Regulated Expression of the BTEB2 Transcription Factor in Vascular Smooth Muscle Cells

Analysis of Developmental and Pathological Expression Profiles Shows Implications as a Predictive Factor for Restenosis

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Background—We have previously shown BTEB2, a Krüppel-like zinc finger transcription factor, to regulate expression of the SMemb/NMHC-B gene, which has been implicated in phenotypic modulation of smooth muscle cells (SMCs). The present study was done to assess the developmental and pathological expression profiles of BTEB2 and to further evaluate the clinical relevance of BTEB2 expression in human coronary artery disease.

Methods and Results—Immunohistochemistry showed developmentally regulated expression of BTEB2 with abundant expression in fetal but not in adult aortic SMCs of humans and rabbits. In balloon-injured aortas, predominant expression of BTEB2 was seen in neointimal SMCs. Atherectomy specimens obtained from primary and restenotic lesions showed predominant expression of BTEB2 to stellate SMCs. The incidence of restenosis in primary lesions was significantly higher in lesions containing BTEB2-positive cells than in lesions without (55.6% versus 25.0%, P<0.01).

Conclusions—The present study shows that BTEB2 expression is developmentally and pathologically regulated. BTEB2 is preferentially expressed in dedifferentiated or activated SMCs. Examination of human coronary artery specimens suggests that primary lesions containing BTEB2-positive cells are associated with higher risk of restenosis than BTEB2-negative lesions. These results suggest that BTEB2 can serve as a molecular marker for phenotypic modulation of vascular SMCs. (Circulation. 2000;102:2528-2534.)

Key Words: angioplasty ■ muscle, smooth ■ restenosis ■ genes

Phenotypic modulation of vascular smooth muscle cells (SMCs) is associated with a variety of vascular diseases ranging from atherosclerosis to restenosis after angioplasty.1–4 Investigation of the molecular mechanisms underlying the proliferation as well as phenotypic changes of vascular SMCs may provide insight into therapeutic interventions.

In our studies to understand the mechanisms underlying phenotypic modulation of SMCs, we have recently identified the DNA-binding BTEB2 transcription factor to regulate the SMemb/NMHC-B gene, which is associated with the dedifferentiated or activated SMC phenotype.5 In vitro experiments indicate that BTEB2 expression is restricted to proliferating vascular SMCs, and BTEB2 mRNA levels are rapidly increased on mitogenic stimulation.5 These data have led us to propose that BTEB2 represents a marker for activated SMCs in the vascular wall.

The aim of the present study was to determine the expression profile of BTEB2 in developmental and pathological vascular processes and, in addition, to examine the expression of BTEB2 in human atherectomy specimens. Our results show that BTEB2 is expressed in fetal and dedifferentiated SMCs in humans and in rabbits and for expression of BTEB2 to correlate with subsequent development of restenosis after angioplasty.

Methods

Tissue Preparation
Thoracic aortas of normal and balloon-injured rabbits were used. Two female rabbits were euthanized in the last month of pregnancy. Thoracic aortas were obtained from both the mother and fetus. Vascular injury was produced by denudation of the thoracic aorta with a balloon catheter in 5 male rabbits (weight 3000 g). Rabbits were killed at 1 or 2 weeks after denudation. All procedures conform
TABLE 1. Clinical Profiles

|                      | BTEB2-Positive, % (n) (n=32) | BTEB2-Negative, % (n) (n=48) | P  
|---------------------|-------------------------------|------------------------------|-----
| Age, y              | 60.2±9.0                      | 63.2±9.9                     | 0.21
| Male sex            | 81.3 (26/32)                  | 77.1 (37/48)                 | 0.66
| Hypertension        | 53.1 (17/32)                  | 39.6 (19/48)                 | 0.23
| Diabetes mellitus   | 18.8 (6/32)                   | 22.9 (11/48)                 | 0.66
| Hypercholesterolemia| 40.6 (13/32)                  | 27.1 (13/48)                 | 0.21
| Smoking             | 56.3 (18/32)                  | 50.0 (24/48)                 | 0.58
| Unstable angina     | 34.4 (11/32)                  | 20.8 (10/48)                 | 0.18
| Lesion in LAD       | 71.9 (23/32)                  | 58.3 (28/48)                 | 0.22

LAD indicates left anterior descending coronary artery.

to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Thoracic aortas of normal fetuses and adults were used as controls for normal tissues. Aortas were obtained from three 9- to 10-week-old fetuses at autopsy. Three adult aortas without atherosclerosis were obtained from 30- to 40-year-old patients during a valvular replacement procedure. This research project was approved by the Ethics Committee of the Gunma University School of Medicine.

