Subnormal Shear Stress-Induced Intimal Thickening Requires Medial Smooth Muscle Cell Proliferation and Migration

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Received November 14, 2001

Arterial intimal thickening is consisted of predominately smooth muscle cells (SMC). The source of these SMCs and mechanisms response for their changes have not been well cleared. Using a model of rabbit common carotid artery (CCA) shear induced intimal thickening, we sought to identify and describe the source of SMCs in intima. The enlarged CCA 28 days after arteriovenous fistula (AVF) creation was subjected to subnormal wall shear stress (WSS) for 1, 3, and 7 days by closure of the AVF. To determine SMC proliferation, BrdU pulse labeling of SMCs was performed. BrdU-labeled SMCs were tracked over time to further confirm SMC migration. In response to subnormal WSS intimal thickening developed progressively. BrdU-labeled SMCs localized in the subendothelial area. When the BrdU-labeled medial SMCs were tracked 1 day after AVF closure, progenies of these BrdU-incorporated SMCs increased by 4.8-fold with 75% of them in the intima. They were 12-fold increased with 83% in the intima 7 days after. En face examination showed an accumulation of SMCs in internal elastic lamina gap after AVF closure, which later migrated into subendothelial area. In situ hybridization revealed increased TGF-β1 mRNA expression in intimal SMCs. This study demonstrates that the medial SMCs are the predominant cells in subnormal WSS-induced intimal thickening. Early expression of TGF-β1 may play an important role in the process of intimal thickening.

Key Words: intimal thickening; smooth muscle cells; proliferation; migration; blood flow; wall shear stress.

INTRODUCTION

Intimal thickening may contribute significantly to degenerative vascular diseases, such as atherosclerosis and restenosis. Smooth muscle cell (SMC) proliferation and migration is thought to be an important process in the formation of intimal thickening. Animal models have been used to study intimal thickening with acute endothelial, medial, or adventitial injury [1–6]. Recently, we developed a novel model of intimal thickening without injury and inflammation in the rabbit carotid artery in response to blood flow and wall shear stress alterations through an arteriovenous fistula (AVF) created between the carotid artery and the corresponding jugular vein [7, 8]. Blood flow and wall shear stress are recognized as important hemodynamic factors [9–12] regulating arterial wall remodeling in our models. Increased blood flow and wall shear stress regulate arterial enlargement without intimal thickening in order to reduce wall shear stress [13–16]. Decreased blood flow and wall shear stress promote arterial intimal thickening, which morphologically consists of SMCs and elastic fibers, and endothelial cell apoptosis in an attempt to reduce the lumen diameter in response to subnormal wall shear stress [7, 17]. Progressive intimal thickening occurred in the previously flow-induced remodeled artery when wall
shear stress was reduced to a subnormal level, but was inhibited by high flow periods [8].

The source or mechanism of the intimal SMCs has not been well delineated. In particular, numerous experimental studies on acute endothelial and medial injuries have repeatedly identified new intimal SMCs originally from the arterial media [1, 4, 5, 18, 19]. Neointima formation has also been observed in response to adventitial injury in various animal models [20, 21], suggesting that arterial SMCs in the intima may originate from the adventitia. A more speculative source of the intimal SMCs is that they may be the putative precursor cells of bone marrow origin that follow a pattern of vascular SMC differentiation on activation [22–27]. But the origin of the intimal SMCs in flow-induced intimal thickening model is still unknown.

Transforming growth factor beta 1 (TGF-\(\beta\)1) has been identified as an important factor for vascular remodeling. Increased expression of TGF-\(\beta\)1 has been demonstrated in human restenotic lesions and in atherosclerotic lesions [28, 29]. Conversely, inhibition of TGF-\(\beta\)1 expression reduced intimal formation [30–32]. Thus, given its role in arterial remodeling and arterial adaptation to hemodynamic changes, TGF-\(\beta\)1 seemed an appropriate marker to identify in a new model of shear-induced intimal thickening.

The purpose of this experiment was to identify the origin of intimal SMCs in a novel subnormal wall shear stress model of intimal thickening and to demonstrate the early time course of SMC proliferation and migration. We hope to identify and understand the role and importance of SMCs in the formation of arterial intimal thickening in response to subnormal wall shear stress and hope to identify the role of TGF-\(\beta\)1 as a potential component to understanding the molecular mechanism behind shear-induced intimal thickening.

