Smooth muscle immaturity in the carotid arterial neointima as a prognostic marker for systemic atherogenic cardiovascular events in the Asian male

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Received September 28, 2015; Accepted October 28, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Although immaturity of neointimal smooth muscle cells (SMCs) in coronary arteries has recently been demonstrated to be associated with acute coronary syndrome, the carotid arterial counterpart has not been investigated. We hypothesized that the same investigation of carotid endarterectomy specimens might contribute to living patients. Carotid endarterectomy specimens from 33 Asian males who underwent a 5-year follow-up were examined. Age, atherosclerotic risk factors, and percentage stenosis were investigated. Histologically, the fibrous cap/lipid core ratio was measured. Maturation of SMCs was assessed by the h-caldesmon/smooth muscle actin (SMA) ratio by immunohistochemistry in 3 different regions (luminal, medial, and opposite side of lipid core) in the neointima. Associations of these factors with preoperative symptoms along with postoperative systemic atherogenic cardiovascular events were analyzed. It was revealed that fibrous cap/lipid core ratio was significantly lower in symptomatic than in asymptomatic patients, while the h-caldesmon/SMA ratio was significantly lower in patients with than without postoperative systemic atherogenic cardiovascular events by the Student's t-test ($P<0.05$). Logistic regression model demonstrated that younger age and a lower h-caldesmon/SMA ratio were associated with postoperative systemic atherogenic cardiovascular events ($P<0.05$). This result was not different when 3 different regions were each analyzed instead. Immaturity of neointimal SMCs shown by a lower h-caldesmon/SMA ratio by immunohistochemistry was associated with systemic atherogenic cardiovascular events. Thus, this finding may be predictive of these events after carotid endarterectomy. Uniform results among different neointimal regions suggest that immaturity of neointimal SMCs causes plaque instability and does not occur secondarily to plaque instability.

Keywords: Carotid endarterectomy, immunohistochemistry, smooth muscle, differentiation, prognosis

Introduction

Stroke is the fourth leading cause of mortality and a major source of chronic disability in the United States and Japan, although its incidence has decreased steadily since the 1960s [1, 2]. Extracranial carotid artery stenosis is a factor in 20%-30% of all strokes [1], and surgical endovascular revascularization such as carotid artery stenting or carotid endarterectomy (CEA) is recommended in patients with stenosis ≥60% and with a life expectancy >5 years [1, 3]. Apart from the risk for stroke, the presence of severe asymptomatic carotid artery stenosis is a predictor of systemic cardiovascular events, reflecting the severity of systemic atherosclerosis [1-4].

The direct trigger of stroke associated with carotid artery stenosis is mainly plaque rupture due to the vulnerability of atherosclerotic plaque leading to thrombus formation and embolization of the intracranial cerebral vasculature [2, 5]. The histological characteristics of rupture-prone vulnerable plaques in the carotid artery were noted to be a large necrotic core, thin fibrous cap, and abundant macrophages [5, 6]; likewise their coronary counterparts were associated with acute coronary syndrome [7, 8].
However, little is known about the association of plaque stability with smooth muscle cells (SMCs), the main component of plaque. Although a smaller quantity of SMCs was reported to be associated with vulnerable plaques than with stable plaque in the coronary arteries [8], the quality of neointimal SMCs in association with plaque stability rarely has been described. Indeed, it is believed that neointimal SMCs have a uniformly "synthetic" phenotype in contrast to a "contractile" phenotype in the media [9]. The synthetic phenotype is immunohistochemically characterized by lack of staining of some differentiated SMC markers such as desmin and smoothelin [10]. Neointimal SMCs were reported to have an almost exclusively synthetic phenotype not only in atherosclerosis but also in Moyamoya disease [11] and restenosis after coronary stent implantation [10]. We formerly demonstrated this phenomenon also in the neointima of Buerger’s disease and thromboembolism [12].

