Shear Stress Regulates Smooth Muscle Proliferation and Neointimal Thickening in Porous Polytetrafluoroethylene Grafts

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High shear stress appears to decrease wall thickening in diseased arteries and vascular grafts. To determine if increased shear stress diminishes smooth muscle (SMC) proliferation, we studied the effect of increased blood flow on neointimal thickening in porous polytetrafluoroethylene grafts implanted in baboons. An aorto-aortic 5-mm graft was placed in tandem with a pair of aorto-iliac 5-mm grafts, so that the proximal graft supplied all flow to both distal grafts. At 12 weeks, calculated luminal shear stress in proximal grafts was twice that in distal grafts (24 ± 8 versus 11 ± 5 dynes/cm²; p < 0.05). All grafts were completely endothelialized. The neointimal cross-sectional area in proximal grafts was about half as large as in distal grafts (3.36 ± 1.61 versus 5.93 ± 0.61 mm²; p < 0.05). Proximal grafts also had significantly less SMC proliferation (0.14 ± 0.05% versus 0.24 ± 0.10%; p < 0.05) and SMC volume (6.1 ± 4.0 versus 12.4 ± 2.6 mm³/cm²; p < 0.01) when compared with distal grafts. We conclude that the elevation in shear stress in the proximal graft, which remained within the physiological range, inhibits SMC proliferation and neointimal thickening in these grafts. (Arteriosclerosis and Thrombosis 1991;11:1844-1852)

Hemodynamic factors are important regulators of vessel wall structure. In native vessels, intraluminal pressure regulates wall thickness through its effects on wall tension, and blood flow regulates vessel diameter through changes in shear. High flow velocity may also minimize atherosclerotic intimal thickening. For example, high-flow regions in the carotid artery are typically free of plaque or thickening, and anecdotal evidence suggests that the high flow in iliac arteries supplying crossover femoro-femoral grafts retards progression of atherosclerosis. In autologous vein grafts transplanted into the arterial circulation, the flow velocity also appears to be important in regulating adaptive intimal thickening. These flow-related phenomena are thought to be mediated by the effects of shear stress on the vessel wall.

In native vessels, the study of the effects of flow and shear stress on wall structure is complicated by concomitant vasomotor responses to changes in hemodynamic conditions. When a change in luminal dimension is observed in response to a change in shear stress, it can be difficult to separate transient changes due to vasoconstriction or vasodilatation from those due to actual remodeling of vascular architecture. In addition, techniques of harvesting and fixation can affect the contractile state of native vessels, resulting in errors of morphometric analysis. Our group has developed a prosthetic graft model of vascular wall building that allows us to study the structural response to alterations in shear stress without any confounding vasomotor response. Since the synthetic graft wall is rigid, any change in luminal dimension must result from a change in the thickness of the neointima.

We use a porous (60-μm internodal distance) polytetrafluoroethylene (PTFE) graft that heals by transmural capillary ingrowth from perigraft tissue as well as ingrowth of endothelium from the ends. By 2 weeks, the luminal surface is entirely covered with endothelium. Between 2 weeks and 3 months, smooth muscle cells (SMCs) proliferate and deposit extracellular matrix beneath this endothelial covering, leading to a thickened neointima. After 3 months, the neointima does not thicken further. The
SMC proliferative rate falls from relatively high levels in the first few weeks (~1%) to about 0.05% at 3 months, but endothelial turnover persists at fairly high rates (1–3%) compared with that of quiescent aortic endothelium (0.15%).

We now report that neointimal thickening, SMC proliferation, and SMC content in porous PTFE grafts can be regulated solely by changes in the magnitude of shear stress. Furthermore, this effect is observed within the physiological range of shear stress magnitude (10–30 dynes/cm²).

