C-reactive protein, transient ischemic attack

Introduction

Cerebral infarction (CI) is associated with some of the highest rates of disability and mortality. Even when promptly treated, most of the patients experience permanent disability. Cerebral infarction and atherosclerosis are closely related; atherosclerosis is a complex chronic inflammatory disease in which multiple factors and systems participate. Carotid atherosclerotic plaque rupture is considered the basis for symptomatic carotid artery stenosis, and may involve local inflammation and angiogenesis[1]. The accompanying platelet aggregation, thrombosis, internal haemorrhage, ulcer and defulvium are considered to be the principal pathophysiologic mechanisms leading to CI.

2 Materials and Methods

2.1 Study population and tissue sampling

A total of 55 patients were recruited for this study from among individuals who had Fifth Hospital of Zhengzhou University (China). All study protocols and procedures were approved by the local ethics committee of Zhengzhou University. The nature, purposes, possible benefits and risks of this study, other treatment options, rights and obligations were sufficiently explained to subjects individually. Written 'Carotid atherosclerosis study informed consent' was obtained from each subject with no compulsion, improper pressure and temptation. Plaques were obtained by surgical excision. Resected tissues were rinsed within 10-20 minutes of removal, and then snap-frozen in liquid nitrogen and stored at -80℃ until analysis.

2.2 Study design

Resected carotid atherosclerotic plaques were divided into two groups according to preoperative clinical symptoms and examination results: Symptomatic group (SP) and Asymptomatic group (AP).

Inclusion criteria for SP included: at least one transient ischemic attack (TIA symptoms such as transient dizziness, amaurosis, limb asthenia and numbness, and salivation) within six months before surgery; and, manifestation of obvious local neurological dysfunction or single blindness, regardless of whether the TIA signs were mild or permanent or if local carotid artery stenosis was >70%. This group was comprised of a total of 30 patients (Patient IDs: #1-#30), including 21 males and 9 females with an

average age of 64.6 ± 12.7. Symptomatic patients were further stratified according to the TIA frequency experienced within six months: I level (1-2), II level (3-5), III level (6 or more).

Inclusion criteria for AP included: no history of TIA within six months before surgery; no clinical signs of nervous system dysfunction; and, presence of local carotid artery stenosis confirmed by CTA at >70%. This group was comprised of a total of 25 patients (Patient IDs: #31-#55), including 19 males and 6 females with an average age of 67.4 ± 7.2.

2.3 Sample preparation and protein analysis

Clear liquid between the upper oil phase and lower cloudy liquid phase was extracted from fully homogenized tissue, and enclosed in the 0.2ml EP and stored at -80℃ until use in analysis. We carried out protein quantification by use of the standard Bradford method using optical density $(OD)_{###}$ on a UV spectrophotometer (MODEL; MANUFACTURER, LOCATION) The CRP expression was assayed by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The OD_{450} value of each spot was determined, then a standard curve with absorbance OD value as ordinate (Y) and corresponding standard fluid concentration as abscissa (X) was generated. Western blotting was used to assay PlGF expression. Gel imaging and processing systems were used to perform scanning and image analysis to determine molecular weight and net value of target.

3 Results

3.1 Comparison of age and gender

χ^2 test with α=0.05 was used to compare the ages from among the AP and SP groups; the age range was not significantly different among the two (P=0.619). Student's *t*-test was used to compare the genders among the two, and again no significant difference was found (P=0.346).

3.2 Analysis of CRP concentration assayed by ELISA

In order to analyze total protein concentrations in samples from our patient cohort, a standard curve was generated first (Figure 1). From this, the standard curve equation was determined to be y = 0.0295x+ 0.0081，R^2 = 0.9867 and was applied to the evaluation of total protein concentration (μg/μl) of the protein extracts. The mean concentration from our 55 patients was 8.91 ± 4.92.

The standard curve was constructed according to the standard fluid absorbance (Table 2) to be used to determine the specific CRP protein concentrations in each sample. The equation formulated from this curve: y = 0.0036x + 0.0036，R^2 = 0.9935 was applied to the OD values of individual samples.

The resultant CRP protein concentrations were compared by student's *t*-test and variance analysis, with the level of significance set at α＝0.05. The mean CRP protein concentration for the SP group was 21.44 ± 9.43μg/ml, and for the AP group was 25.27 ± 4.20μg / ml, (P =0.052). There were no significant differences (P>0.05) in CRP content of plaque tissues from the AP and SP groups.

3.4 PlGF expression assayed by Western blotting

The mean relative PlGF expression in the AP group was determined to be 2.02 ± 0.53, and that in the SP group was 0.58 ± 0.23. When the level of significance was set at α＝0.05, P=0.000, indicating that there was a significant difference in relative PlGF expression between the two groups. Specifically, the relative PlGF expression in AP was lower than that in SP (Figure 2).

3.5 Association of TIA frequency with instability of plaque

Stratification was performed in SP according to TIA frequency. Eight patients were characterized as level I, nine as level II, and thirteen as level III (Table 3).