MATERIALS AND METHODS

Animal Operative Procedures

To induce high blood flow, AVF was created between the left common carotid artery (CCA) and left jugular vein of Japanese white male rabbits by sterile techniques as described previously [7, 8, 16, 17]. The remodeled arteries were then subjected to reduction in blood flow by closure of the AVF using the same techniques we used before [8, 17]. Animals were housed individually and cared in accordance with Japanese Community Standard on the care and use of laboratory animals. The Animal Research Committee, Akita University School of Medicine, approved the protocols for animal experimentation. All experiments adhered to the Guidelines for Animal Experimentation of the University.

Experiment Design

BrdU administration: Animals received an injection intraperitoneally of BrdU at 50 mg/kg [16, 33] in physiologic saline solution (10 mg/ml). It had been tested that BrdU saline solution (20–25 ml) was absorbed almost completely in the peritoneal cavity within 1 h. Incorporation of BrdU into a nucleus occurs when a cell enters DNA synthesis phase (S-phase) of the cell mitotic cycle. Because BrdU remains in blood flow for approximately 10 min before being degraded [34] and the S-phase of a cell cycle usually lasts for 6–8 h, a single intraperitoneal injection of BrdU will label all the cells in S-phase, while cells entering the S-phase at any time rather than during the BrdU labeling period will not be labeled. Thus, the BrdU-labeling method used in this experiment is consistent to pulse labeling. The labeled SMCs continue their cell cycles to complete mitosis, generating two daughter cells. The daughter cells will certainly carry the incorporated BrdU in their nuclei and so will the next progeny of them should these daughter cells enter another cell cycle. This method of BrdU labeling enabled us to track SMC proliferation and migration.

Pulse-labeled group. BrdU was given to control animals; at 7 and 28 days of AVF; and at 1, 3, and 7 days of AVF closure after 28 days of AVF open (\(n = 5\) each). Animals were sacrificed 1 h after BrdU administration.

Tracking group I. BrdU was administered at 28 days of AVF, and animals were sacrificed at 1, 3, and 7 days of AVF closure (\(n = 5\) each).

Tracking group II. BrdU was administered at 7 days of AVF, 1 h later the AVF was closed, and then animals were sacrificed at 0.5, 1, and 28 days of AVF closure (\(n = 4\) each).

Blood Flow Measurements

Blood flow rate of left CCA was measured with an electromagnetic flow meter (Nihon Kohden Co., Japan) at a standardized location, 10–15 mm proximal to the thyroid artery branch. Measurements were taken before and after construction of AVF, before and after the closure of AVF, and before sacrifice.

Artery Perfusion, Fixation, and Preparation

A laparotomy was performed under anesthesia. The abdominal aorta distal to the renal branches was cannulated
TABLE 1
Blood Flow, Dimensions, and Wall Shear Stress of the Left CCA

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AVF-7d</th>
<th>AVF-28d</th>
<th>CAVF-1d</th>
<th>CAVF-3d</th>
<th>CAVF-7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>BFR (ml/min)</td>
<td>20 ± 2</td>
<td>160 ± 10*</td>
<td>380 ± 21*†‡</td>
<td>22 ± 2</td>
<td>19 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>R (mm)</td>
<td>1.04 ± 0.03</td>
<td>1.16 ± 0.05*</td>
<td>1.95 ± 0.05*†‡</td>
<td>1.76 ± 0.05*</td>
<td>1.63 ± 0.13*</td>
<td>1.48 ± 0.16*§</td>
</tr>
<tr>
<td>WSS (dynes/cm²)</td>
<td>12.01 ± 2.14</td>
<td>65.11 ± 7.26*</td>
<td>32.08 ± 1.96*†‡</td>
<td>2.58 ± 0.32*</td>
<td>2.88 ± 0.96*</td>
<td>3.82 ± 1.19*</td>
</tr>
<tr>
<td>CSAm (mm²)</td>
<td>0.55 ± 0.06</td>
<td>0.52 ± 0.09</td>
<td>0.68 ± 0.11*†</td>
<td>0.68 ± 0.06*†</td>
<td>0.78 ± 0.17*†</td>
<td>0.99 ± 0.09*§¶</td>
</tr>
<tr>
<td>CSAi (mm²)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0.06 ± 0.02</td>
<td>0.18 ± 0.03†</td>
<td>0.34 ± 0.08§</td>
</tr>
</tbody>
</table>