Methods

Operative Technique

Vascular prostheses made of 5-mm-diameter, porous (60-μm intermodal distance), wrapped PTFE were implanted in five 10-kg, 2-year-old male baboons (Papio cynocephalus). All procedures were performed by standard vascular surgical techniques in the operating suites of the Regional Primate Research Center at the University of Washington. The animals were sedated with ketamine (10 mg/kg i.m.) and xylazine (3 mg/kg i.m.) and then anesthetized with halothane. The supraceliac aorta, infrarenal aorta, and inferior mesenteric and iliac arteries between the renal vessels and aortic bifurcation were ligated. The animals were heparinized (3,000 units i.v.), and end-to-side anastomoses were constructed between the renal vessels and aortic bifurcation were exposed and controlled. Lumbar arteries bilaterally distal to the grafts to ensure the absence of a pressure drop across the prosthesis. The aorta was ligated to divert bilateral lower-extremity and inferior mesenteric and iliac arteries were exposed and controlled. Lumbar arteries between the renal vessels and aortic bifurcation were ligated. The animals were heparinized (3,000 units i.v.), and end-to-side anastomoses were constructed between graft and artery with running 6-0 polypropylene suture. A proximal graft extended from the infrarenal to the infrarenal aorta, and two other distal grafts were placed bilaterally in the aorto-iliac position (Figure 1). The aorta was ligated to divert bilateral lower-extremity and inferior mesenteric flow through the proximal graft while lower-extremity flow coursed through individual distal grafts. The proximal graft thus carried at least twice the blood flow of a single distal graft. Intra-arterial pressure was measured by direct puncture proximal and distal to the grafts to ensure the absence of a pressure drop across the prosthesis.

A separate group of four animals received only bilateral aorto-iliac 5-mm grafts to assess neointimal development in the absence of a proximal graft.

Animal care was in compliance with the Principles of Laboratory Animal Care from the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals as put forth in NIH publication No. 80-23 (revised, 1985).

Hemodynamic Measurements

Twelve weeks after implantation, the animals with three grafts were sedated only with ketamine (10 mg/kg i.m.), and hemodynamic measurements were made with a duplex scanner (Acuson 128, Mountain View, Calif.). Center-stream time-averaged velocity ($V_{uw}$) was determined by software contained within the duplex scanner.

Mean shear stress ($\tau$) was approximated with a variation of the Hagen-Poiseuille equation:

$$\tau = 4\eta Q / \pi r^3$$

where $\eta$ is blood viscosity (0.035 poise), $Q$ is volume flow (milliliters per second), and $r$ is the vessel radius in centimeters (derived from the luminal circumference determined as described below). If time-averaged velocity multiplied by cross-sectional area ($V_{uw} \pi r^2$) is substituted for $Q$, mean shear stress can be expressed in terms of velocity:

$$\tau = 4\eta V_{uw}/r$$

Studies from the Regional Primate Research Center of implantable Doppler crystals and telemetric monitoring have shown that velocity data obtained from resting, awake baboons closely approximate measurements made under ketamine sedation (Smith OA, personal communication). Thus, shear stress calculations based on velocity data obtained under ketamine sedation should be reasonably close to the true values in resting, awake animals. We have also verified in this baboon model that volume flow calculated with time-averaged velocity and luminal diameter values measured by duplex scanning agree with flow values measured by timed collection (Zierler B et al, unpublished results).

Morphological Studies

At the time the animals were killed, they were deeply anesthetized (ketamine 30 mg/kg i.m. and xylazine 4 mg/kg i.m.), anticoagulated with heparin (3,000 units i.v.), and then exsanguinated by perfusion with lactated Ringer's solution at 100 mm Hg systemic pressure for 15 minutes followed by 15 minutes of perfusion-fixation with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The grafts were excised and immersion-fixed for at least 24 hours.

All grafts were then extensively rinsed first in 0.1 M phosphate buffer (pH 7.4) followed by 0.1 M glycine in 0.1 M phosphate buffer (pH 7.4). One entire distal graft and half of the proximal graft were cut into 0.5-cm rings for paraffin embedding and morphometry. At least one representative ring from the contralateral distal graft was similarly processed.

Before the sections were embedded, the luminal circumference was traced (to obtain the luminal...
radius for calculation of shear stress) by use of a camera lucida and digitizing pad, thus avoiding any shrinkage artifact introduced by paraffin embedding. Hematoxylin- and eosin-stained cross sections were again projected onto a digitizing pad for planimetric determination of neointimal area. Anastomotic cross sections were not analyzed because neointimal thickening in the perianastomotic region may be regulated by factors other than shear stress, such as compliance mismatch between graft and artery.13

The remaining material was processed for scanning electron microscopy (SEM). Grafts were biseected longitudinally, pinned out on Teflon sheets, and dried by the critical-point method after dehydration with graded alcohol solutions. Portions of the dried specimens were then mounted on studs and sputter-coated with gold-palladium. SEM was performed with a JEOL 35 C instrument.