We assessed the correlation between the grey level of symptomatic patients and TIA frequency by rank sum correlation test, R^2=0.915 when α=0.01.Correlation was statistically significant; thus, PlGF expression in plaques was highly correlated with TIA frequency in symptomatic patients.

4 Discussion

Our study found that there was a significant difference in PlGF expression in human carotid atherosclerotic plaque tissues from asymptomatic and symptomatic patients. PlGF expression was positively correlated with TIA frequency, which suggests that PlGF could also be correlated with plaque instability. There were no differences found in the CRP expression in plaque from AP and SP. Moreover, CRP

expression was not correlated with PlGF local expression in plaques. We inferred that CRP mainly reflects the condition of systemic inflammatory response, and has no significant effects on local plaque instability. PlGF is known to be involved in local angiogenesis of plaques, which can lead to plaque instability and the symptoms which characterize SP. Although the extent of carotid stenosis has been an important criterion for surgery, the carotid plaque instability is closely related with ischemic cerebrovascular disease. Therefore, the study of carotid plaque stability is of great significance to the prevention of ischemic stroke[2].

Carotid atherosclerosis is a part of general arteriosclerosis characterized by subintimal plate thickening of medium and large arteries. It may reduce or block blood flow. Together, the arterial wall thickening, hardening in the plaque site, lipid deposition and plaque necrosis characterize atherosclerosis. The subsequent events of plaque rupture and secondary thrombosis are the major pathophysiological basis for acute cardiovascular events.

CRP is a member of the pentamer protein family, which is secreted by liver epithelial cells in response to the presence of cytokine interleukin (IL)-6. Thus, as a sensitive inflammatory biomarker, CRP is considered a powerful predictor of cardiovascular events[3]. Previous studies have focused on the relationship between serum CRP and inflammation. We confirmed that CRP can also be detected in atherosclerotic plaques. Unfortunately, we did not find there were significant differences in CRP between SP and AP, which differed from our hypothesis, but was consistent with previous studiess[4]. The results of repeated assay comparisons of serum CRP between high and low risk people in were not consistent with our comparison between SP and AP. Different isoforms and post-translationally modified versions of CRP may explain this inconsistency [5].

PlGF is a kind of peptide growth factor that is known to bind with Flt-1/VEGFR-1[6], neural cilia protein -1 (NRP1) and neural cilia protein -2 (NRP-2) receptor, but specifically not with vascular endothelial growth factor (VEGFR-2)[7]. Studies into the molecular mechanisms underlying atherosclerosis have rarely involved PlGF. The As a result, the effects of PlGF on atherosclerotic plaque development remains unclear. Some studies have shown that PlGF, unlike VEGFR, is not necessary for embryonic development, despite the fact that it is highly expressed during pathological angiogenesis, such as in tumorigenesis or repair in response to trauma[8]. This finding suggested that PlGF may play a specific role in pathological angiogenesis.

Experience of a TIA event is a strong indicator of upcoming stroke. If the blood supply decreases for more than several minutes, the nerve cells in the ischemic region will die, leading to permanent neurologic impairment[10]. Recent studies have proven that in patients with carotid atherosclerosis, plaque rupture and thrombosis play vital roles in ischemic attack. The pathological angiogenesis may cause carotid atherosclerotic plaque instability, then rupture and thrombosis, which is relative to symptoms. Recent studies showed that angiogenesis may promote atherosclerotic lesions vulnerable to mechanical stress, leading to plaque internal hemorrhage[9]. Furthermore, our study found that TIA frequency was significantly related with PlGF expression levels in plaques. We inferred that the increased PlGF may lead to local plaque instability by encouraging pathological angiogenesis. Therefore, we may indirectly predict the outcome of stable patients by assaying serum PlGF level.

In addition, carotid atherosclerosis tends to occur in the initial segment of the carotid bifurcation, which suggests carotid atherosclerosis involves local factors. These factors that effectively increase intimal damage include the high shear stress of carotid blood flow and the mechanical damage of turbulence. When the sympathetic nervous system is activated by aggravating activities, agitation, cold or drugs, blood pressure becomes elevated, heart rate accelerated, and myocardial contraction enhanced; all leading to significant increase in mechanical stress suffered by plaques, including circumferential stress, shear stress, extrusion pressure which local artery spasm exerts on plaque, turbulence and so on. These stresses lead to plaque rupture. Circumferential stress is directly related to lumen diameter and intravascular pressure, while inversely proportional to vascular wall thickness. Recent studies on the shear stress of blood flow have revealed that complex shear stress is involved in endothelial cell activation. Different mechanical movements in the blood stream have different effects on endothelial cells. Turbulence promotes cell proliferation and apoptosis, inflammatory reactions, absorption synthesis, and monocyte and lipid deposition.

Thus, the possibility of plaque internal hemorrhage and rupture is supported. Increased apoptosis further encourages thrombosis and reduces the atherosclerotic plaque stability [11].

The commonly used treatments to stabilize atherosclerotic development include: 1) abstinence from cigarette smoking and wine intake; 2) changing diet style, such as eating more fish, and foods low in salt; 3) reducing abdominal fat; 4) performing more aerobic exercise; 5) inhibiting cholesterol synthesis by taking prescribed statins; 6) maintaining a normal hypoglycemic level; and, 7) supplementing the trace elements, such as vitamins and magnesium[12].