We previously evaluated SMC maturation in uterine muscular neoplasm by immunostaining for h-caldesmon [13], and found that this marker was useful for assessment of SMCs beyond intermediate maturation. Also, we recently showed by an autopsy study that neointimal SMCs have divergent phenotypes in differentiation in the coronary artery [14]. That is, an h-caldesmon-negative relatively immature phenotype

Figure 1. Methods of the present study. A. Exclusion criteria of the subjects are illustrated. B. Representative carotid endarterectomy specimen with elastic Masson staining (×20). The narrowest thickness of the fibrous cap (blue arrow) and greatest thickness of the lipid core (black arrow) were measured. Three fields from the luminal side (red square), medial side (yellow square), and opposite side (white square), respectively, of the lipid core were selected from the neointima for the assessment of immunohistochemical results. C. Postoperative atherogenic cardiovascular events are presented.
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and an h-caldesmon-positive relatively mature phenotype exist, and the former was associated with acute coronary syndrome. Autopsy studies, however, are not beneficial to pre-mortem patients. In contrast, CEA specimens presented to pathological departments may benefit living patients. To the best of our knowledge, evaluation of neointimal SMCs by immunostaining of h-caldesmon in the carotid artery has not been performed. Therefore, the purpose of the present study was to assess whether results of immunohistochemistry for h-caldesmon in CEA material are associated with pre-operative symptoms and are predictive of systemic cardiovascular events.

Material and methods

Patients

The local Ethics Committee approved the protocol of the present study. A total of 51 consecutive CEAs from 48 patients (44 males and 4 females, aged 52-84 years) performed between November 2003 and December 2008 at NTT Medical Center Tokyo were identified from the database at that institution. Detailed clinical data had been recorded for each patient, including age and sex, along with past history.

Figure 2. Elastica Masson staining (A, B) as well as Immunohistochemistry for α-SMA (C, D, G, H) and h-caldesmon (E, F, I, J) in case with postoperative cardiovascular events (A, C, E: identical fields) and in a non-event case (B, D, F: identical fields). (A, B) Lipid core, fibrous cap, and other neointimal components are observed. (C, D) Abundant α-SMA-positive SMCs are identified in the neointima. (E) Most of the neointimal SMCs are not immunostained by h-caldesmon. (F) Most of neointimal SMCs are also positive for h-caldesmon. (G-J) High-power field of the medial side of the lipid core in (C-F), respectively. Most SMCs in (G) are not immunostained by h-caldesmon in (I) while most SMCs in (H) are immunostained by h-caldesmon in (J).
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and/or presence of smoking, hypertension, diabetes, or dyslipidemia based on current Japanese criteria. Percentage stenosis had been measured by ultrasonic and/or magnetic resonance angiography according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET). Preoperative occurrence of cerebral infarction and transient ischemic attack (TIA) had been recorded. Furthermore, we searched for systemic cardiovascular events that had occurred during a postoperative follow-up period of 5 years. Of note, in the present study, cardiovascular events associated with atherosclerosis were adopted, such as re-stenosis, cerebral infarction, contralateral carotid stenosis, acute coronary syndrome, arteriosclerosis obliterans, and aortic aneurysm. Eight paraffin-embedded specimens in which the follow-up period was less than 5 years were excluded from the present study. However, specimens from patients in whom the systemic atherogenic cardiovascular events were recognized within 5 years were included, even if the follow-up period was less than 5 years. Two specimens from patients with the complication of atrial fibrillation were excluded, since thromboembolism regardless of atherosclerosis may occur. One specimen from a postoperative death from another cause, 1 from a patient with a postoperative local complication (early rupture), and 1 specimen that had been borrowed and not returned were excluded. Three specimens from a CEA of contralateral carotid stenosis were excluded, but preceding CEA specimens were used and regarded as from cases with postoperative systemic atherogenic cardiovascular events. Thus, 35 specimens from 35 patients (33 males and 2 females) were adopted. Due to the remarkable sex difference, only the 33 specimens from the males were included in the present study (Figure 1A). In all 33 cases, antiplatelet drugs had been prescribed post-operatively.

Grouping

Grouping of the cases was performed by 2 individual methods. First, cases were divided into a symptomatic or asymptomatic group based on the presence or absence, respectively, of preoperative symptoms including cerebral infarction and TIA. Second, cases were divided into an event or non-event group by the presence or absence, respectively, of postoperative occurrence of one or more of the systemic atherogenic cardiovascular events thus far mentioned.