Note. BFR, blood flow rate (ml/min); R, luminal radius (mm); CSAm, cross-sectional area of media (mm²); CSAi, cross-sectional area of intima (mm²); WSS, wall shear stress (dynes/cm²).
* Significant difference vs control (P < 0.01).
† Significant differences vs AVF-7d (P < 0.01).
‡ Significant difference vs CAVF-1d, CAVF-3d, and CAVF-7d (P < 0.01).
§ Significant difference vs AVF-28d (P < 0.01).
¶ Significant difference vs AVF-28d.

with a sheath catheter. Animals were sacrificed with an overdose injection of pentobarbital solution (100 mg/kg). The arteries were pressure perfusion fixed with 4% paraformaldehyde phosphate buffer (pH 7.4) via the catheter under an intraarterial pressure of 100 mmHg at 20°C for 30 min. After the arteries were carefully dissected, a segment of the left CCA (9 mm in length) proximal to the thyroid artery branch was taken for histological and immunohistochemical analyses. Specimens were dehydrated with alcohol and embedded in paraffin. Sections of 4 μm in thickness were stained with hematoxylin and eosin and Elastica Masson’s trichrome.

Immunohistochemical Stains

Histological sections (4 μm) were deparaffinized in a xylenes–ethanol series and incubated in 2 N HCl (for BrdU stain) for 30 min. Washing with water, and they were incubated with 1% H₂O₂ in methanol for 30 min. After washing with PBS, the sections were incubated with goat serum (1:200 diluted in PBS) for 20 min. The sections were then incubated either with mouse anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA, 1:50 dilution in 1% BSA in PBS) or with anti-smooth muscle (-SM) actin monoclonal antibody (mouse antihuman) (Sigma, St. Louis, MO; 1:400 dilution in 1% BSA in PBS) or with RAM11 antibody (mouse antirabbit) (DAKO, M0633; 1:50 dilution in 1% BSA in PBS) at 20°C for 30 min, separately. After rinsing in PBS, the sections were incubated with biotinylated antimouse IgG antibody for 30 min followed by the ABC method (Vector Laboratories, Inc) according to the manufacturer’s protocol. The sections were counterstained with hematoxylin.

Quantitation of BrdU-Labeled SMCs

BrdU-labeled-SMCs in the intima and in the media were counted and cataloged on the entire cross-section separately (five sections/animal) as follows: subendothelial BrdU-labeled SMCs (within one to two layers of SMCs beneath endothelial cells in intima), deep intima BrdU-labeled SMCs (all other layers of SMCs in intima), subinternal elastic lamina (sub-IEL) BrdU-labeled SMCs (with one to two layers of SMCs beneath IEL in media), and deep media BrdU-labeled SMCs (all other layers of SMCs in media). The sub-IEL BrdU-labeled SMCs were further divided into BrdU-labeled SMCs beneath IEL and within IEL gap area [16].

Fluorescing Immunohistochemical Stain

Segments of left CCA (3 mm in length) were prepared in the tracking II group immediately after the perfusion fixation. The segments were followed by wash in PBS. They were incubated with 0.5% Triton X-100 in 2 N HCl for 30 min followed by wash in PBS and were blocked with goat serum (1:100) for 30 min at room temperature. They were then incubated with either antimouse BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA) or rhodamine phalloidin (Molecular probe Company) at 1:20 dilution in 1% BSA in PBS for 1 h at room
FIG. 1. Histological changes in the left CCA after flow alteration. Gaps in the internal elastic lamina (IEL gaps) were frequently observed (A, AVF 7 days). After AVF closure intimal thickening (IT) occurred (B, Rev 1 days; C, Rev 7 days; Elastica Masson’s trichrome staining; original magnification, ×400). The neointima was consisted of smooth muscle cells (D, Rev 1 day; E, Rev 3 days; F; Rev 7 days; immunostaining of α-smooth muscle actin showing brown color; original magnification, ×1000). There were no macrophages in the media and intima (G, Rev 12 h; H, Rev 1 day; I, rabbit lung positive control; immunostaining of RAM11, original magnification, ×1000).