Collection of Human Coronary Atherectomy Samples

Samples were obtained from patients undergoing directional coronary atherectomy (DCA) between January 1993 and December 1994 at Saiseikai Maebashi Hospital (80 primary lesions and 136 restenotic lesions). Clinical variables documented include age, sex, presence of current angina pectoris and risk factors for coronary artery disease (history of hypertension, hypercholesterolemia, diabetes mellitus, and cigarette smoking), and type of angioplasty (stable versus unstable) (Table 1).

Atherectomy Procedures and Angiographic Analysis

DCA procedures and criteria for successful DCA were done as described. Follow-up angiography was performed at 4 months after atherectomy. Cineangiograms were evaluated with digital calipers by three cardiologists blinded to the patient’s backgrounds. The minimal lumen diameter (MLD) was determined from magnified images obtained in the single most stenotic view before and after coronary atherectomy, and the reference diameter (RD) was defined as the diameter of the proximal or distal segment without stenosis. A 10F guiding catheter was used as the reference object. Angiographic parameters were calculated as follows: % diameter stenosis=MLD/RD; acute gain=MLD (at DCA)−MLD (immediately after DCA); gain index=acute gain/RD; late loss=MLD (immediately after DCA)−MLD (follow-up); and loss index=late loss/acute gain. Restenosis was considered to be present if the treated vascular segment contained a lesion of >50% stenosis or if >50% of the acute gain was lost.

Histological Definitions

Atherectomized tissues were histologically classified into 3 types: (1) diffuse intimal thickening (DIT), (2) atherosclerotic tissue, and (3) lesion containing stellate cells. Lesions classified as DIT were defined as having proliferation of the intima without atherosclerotic change; lesions classified as atherosclerotic tissue included samples consisting of fibrous connective tissue, with or without associated foam cells, cholesterol clefts, lipid cores of pale granular material, or lymphocytes; and lesions containing stellate cells were characterized by the proliferation of stellate-to-spindle-shaped cells with loose to mildly fibrotic connective tissues.

Immunohistochemistry

All samples were immediately fixed in 10% buffered formalin and embedded in paraffin. Immunoenzymatic staining was carried out as previously described. All tissues were stained with antibodies against SM1 smooth muscle myosin heavy chain isoform (hereafter referred to as SM1) (dilution 1:1000 in PBS).7–10 von Willebrand factor (vWF) (1:100 dilution, Atlantic Antibodies)11,12 or BTEB2 (1:1000 dilution).5 Negative control sections were stained with normal mouse IgG (dilution 1:100 in PBS, Santa Cruz Biotechnology) in lieu of BTEB2 antiserum.

Double-Labeling Immunohistochemistry

Atherectomized specimens were stained by anti-BTEB2 antibody and monoclonal antibody against human proliferating cell nuclear antigen (PCNA) (1:50 dilution in PBS, Santa Cruz Biotechnology). Pretreatment of atherectomized tissues by microwave irradiation (400 W, 5 minutes ×2) was performed after deparaffinization. After incubation with anti-PCNA antibody, specimens were covered with peroxidase-conjugated anti-mouse immunoglobulin (1:100 dilution in PBS, Santa Cruz Biotechnology). After being stained with DAB, sections were treated with 0.1 mol/L glycine–hydrochloric acid buffer (pH 2.2) for 120 minutes with 4 changes. Next, samples were incubated for 60 minutes with monoclonal antibody against BTEB2. After being washed with Tris-buffered saline (pH 7.6), sections were treated by the alkaline phosphatase/anti–alkaline phosphatase (APAAP) method (APAAP kit, DAKO). Sections were visualized by addition of a solution of 0.2 mmol/L naphthol AS-MX phosphate, 1 mmol/L Fast Red TR, and 1 mmol/L levamisole in 0.1 mol/L Tris-HCl buffer (pH 8.2) for 20 minutes. After being counterstained with methyl green, samples were mounted in glycerol-gelatin medium.