Carotid endarterectomy is a surgery routinely used to correct carotid stenosis and occlusions. It has become the gold standard for revascularization of the extracranial carotid occlusive disease. The CEA surgery may eliminate severe atherosclerosis stenosis, and also act as the second-level prevention for symptomatic patients[13]. In addition, CEA effectively reduces the stroke risk for asymptomatic patients[16]. With the development of science, carotid angioplasty and stenting (CAS) has provided a minimally invasive method for the treatment of extracranial carotid artery occlusive disease; trauma is minimal and patients receiving CAS recover quickly. Moreover, CAS facilitates treatment within the forbidden physical area known as the 'siphon segment', which CEA cannot reach. Maturity of CAS technology and equipment has led many physicians to consider CAS as having completely replaced CEA. However, a large-scale study showed that stent implantation was not equally beneficial for the treatment of carotid atherosclerosis plaques[14]. For patients who experience recurrent stroke, the optimal time window for surgery is two to five weeks after the condition is stabilized. Waiting more than 12 weeks may lead to further loss of brain cells[15].

5 Conclusions

The local PIGF level is able to better reflect plaque instability than CRP dose. Increased amounts of PlGF may promote plaque instability by encouraging pathological angiogenesis. Therefore, we may indirectly predict the outcome of stable patients by assaying serum PIGF level.

Table 1. Characteristics of experimental subjects

Group	Male (*n*)	Female (*n*)	Age (χ ± s)
SP	21	9	64.63 ± 12.73
AP	19	6	67.36 ± 7.18

Figure 1. Standard curve for protein quantification by Bradford method

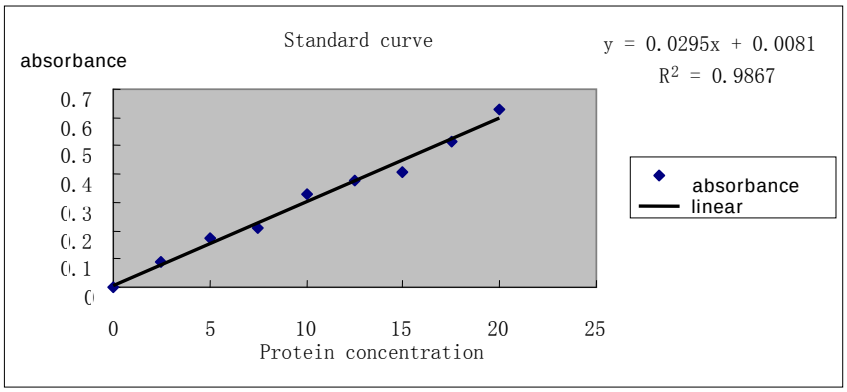

Table 2. ELISA standard curve

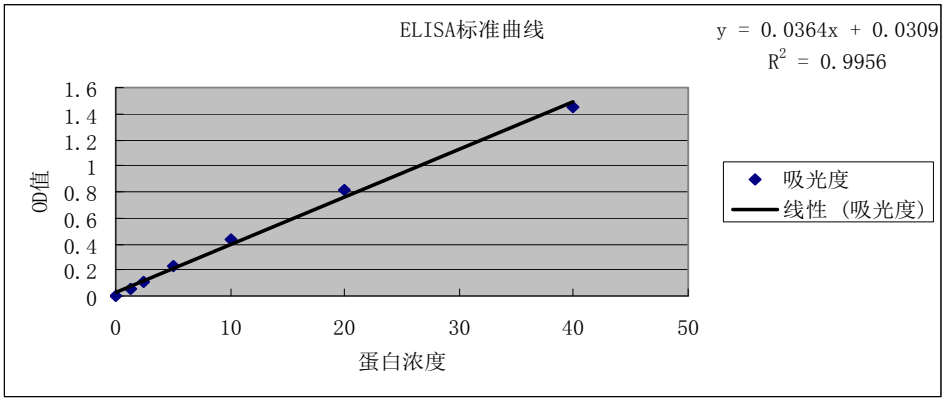

Figure 2. The expression image of standard protein β-Actin and PlGF.

Note: 5, 18 and 27 were in the SP group; 39, 35 and 46 were in the AP group.

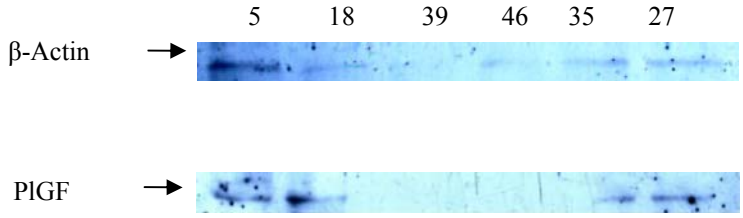

Table 3. TIA stratification and mean grey levels

TIA stratification	*n*	Mean grey level of SP group
□level	8	1.45 ± 0.30
□level	9	2.05 ± 0.45
□level	13	2.44 ± 0.35

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