FIG. 2. BrdU incorporated SMCs were observed in the left CCA (immunostaining of BrdU; original magnification, ×1000). In pulse-labeled group, a few BrdU-labeled SMCs (stars) were observed in the lumen side of the media at 7 days of AVF (A). Many BrdU-labeled SMCs (arrowheads) were located beneath endothelial cells (EC) at Rev 3 days (B) and Rev 7 days (C). In the tracking group, BrdU-incorporated SMCs appeared in the intima (arrowheads) and in the media (stars) at Rev 1 day (D), Rev 3 days (E), and Rev 7 days (F). In the tracking group II, BrdU-incorporated SMCs appeared in IEL gap areas (stars) at Rev 12 h (G) and Rev 1 day (H), and a few of them were beneath endothelial cells (arrowhead). After 28 days of AVF closure, they appeared mainly in the intima (arrowheads) (I).
temperature, separately. After washing in 1% BSA in PBS, they were incubated with a fluorescent isothiocyanate (FITC) (1:20) conjugated antimouse IgG (KPL Lab Inc.) at room temperature for 1 h. The samples were then stained for 1 min with propidium iodide (PI) for counterstaining of nuclei. After washing again in PBS, they were opened longitudinally for en face examination. The whole wall of the opened segments was mounted on the glass slide with the lumen side facing up and covered with a cover slip for observation under laser scanning confocal microscopy (LSCM) (Carl Zeiss LSM 410) using HeNe laser (488 nm) and Argon laser (530 nm).

In Situ Hybridization

In situ hybridization was performed on cross sections of Rev 1, 3, and 7 days following the methods as previously described [35] with modification. Sections (4 μm) were deparaffinized in a xylene–ethanol series and incubated in 0.2 N HCl for 20 min. After washing in PBS, sections were incubated with proteinase K (1 μg/ml in PBS) for 5 min at 37°C. Prehybridization was performed for 1 h at 55°C after washing again in PBS. Hybridization was performed with 1 μg/ml of biotin-labeled rabbit TGF-β1 antisense or sense RNA probe in hybridization solution overnight at 55°C in a humidified chamber. The RNA probes were generated by in vitro transcription using Riboprobe Gemini System II (Promega, Madison, WI) with the addition of biotin-16-UTP. The template cDNAs for TGF-β1 probes were sequence confirmed, and the specificity of the probes was confirmed by ribonuclease protection assay before using. Following a thorough wash in 0.1 × SSC for 2 h at 55°C, the hybridized signals were detected using the ABC method (Vector Laboratories, Inc.) according to the manufacturer’s protocol. The sections were counterstained with hematoxylin, dehydrated, and coverslipped. The detection was expected to be a brown precipitation in the cytoplasm.

Histometric Measurements

Histomorphometry was performed on cross-sectional histological sections stained with Elastica Masson’s trichrome (one section per case). Histological images of the sections at 50× magnification were obtained with a profile projector (Nikon V-16, Nikon Co., Japan). Circumferential length of lumen (CL), circumferential length of internal elastic lamina (CIEL), and circumferential length of external elastic lamina (CEEL) were digitized from the images with a digitizer (Cosmozone-1, Nikon Co.). Calculations were made for corrected lumen radius [$r = 1.25 \times CL/2\pi$ (mm)], corrected cross-sectional area of intima [$CSAi = \pi \times (1.25 \times CIEL/2\pi)^2 - \pi r^2$ (mm$^2$)], and corrected cross-sectional area of media [$CASm = \pi \times (1.25 \times CIEL/2\pi)^2 - \pi \times (1.25 \times CIEL/2\pi)^2$ (mm$^2$)]. Calculations assumed that the artery was tubular; a correction factor of 1.25 was used to account for the shrinkage index due to the fixation process [7, 8, 13–17].

Wall shear stress. Assuming laminar flow, wall shear stress (dyne/cm$^2$) was calculated using the poiseuille formula as follows: wall shear stress = $4\mu BFR/60\pi r^3$, where $\mu$ is the blood viscosity (taken to be 0.03 in poise), BFR is blood flow rate (milliliters per minute), and $r$ is the radius expressed in centimeters [7, 8, 13–17].

Statistical analysis. All data were expressed as the mean ± SD. Differences between groups were analyzed by ANOVA with Bonferroni correction for multiple comparisons where appropriate. Differences were considered significant when $P < 0.05$.

RESULTS

Blood Flow

Blood flow increased immediately and significantly after AVF creation. It increased 8-fold after 7 days of AVF creation and more than 15-fold after 28 days. The increase in blood flow coincided well with our previous data [7, 8, 16, 17]. After AVF closure, blood flow decreased to 40 ml/min immediately and returned to the control level 1 day after AVF closure (Table 1).