In Situ Hybridization

In situ hybridization using rabbit-specific 35S-labeled riboprobes was performed as previously described.13,14 A 660-bp fragment (full length) from the rabbit BTEB2 cDNA was subcloned into the pSP72 vectors (Promega). Sense and antisense riboprobes were prepared by linearization of the constructs with the respective restriction endonucleases EcoRI and XhoI and then use of either SP6 or T7 RNA polymerase. In situ results were evaluated by polarized light epiluminescence microscopy (Leitz).

Statistical Analysis

Statistical analysis of frequency was performed with the χ2 test or Fisher’s exact test for small samples, and means were compared by unpaired 2-tailed t test. The odds ratio was used as a measure of risk. Modeling of dichotomous variables was done by logistic-regression modeling. Values are reported as mean±SD. A value of P<0.05 was considered statistically significant.

Results

BTEB2 Expression Is Developmentally Regulated

We first investigated BTEB2 expression during aortic development. Immunohistochemistry using tissues of human and rabbit normal fetal aortas showed prominent BTEB2 staining in the media of both humans and rabbits (Figure 1). In contrast, staining of BTEB2 was not seen in either intimal or medial SMCs in adult aortas, as confirmed by SM1 immunostaining to identify SMCs (Figure 1). Interestingly, BTEB2 staining was also seen in endothelial cells of rabbits and human aortas as confirmed by vWF staining (data not shown). Collectively, our results show developmentally regulated expression of BTEB2 in aortic SMCs with predominant expression in the fetus.
BTEB2 Expression Is Pathologically Regulated With Induction in Neointima

We next investigated whether BTEB2 is expressed in neointimal cells containing dedifferentiated SMCs in balloon-injured rabbit aortas. Neointimal cells which developed after denudation were positive for BTEB2 (Figure 2A). SMCs in neointima as well as endothelial cells were BTEB2-positive as assessed by SM1 (Figure 2A) and vWF expression (data not shown). Interestingly, a large number of BTEB2-positive cells were found in the adventitia after balloon injury, although these cells stained only weakly for BTEB2 in normal rabbit aorta (data not shown). In situ hybridization experiments using the rabbit balloon-injured aorta at 2 weeks after injury showed that the silver grain dots on the neointima yielded by antisense BTEB2 probe seemed to be stronger than those formed by sense BTEB2 probe (Figure 2B). Although some grains were present in the media, they seemed to be nonspecific because many of them were not localized to the cytoplasm where mRNAs should be present but rather were distributed over nuclei.

Human Coronary Lesions Express BTEB2

We further investigated BTEB2 expression in coronary lesions by use of atherectomy tissues. All atherectomy specimens, including restenotic lesions and primary lesions, contained atherosclerotic tissue and/or DIT lesions. As shown in Figure 3A and 3B, both restenotic and primary lesions contained stellate cells, which we refer to as stellate SMCs (St-SMCs) on the basis of their being SM1-positive (Figure 3). One hundred twenty of 136 restenotic lesions (88%) and 32 of 80 primary lesions (42%) were positive for these cells. In addition, in both restenotic and primary lesions, BTEB2 was exclusively positive in St-SMCs; few if any cells in DIT lesions and atherosclerotic tissues were positively stained with BTEB2 antibody (Figure 3C). Furthermore, double-labeling immunohistochemistry showed St-SMCs to be positive for both PCNA and BTEB2, in contrast to DIT and atherosclerotic lesions, in which either protein was barely detectable (Figure 3C). These findings show that BTEB2 is preferentially expressed in PCNA-positive St-SMCs. In contrast, neither PCNA nor BTEB2 was detectable in DIT lesions (Figure 3C).