Pathological study and immunohistochemistry

The surgically removed carotid plaques were fixed in 10% formalin solution for 24 h, decalcified by formic acid, and embedded in paraffin. The specimen with the most narrowed artery was selected for each case. Prior to immunostaining, selected tissue sections were re-stained with hematoxylin-eosin and elastic Masson staining. Immunohistochemistry was performed using paraffin-embedded sections, 4 μm thick, using the avidin-biotin-peroxidase complex according to standard methods. The monoclonal antibody to alpha-smooth muscle actin (α-SMA, clone 1A4, working dilution 1:100, Dako, Glostrup, Denmark) was used to identify all SMCs, and h-caldesmon (clone h-CD, working dilution 1:100, Dako) was used to identify SMCs beyond intermediate differentiation. Deparaffinized and dehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. For immunostaining of h-caldesmon, sections were autoclaved in 10 mmol/L sodium citrate buffer (pH 6.0) at 121°C for 10 min to expose antigens and were cooled for 30 min. After rinsing in 0.1 mol/L phosphate buffered saline (PBS, pH 7.4), the sections were incubated with affinity purified primary antibodies overnight at 4°C. Thereafter, they were incubated with Envision (+) rabbit peroxidase (Dako, Carpinteria, CA, USA) for 30 min. The peroxidase reaction was performed using 0.02% 3,3’-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.1 mol/L PBS (pH 7.4). Finally, nuclear counterstaining was performed with Mayer’s hematoxylin.

Assessment

For each patient, the number of risk factors present was counted from among past history and/or presence of smoking, hypertension, diabetes, and dyslipidemia.

Light microscopy performed with a Carl Zeiss HAL 100 instrument and a W-PI 10×/23 ocular lens was used to analyze and quantitate the histological data. The narrowest thickness of the fibrous cap and widest thickness of the lipid
Figure 3. Comparisons of representative data (average ± standard deviation) according to preoperative symptomatic vs. asymptomatic patients, as well as postoperative event vs. non-event patients. *P < 0.05 by Student’s t-test.
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The core of each vessel were measured using elastic Masson staining in the region where the lipid core was widest (Figure 1B). In specimens with obviously ruptured plaque, the thickness of the fibrous cap was counted as 0. Then, the ratio of fibrous cap thickness to lipid core thickness was calculated (fibrous cap/lipid core ratio).

In assessing immunohistochemical results, the luminal, medial, and opposite (neointima but devoid of lipid core) sides of the lipid core were identified from the neointima. Then, 3 different fields from each of these 3 regions were selected in each specimen (Figure 1B). Identical fields were selected from α-SMA and h-caldesmon immunostaining. Digital images of immunohistochemistry of these 9 fields (×200) in 2 immunostainings were captured by the NY-D5000 super system (Microscope Network, Nikon, Tokyo, Japan) and were printed by a true-color printer (IPSiO SP C420, Ricoh, Tokyo, Japan). In every image, a separated positive area was regarded as individual positive cells, but a positive area less than the size of a muscular nucleus was discarded. Total number of positive cells was counted in each α-SMA and h-caldesmon immunostained image, respectively. Then, the average ratio of h-caldesmon+ cells to α-SMA+ cells (h-caldesmon/SMA ratio) was counted in each of 3 regions. Further, the total average h-caldesmon/SMA ratio was calculated in each specimen.

Statistical analyses

As statistical analyses, comparison of average values for data including clinicopathological and immunohistochemical results was first performed by the Student’s t-test between symptomatic and asymptomatic groups as well as between event and non-event groups. Then, logistic regression analyses were performed using the SPSS software package (ver17.0, Tokyo, Japan) since outcome variables were dichotomous, and multiple explanatory variables were to be analyzed simultaneously. Outcome variables were established as symptomatic vs. asymptomatic groups (symptomatic =1, asymptomatic =0) as well as event vs. non-event groups (event =1, non-event =0). As explanatory variables, 5 variables were established and used in each analysis: 1, age; 2, number of atherosclerotic risk factors; 3, percentage stenosis by NASCET; 4, fibrous cap/lipid core ratio; and 5, total average h-caldesmon/SMA ratio. These analyses were also performed by changing total average h-caldesmon/SMA ratio to the average ratio in each of 3 different regions. The level of significance was set at P<0.05.

Results

Patients’ baseline characteristics and average values of assessed data

Specimens for the present study were obtained from 33 males (age 52-84, mean 68.1±6.8 y). Past history and/or presence of smoking, hypertension, diabetes, and dyslipidemia were identified in 30, 29, 15, and 17, respectively, of the 33 patients. The number of these risk factors ranged from 1 to 4 (mean 2.76±0.83) among the 33 patients. Percentage stenosis of the carotid artery by NASCET ranged from 50 to 99% (mean 80.2±12.1%). Fibrous cap thickness ranged from 0 to 1200 (mean 267±274) μm, while lipid core thickness ranged from 200 to 7500 (mean 2249±1916) μm. Fibrous cap/lipid core ratio ranged from 0 to 400% (mean 31±71%). Immunohistochemical results of SMA+ cells ranged from 2068-7861 (mean 4828±1563), while h-caldesmon+ cells ranged...
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Table 2. Odds ratios (ORs) of h-caldesmon/SMA ratio according to different evaluated regions