Wall Shear Stress

Wall shear stress increased sharply 7 days after AVF ($P < 0.01$ vs controls) and decreased significantly at 28 days of AVF ($P < 0.01$ vs 7 days of AVF) (Table 1) as the artery adapted. After successful closure of AVF, wall shear stress dropped markedly to 2.6 dynes/cm$^2$, which was far below ($P < 0.01$) the control level of 12 dynes/cm$^2$. Wall shear stress gradually elevated at 3 and 7 days after AVF closure, but it was still below 5 dynes/cm$^2$ (Table 1).

Dimensions of the Left CCA

Lumen radius increased at 7 days of AVF ($P < 0.01$ vs control) and it was 1.9-fold greater at 28 days of AVF than...
that of controls ($P < 0.01$) (Table 1). Lumen radius gradually decreased after AVF closure ($P < 0.01$, vs AVF 28 days) (Table 1). CSAm increased after 28 days of AVF and continued to increase after AVF closure (Table 1). CSAi appeared at 1 day of AVF closure and significantly increased at 3 and 7 days of AVF closure.

**Histological Findings**

Flow-loaded left CCA showed a distinct IEL gap at 7 and 28 days of AVF but without intimal thickening development (Fig. 1A). There were one or two layers of SMCs between endothelial layer and IEL at 1 day after AVF closure (Fig. 1B), which marked the initial development of intimal thickening. The number of SMC layers in the intima seemed to increase progressively after AVF closure (Fig. 1C).

**Immunohistochemical Stain of a-SM Actin and RAM11**

All cells in the intima and media showed a-SM actin positive stain, which demonstrated that these cells were SMCs (Figs. 1D, 1E, and 1F). There were no RAM11-positive cells found in the intima and media, showing no evidence of inflammatory cell infiltration in these arteries (Fig. 1G).

**BrdU-Labeled SMCs**

**Pulse-labeled group.** Labeling of SMCs in vessel wall is normally very low [36–38]. Our data showed that only one to two media SMCs per section were labeled in normal left CCA. Medial SMCs proliferated significantly after high flow, showing a high BrdU-labeled SMCs index (Table 2 and Fig. 2A). Half of the BrdU-labeled SMCs located in the inner media, which were mainly in the IEL gap areas. After AVF closure, the number of BrdU-labeled SMCs increased significantly and peaked at 3 and 7 days of AVF closure. More than 70% of these cells located in the intima were mainly in the subendothelial area (Table 2 and Figs. 2B and 2C).

**Tracking group I.** BrdU-incorporated SMCs were observed in media and intima after AVF closure (Table 2 and

<table>
<thead>
<tr>
<th>Pulse-labeled group</th>
<th>Total I (Sub-EC)</th>
<th>Total M (Sub-IEL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5 ± 1.0</td>
<td>0</td>
</tr>
<tr>
<td>AVF7 day</td>
<td>24.6 ± 2.7*</td>
<td>(12.8 ± 3.1*)</td>
</tr>
<tr>
<td>AVF 28 day</td>
<td>15.0 ± 2.8†</td>
<td>(10.8 ± 2.8*)</td>
</tr>
<tr>
<td>Rev 1day</td>
<td>17.4 ± 0.5</td>
<td>(12.6 ± 1.1)</td>
</tr>
<tr>
<td>Rev 3day</td>
<td>91.0 ± 9.3¶</td>
<td>(82.4 ± 8.6¶)</td>
</tr>
<tr>
<td>Rev 7 day</td>
<td>106.3 ± 6.9¶§</td>
<td>(94.8 ± 9.0¶§)</td>
</tr>
<tr>
<td>Tracking group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev 0.5 day</td>
<td>51.8 ± 7.3</td>
<td>(19.0 ± 6.6)</td>
</tr>
<tr>
<td>Rev 1 day</td>
<td>106.4 ± 14.7¶</td>
<td>(34.0 ± 6.3¶)</td>
</tr>
<tr>
<td>Rev 7 day</td>
<td>152.3 ± 11.4¶§</td>
<td>(47.8 ± 5.1¶§)</td>
</tr>
<tr>
<td>Tracking group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev 0.5 day</td>
<td>28.8 ± 2.2</td>
<td>(21.3 ± 2.9)</td>
</tr>
<tr>
<td>Rev 1 day</td>
<td>32.5 ± 3.1</td>
<td>(26.5 ± 2.7)</td>
</tr>
<tr>
<td>Rev 28 day</td>
<td>38.0 ± 5.7</td>
<td>(10.0 ± 1.8)</td>
</tr>
</tbody>
</table>