High Incidence of Restenosis in Patients With BTEB2-Positive Lesion

To assess the clinical relevance of BTEB2 expression in human coronary disease, we further correlated the restenosis rate after DCA with the immunoreactivity of BTEB2 staining. We classified patients into two groups depending on the basis of the presence of BTEB2-positive lesions. Of 80 primary lesions, 32 showed presence of St-SMC lesions with positive BTEB2 staining, in contrast to 48 patients for those without (the lesions have absolutely no St-SMCs). The restenosis rate was significantly higher at 55.6% for the BTEB2-positive lesion group.
group compared with 25.0% for the BTEB2-negative group ($P=0.01$) (Table 2 and Figure 4A). Interestingly, among the 25 patients with restenosis, 7 patients, all of whom had BTEB2-positive lesions, repeatedly developed restenosis and needed coronary intervention more than three times. The incidence of recurrent restenosis was remarkably higher in the BTEB2-positive than in the BTEB2-negative group (46.7% versus 0%, $P=0.02$) (Table 2 and Figure 4A). Univariate analysis showed the presence of BTEB2-positive cells to be a significant predictor of restenosis. Other than the presence of BTEB2-positive cells, the presence of a left anterior descending coronary artery lesion was also a significant predictor of restenosis. Odds ratios for restenosis and recurrent restenosis associated with the presence of a BTEB2-positive lesion were 3.75 ($P=0.01$) and infinity ($P=0.02$), respectively (Table 3). The relation between the presence of BTEB2-positive lesions and the risk of restenosis did not change in the multivariate logistic regression model (odds ratio 4.79, 95% CI 1.73 to 13.24, $P=0.003$). MLD, percent diameter stenosis, late loss, and loss index also differed significantly between the BTEB2-positive and BTEB2-negative groups (Table 2 and Figure 4B). Baseline patient profiles and angiographic results before and immediately after atherectomy did not differ between the two groups (Tables 1 and 2). In contrast, there was no correlation between restenotic lesions containing BTEB2-positive cells and recurrent restenosis (data not shown).

**Discussion**

Phenotypic modulation of SMCs is associated with developmental and pathological vascular processes. Whereas the contractile phenotype is characteristic of quiescent differentiated SMCs, the synthetic or activated phenotype reflects a dedifferentiated and proliferative SMC phenotype. The hallmark of vascular processes including neointimal formation that follows arterial injury is the presence of activated vascular SMCs.9,15 Despite the importance of phenotypic modulation of SMCs, the molecular mechanisms underlying this process are poorly understood. As a first step toward understanding of the molecular mechanisms underlying phenotypic modulation, we have previously investigated the regulatory mechanisms governing expression of the SMemb/NMHC-B gene8,10,16,17 and demonstrated that the transcription factor BTEB2 plays a role in its transcriptional regulation.5 In the present study, we examined the expression of BTEB2 in developmental and pathological states. Our results suggest that BTEB2 may be a possible molecular marker of phenotypic modulation of vascular SMCs.

**Developmental Change in BTEB2 Expression**

BTEB2 was expressed in fetal aortic SMCs but not in adult rabbits or humans. Expression of BTEB2 was regulated in a developmental manner. Although BTEB2 expression in the present study was examined in two distinct states (fetal and...
adult stages), prominent expression in the fetal stages suggests that BTEB2 may play a role in aortic development. Because BTEB2 was isolated as a DNA-binding factor that regulates the SMemb/NMHC-B gene and shows a temporal profile of in vivo expression levels being most prominent in the fetal stages, it is tempting to envision that developmentally coordinated expression of BTEB2 regulates the temporal expression of genes associated with the activated SMC phenotype including the SMemb/NMHC-B gene, although BTEB2 may not be the sole regulator of the SMemb/NMHC-B gene. Because transcription is a result of the combinatorial effect of numerous factors and regulators, it is likely that it is a candidate for being a dominant regulator of expression of this gene. In fact, the expression of BTEB2 was not limited to smooth muscle but was also found in endothelial cells. Conversely, BTEB2 was not expressed in the medial layer of normal human aorta or coronary arteries in contrast to SMemb/NMHC-B. These results suggest that BTEB2 expression does not necessarily parallel the expression of SMemb/NMHC-B and that BTEB2 alone is not responsible for the regulation of the SMemb/NMHC-B gene. The role of BTEB2 in SMemb/NMHC-B regulation in vivo deserves further investigation.