<table>
<thead>
<tr>
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<th>Symptomatic vs. Asymptomatic</th>
<th>Event vs. Non-event</th>
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<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P</td>
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<tr>
<td>Average of 3 regions</td>
<td>1.02 (0.95-1.10)</td>
<td>0.53</td>
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<tr>
<td>Luminal side of lipid core</td>
<td>1.05 (0.96-1.16)</td>
<td>0.31</td>
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<tr>
<td>Medial side of lipid core</td>
<td>1.02 (0.94-1.11)</td>
<td>0.57</td>
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<tr>
<td>Opposite side of lipid core</td>
<td>1.01 (0.96-1.07)</td>
<td>0.67</td>
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SMA, smooth muscle actin; CI, Confidence interval.

Differences in assessed values according to postoperative events

Average age ± S.D. in the event group vs. non-event group was 66.4±9.2 vs. 68.9±5.2 y. Differences in average values ± S.Ds. between the event vs. the non-event group were 2.91±0.83 vs. 2.68±0.84 for atherosclerotic risk factors, 82.5±15.4% vs. 79.1±10.3% for stenosis by NASCET, 173±182 vs. 314±317 μm for fibrous cap thickness, 2632±2115 vs. 2057±1831 μm for lipid core thickness, 14±18% vs. 39±86% for the fibrous cap/lipid core ratio, 5086±1644 vs. 4698±1544 for SMA+ cells, 1072±777 vs. 1663±1110 for h-caldesmon+ cells, and 20.1±10.0% vs. 34.3±18.6% for the h-caldesmon/SMA ratio. Of these comparisons, only the h-caldesmon/SMA ratio was statistically significant by the Student’s t-test (P<0.05).

Results of logistic regression analyses

The results of logistic regression analyses using the total average h-caldesmon/SMA ratio are summarized in Table 1. There was no significantly related variable in the analysis of the symptomatic versus the asymptomatic group. However, the analysis of the event vs. the non-event group showed that age and the h-caldesmon/SMA ratio were significantly related to each other (OR 0.86, 95% CI 0.74 to 1.00, P=0.047; and OR 0.90, 95% CI 0.81 to 1.00, P=0.045, respectively), meaning that younger age and a lower h-caldesmon/SMA ratio were...
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significantly related to postoperative systemic atherogenic cardiovascular events.

The above-mentioned 2 logistic regression analyses were further performed by changing the total average h-caldesmon/SMA ratio to the ratio in each of 3 different regions. As shown in Table 2, no significant results were obtained concerning analyses of symptomatic versus asymptomatic groups. Although no significant results were obtained concerning analyses of the event vs. the non-event group either, similar results were obtained for analyses based on the luminal side (OR 0.90, 95% CI 0.81 to 1.00, P=0.056), medial side (OR 0.94, 95% CI 0.87 to 1.01, P=0.11), and opposite side (OR 0.93, 95% CI 0.86 to 1.00, P=0.057).

Discussion

In the present study, we showed heterogeneity in the degree of neointimal SMC maturation assessed by the immunohistochemical h-caldesmon/SMA ratio in the carotid artery for the first time, even if the extent of stenosis in the arteries was to the same degree. An especially important finding was that this ratio was significantly associated with postoperative systemic atherogenic cardiovascular events. This means that when we regard atherosclerosis as a systemic disease [2-4], we can predict the cardiovascular prognosis by examining CEA material. Therefore, immaturity of neointimal SMCs may not only be associated with acute coronary syndrome when assessed in the coronary arteries as we have recently reported [14], but also may be an indicator of systemic complications when assessed in the carotid arteries.

The Student's t-test demonstrated that only the fibrous cap/lipid core ratio was significantly different between symptomatic and asymptomatic patients, with this ratio being lower in the symptomatic patients. This result is in accordance with a histological study of a large cohort by other authors in which unstable plaques indicated by a thin fibrous cap and large lipid core were significantly more prevalent in symptomatic cases than in asymptomatic cases [5]. Another study also showed that a large lipid core and macrophage infiltration were associated with plaque instability [6].