Note. Total I, total numbers of BrdU incorporated SMCs in the intima (cells/section); Sub-EC, total numbers of BrdU incorporated SMCs in subendothelial area (cells/section); total-M, total numbers of BrdU incorporated SMCs in the media (cells/section); Sub-IEL, total numbers of BrdU incorporated SMCs in sub-IEL area (cells/section).

* Significant difference vs control ($P < 0.01$).
† Significant difference vs AVF-7d ($P < 0.01$).
‡ Significant difference vs AVF 28 day ($P < 0.01$).
¶ Significant difference vs Rev 1 day ($P < 0.01$).
§ Significant difference vs Rev3d ($P < 0.01$).
£ Significant difference vs Rev 0.5 and 1 day ($P < 0.01$).
Figs. 2D–2F). These SMCs ought to be the progeny of BrdU-labeled SMCs at 28 days of AVF. BrdU incorporated SMCs increased by times after AVF closure and became 12-fold after 7 days of AVF closure as compared with that at 28 days of AVF. Most of them (>80%) were located in the intima diffusely. More than half of BrdU incorporated SMCs in the media were in the sub-IEL area and mainly in the IEL gap areas.

**Tracking group II.** Within 1 day of AVF closure the BrdU incorporated SMCs almost located in the inner media (80%). They were mainly in the IEL gap areas (Figs. 2G and 2H). After 28 days of AVF closure the BrdU incorporated SMCs became 2.5-fold increased with 54% of them in the intima. The others were located in the media (Table 2 and Fig. 2I).

**Fluorescing Immunohistochemical Stain**

Endothelium was well preserved with several BrdU-labeled endothelial cells (Figs. 3A and 3F). After 0.5 and 1 day of AVF closure the IEL gaps were observed frequently (Figs. 3B and 3G). The media SMCs beneath IEL gaps or surrounding them changed their orientation and tended to accumulate in the IEL gaps (Figs. 3B, 3C, 3G, and 3H). A lot of these SMCs showed BrdU incorporation. The deeper media SMCs near the bottom of IEL gap showed similar changes, but the SMCs in the surrounding area did not show the changes in their orientation (Figs. 3D and 3I). Several SMCs appeared in the subendothelial area near the IEL gap after 1 day of AVF closure (Fig. 3J).

**TGF-β1 mRNA expression.** The results of in situ hybridization showed strong expression of TGF-β1 mRNA after AVF closure. It was expressed mainly by intimal SMCs (Figs. 4B–4D) and medial SMCs located in the sub-IEL zone, which showed strongest 3 days after AVF closure. Deeper medial SMCs had weak expression of TGF-β1 mRNA.

**DISCUSSION**

As we previously described, subnormal wall shear stress in rabbit CCA results in intimal thickening without arterial injury and inflammatory. [7] In this model previously enlarged arteries due to high flow develop intimal thickening when exposed to subnormal wall shear stress. These findings have also been confirmed with sequential increases and decreases in flow leading to intimal thickening only with subnormal wall shear stress. [8] In other animal models of intimal thickening, injury to endothelial cells or media and adventitia is major requirement. [1–6] We documented intact endothelial cells at arterial enlargement and intimal thickening points throughout the experiment. Thus, our model is a unique noninjury and no inflammatory model of intimal thickening.

Intimal thickening in this experiment develops 1 day after decreased wall shear stress. According to the pulse-labeling data, we reported significant SMC proliferation after 3 days of high flow normalization and these labeled SMCs were mainly in subendothelial and sub-IEL areas. It is possible that these SMCs are more susceptible to flow changes than those in the outer layers of the vessel wall. Due to their location it is possible that modulating factors for SMC proliferation and migration in response to subnormal wall shear...
stress are more concentrated to the inner zones of the vessel wall with subnormal wall shear stress.