Pathological Regulation of BTEB2 Expression

Consistent with the developmentally regulated expression profile of BTEB2, we further showed that BTEB2 is highly expressed in dedifferentiated neointimal cells in a rabbit model of balloon injury. Given that the neointima is the site at which numerous injury-induced genes are expressed and proliferation of SMCs occurs during the first 4 weeks after injury, these findings suggest that BTEB2 expression is strongly correlated with the activated vascular SMC phenotype.

Studies using human samples also showed expression of BTEB2 in coronary lesions. Interestingly, BTEB2 expression in both restenotic and primary lesions was restricted to St-SMCs. On the basis that these St-SMCs were also positive for PCNA, we consider that these cells represent the proliferative or activated SMCs. In contrast, SMCs in DIT or media were negative for both BTEB2 and PCNA. Preferential expression of BTEB2 in stellate cells may be associated with phenotypic modulation of vascular SMCs which suggests that BTEB2 can serve as a molecular marker for St-SMCs.

St-SMCs are found in a large number of restenotic lesions but are also present in primary lesions. In a previous analysis of atherectomy specimens, lesions containing St-

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**Figure 3.** Photomicrographs of restenotic and primary lesions from human coronary arteries. A, St-SMCs in restenotic lesions were stained with hematoxylin-eosin counterstain (H&E) and anti-BTEB2 antibodies. Bar=50 μm. B, Primary lesion containing St-SMCs and DIT lesions. Bar=500 μm. C, Atherectomy specimens obtained from primary lesions were stained with hematoxylin-eosin counterstain (H&E), anti-BTEB2, and anti-PCNA (double-labeling) or anti-SM1 antibodies. Double-labeling immunohistochemistry was used to determine whether BTEB2-expressing cells (shown by red reaction product) were PCNA-positive (brown reaction product). i indicates intima; m, media. Bar=50 μm.
SMCs have not been distinguished from other types of atherectomized tissues, and St-SMCs do not appear to be actively proliferative. In contrast, we demonstrate that St-SMCs express PCNA as well as BTEB2. However, it should be noted that the frequency of PCNA-positive St-SMCs in this study is much higher than that reported in the previous study. One of the major reasons for this discrepancy would be that we used microwave irradiation to enhance the antigenicity of the sample. This technique may cause overestimation of the number of PCNA-positive cells. Thus, the question regarding PCNA expression and restenosis cannot be addressed without further investigation.

Correlation Between BTEB2 Expression and Restenosis

An important clinical implication of our studies is the correlation between positive BTEB2 staining and an increased risk of restenosis in primary lesions. The fact that recurrent restenosis is strongly correlated with BTEB2 expression is further suggestive that BTEB2 expression may reflect a proliferative SMC state. Although further studies are needed to understand the molecular mechanisms underlying the association between restenosis and BTEB2 expression, it is tempting to envision that BTEB2 is at least one of the important transcription factors involved in regulating the specific subset of genes that are activated in restenosis. Persistent activation of these genes by continuously elevated levels of BTEB2 may lead to the pathogenic state associated with recurrent restenosis. This is reminiscent of cancerous cells in which gene regulation, including regulation at the transcription level, is not properly coordinated, leading to an autostimulatory and proliferative state.

In summary, our studies have shown that BTEB2 expression is developmentally and pathologically regulated. BTEB2 appears to be preferentially expressed in activated vascular SMCs in balloon-injured rabbit aortas and in human coronary arteries. BTEB2 expression in primary lesions also correlated with development of restenosis after atherectomy. Further investigation of BTEB2 as a predictive factor for coronary

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<th>BTEB2-Positive (n=32)</th>
<th>BTEB2-Negative (n=48)</th>
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<tr>
<td>At atherectomy</td>
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<td>2.87±0.10</td>
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<td>Percent diameter stenosis</td>
<td>14.3±12.3</td>
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<td>No. of patients at follow-up, % (n)</td>
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<td>25.0 (10/40)</td>
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<td>Recurrence of restenosis, % (n)</td>
<td>46.7 (7/15)</td>
<td>0 (0/10)</td>
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</table>

Recurrence of restenosis indicates that restenosis occurred more than twice.

*Fisher’s exact test.
restenosis may provide important insight into targeted therapeutic interventions for coronary restenosis.

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References
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