Although our previous study demonstrated that immaturity of neointimal SMCs assessed by the h-caldesmon/SMA ratio was associated with acute coronary syndrome in coronary arteries [14], this ratio did not differ significantly between symptomatic and asymptomatic patients. This may be due to a difference in pathogenesis between cerebral and myocardial infarction. That is, the major pathogenesis of cerebral infarction associated with carotid stenosis is not local acute thrombotic occlusion as in myocardial infarction, but artery-to-artery embolism [2]. In addition, hemodynamics, including the development of collaterals, is also concerned with the occurrence of cerebral infarction [2]. Furthermore, sample bias due to investigation of only CEA material may be of concern since extracranial carotid artery stenosis is a factor in only 20%-30% of all strokes [1].

When data based on the grouping according to postoperative events were compared, the fibrous cap/lipid core ratio was not significantly different between event and non-event patients as verified by a higher standard deviation. This may be due to the relatively smaller number of enrolled subjects, but, alternatively, this parameter may not influence systemic complications although it affects local manifestations. Instead, the Student’s t-test demonstrated that only the h-caldesmon/SMA ratio was significantly different between the event and non-event groups. Furthermore, logistic regression analysis revealed that younger age and a lower h-caldesmon/SMA ratio were significantly related to postoperative systemic atherogenic cardiovascular events. Although the average age was not greatly different between the event and non-event groups, the lower standard deviation may have resulted in a significant value for this variable in the logistic regression analysis. Indeed, other authors have also reported results of CEA studies showing that younger patients were more likely to develop recurrent carotid stenosis and cardiovascular events than their older counterparts [15, 16]. On the other hand, independently of age, the h-caldesmon/SMA ratio was significantly related to postoperative atherogenic events. Therefore, the degree of maturation of neointimal SMCs in the carotid artery may be paralleled by that in the systemic vasculature, where immature neointimal SMCs are likely to be associated with atherogenic events.

With regard to the etiology of the divergent maturation of neointimal SMCs, we hypothesize
that at least 2 possibilities exist. First, intra-plaque leukocytes, including macrophages, secrete enzymes such as matrix metalloproteinase, which inhibit maturation of SMCs in the same manner as the development of plaque instability through fragilization of the fibrous cap [2, 5]. Second, a genetic or acquired unknown factor regulates maturity or at least the maturation speed of SMCs. Then, immature SMCs cannot inhibit the secondary enlargement of the lipid core in contrast to mature SMCs that suppress growth of the lipid core. We postulate that the second hypothesis is likely, since additional logistic regression analyses in which 3 different neointimal regions were individually analyzed demonstrated similar results among analyses based on luminal, medial, and opposite side of the lipid core. If the first hypothesis were true, the luminal or medial side of the lipid core, where macrophages were near SMCs, should have been more significantly related to the outcome than the opposite side of the lipid core. Genetic predisposition has been suggested to account for 40% to 60% of human susceptibility to coronary artery disease and thus to atherosclerosis, but genome-wide association studies demonstrated that most genetic risk variants are mediated through unknown mechanisms [17]. Whether or not maturation of SMCs is associated with these unknown mechanisms will be elucidated in the future.

The limitation of the present study is that analyses of the individual atherosclerotic risk factors of smoking history, hypertension, diabetes, and dyslipidemia were not performed since the number of enrolled patients was relatively small, aggregation bias was relatively higher than in usual studies in which the positivity ratio of each factor is around 50% (e.g. smoking was found 30 out of 33 subjects), and pathological risk factors other than atherosclerotic risk factors were mainly analyzed. However, one study reported that none of these factors was significantly different between a symptomatic and asymptomatic group [5]. In addition, female subjects were excluded from the present study because of the extremely small number of females identified in our examination of the database. This small number was probably because of estrogen-related anti-atherosclerotic mechanisms. Future study is necessary to investigate SMC maturation and sex differences.

In conclusion, we have conducted a clinicopathological retrospective cohort study using CEA material from Asian males. Plaque instability indicated by a lower fibrous cap/lipid core ratio was shown more frequently in symptomatic than in asymptomatic patients, whereas immature neointimal SMCs indicated by a lower h-caldesmon/SMA ratio was associated with postoperative systemic atherogenic cardiovascular events. Thus, immunostaining of h-caldesmon and SMAs in CEA material may predict systemic atherogenic events. Uniform results among different neointimal regions suggested that immaturity of neointimal SMCs is not a result of but a cause of plaque instability.

Acknowledgements

We thank Goichiro Yanagi for his skilful technical assistance. Further, we thank Dr. Yumiko Yamaoka for her academic advice.

Disclosure of conflict of interest

None.

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