The origin of SMCs in the intima is still unknown. In injury models of intimal thickening it was proposed that intimal SMCs migrated from media or adventitia. In our experiment, the tracking data revealed that media SMCs labeled at 7 or 28 days after AVF creation migrated from the media into the intima after AVF closure. After migration into the intima, these SMCs were still proliferating to develop neointima. Thus, the SMCs in the intima not only migrated from the media but also proliferated further in the intima in response to decreased flow and wall shear stress. This observation is consistent with the proposal that both proliferation and migration are important mechanisms for accumulation of SMCs in the intima. [39]

This experiment also demonstrated that the inner part of the media SMCs contributed to the intima thickening. The 80–90% of BrdU-labeled SMCs located in the IEL gap area demonstrated SMC migration in this region. [16] SMCs in the sub-IEL area seem to respond very actively to the stimulation of flow changes because of their location in the proximally to the intima and endothelial cells, and may be strongly influenced by molecular signals like growth factors. [40] Not all of proliferating SMCs in the media seem to migrate into the intima. Our data showed that some BrdU incorporated SMCs appeared in the intima after AVF closure and some of them remained in the media, contributing to medial hyperplasia.

Endothelial cells and SMCs beneath endothelial cells play a role in modulating the remodeling of vessel wall by producing growth-promoting and growth-inhibitory factors. [41, 42] Low wall shear stress could influence smooth muscle cell proliferation by modulating production of SMC regulatory molecules. With our model, we have successfully demonstrated the progressive nature of arterial intimal thickening in response to decreased wall shear stress. [8] The mechanisms describing this observed arterial intimal thickening are also complex and poorly understood. Recently, TGF-β has been identified as an important regulator of intimal thickening. Many experiments have demonstrated the expression of TGF-β in injury models by intimal thickening. [43, 44] Inhibition of TGF by local application of antisense S-oligonucleotides [45] has demonstrated a reduction in intimal proliferation in similar injury models. Thus, TGF-β may play an important role in early arterial adaptation to hemodynamic changes.

We identified the early string expression of TGF-β1 mRNA in the new intimal SMCs and medial SMCs during the formation of intimal thickening in response to subnormal wall shear stress. It is possible that TGF-β1 responds to decreased wall shear stress and initiates the molecular cascades that are required to allow the artery to remodel and adapt to its new hemodynamic environment. This may involve smooth muscle proliferation and migration along with matrix changes. While TGF-β1 may be an important factor for the initial cascades, it may not be the only factor. Other growth factors, such as PDGF integrins, MMPs, and nitric oxide, may also play a role along with TGF-β1. Although many growth factors are known to regulate SMC proliferation and migration, it is still uncertain how these growth factors play their roles each other in this flow and shear induced arterial intimal thickening. Further investigation addressing these aspects will greatly enhance our understanding of molecular mechanisms of the flow and shear induced intimal thickening. Our experimental model of intimal thickening has proven to be a useful tool to analyze arterial intimal thickening. Our model provides a unique environment for study of vessel remodeling under low flow and wall shear stress condition, such as remodeling in atherosclerotic arteries and arteries distal to an anastomosis site.

In summary, the source of SMCs in flow-induced intimal thickening seems to be the medial SMCs, which localize mainly in the sub-IEL area, rather than in a deeper zone of the media. These SMCs may migrate through the IEL gap area to the intima. After migration, SMCs further proliferate in the intima, especially beneath endothelial layer, resulting in progressive intimal thickening. TGF-β1 may play an important role in initiating this process of intimal thickening. Thus, reduction in blood flow and wall shear stress is important in regulating medial SMC migration into the intima as well as further proliferation in the intima.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Morris and Alvilda Hyman Aneurysm Research Program; a grant from the Pacific Vascular Research Foundation (San Francisco, CA).

FIG. 4. Localization of gene expression of TGF-β1 in rabbit left CCA by in situ hybridization. (A) In the control condition, TGF-β1 mRNA was not detected. (B) One day after AVF closure, TGF-β1 mRNA expressed and mainly localized in intimal SMCs and medial SMCs, which located in the sub-IEL zone as shown by the brown precipitation. (C) Three days after AVF closure, TGF-β1 mRNA expression was at the peak and mainly in SMCs in the intima and in the sub-IEL zone of the media. SMCs in the deeper media had weak expression. (D) At 7 days of AVF closure, TGF-β1 mRNA expression decreased and was mainly localized in intimal SMCs. Abbreviations: EC, endothelial cell; IEL, internal elastic lamina; M, media. (Original magnification, ×1000).
REFERENCES