Determination of Smooth Muscle Cell Proliferative Rate (Autoradiography)

Four animals with three grafts were given intramuscular injections (0.5 mCi/kg/dose) of tritiated thymidine (New England Nuclear, Boston, Mass.) 1, 9, and 17 hours before they were killed. Unstained, deparaffinized histological cross sections were dipped in NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, N.Y.), allowed to dry, and protected from light for 14 days at 4°C. They were then developed with D-19 developer (Kodak), fixed, and stained with hematoxylin. The slides were viewed with an oil-immersion objective at ×1,000 power, and the number of labeled nuclei (at least five silver grains over the nuclear profile) in the neointima was counted. The thymidine labeling index was defined as the number of labeled nuclei divided by the total number of nuclei in the neointimal cross section multiplied by 100. This value represents the percentage of neointimal cells synthesizing DNA in preparation for cell division during the 24 hours before the animals were killed. A cross section from each paraffin block (excluding the anastomoses) was evaluated in this fashion.

Determination of Neointimal Smooth Muscle Cell Content

The point-hit method14 was used to determine the fraction of neointima occupied by SMCs. Previously embedded tissue from eight grafts (one proximal and one distal from each of four animals) was removed from the paraffin block. The neointimal ring was separated from the surrounding PTFE material and reembedded in Epon for thin-sectioning. Transmission electron photomicrographs (JEOL S100) were taken (initial magnification, x 3,000) of four regions 90° apart around the circumference of each neointimal cross section. At each region, serial nonoverlapping fields were photographed in a radial fashion, so that the entire depth of neointima from the lumen to graft was imaged. The negatives were enlarged to 8×10-in. prints (final magnification, ×9,000). A 9×12 grid (108 points of intersection) was then placed over each print, and the number of points superimposing SMCs or extracellular matrix was determined.

The proportional area occupied by SMCs was determined by the equation:

\[
\text{% SMC area} = \frac{\text{SMC points}}{\text{SMC + extracellular matrix points}} \times 100
\]

SMC volume per centimeter of graft was calculated by multiplying the proportional SMC area by the neointimal area for each graft times 10 mm/cm.

Statistical Analysis

Differences between groups were analyzed with a paired or unpaired t-test (two-tailed) as indicated. Statistical significance was assumed for \(p<0.05\).
Results

Hemodynamic Measurements

The experimental graft configuration was designed to accomplish two hemodynamic objectives: 1) to produce significantly different hemodynamic environments in separate grafts of the same size in the same animal without the use of an arteriovenous fistula (thus preserving the typical oscillating directional behavior of the shear and velocity vectors during the cardiac cycle) and 2) to maintain shear stress values within the physiological range (10–30 dynes/cm²). Velocity waveforms in proximal and distal grafts were similar in contour, indicating that the directional behavior of the velocity and shear vectors were similar at the two locations (Figure 2). It is also apparent that forward flow during diastole was minimal or nonexistent at both locations.

Intra-arterial pressure measurements at the time of implantation showed no decrease in pressure across the proximal or distal grafts. Table 1 shows that the time-average velocity in the proximal aorto-aortic graft was roughly twice that in the distal aorto-aortic graft. There was also a twofold difference in computed shear stress (Figure 3). The differences in velocity and shear stress between graft locations are statistically significant (p<0.05). Note also that the group mean shear stress value for the proximal graft is only slightly greater than 20 dynes/cm².

Neointimal Area

All proximal and distal grafts were completely covered by endothelium when studied by SEM. The neointimal area of proximal aorto-aortic grafts was less than that of the distal aorto-iliac grafts in each animal (Table 1 and Figure 4). These differences were statistically significant (p<0.05). The difference in neointimal area was also evident along the entire length of the grafts (Figure 5). Histological cross sections of proximal and distal grafts illustrating the differences in neointimal thickening are shown in Figure 6.

The mean neointimal area for 5-mm aorto-iliac grafts (n=6) not in tandem with a proximal graft was 4.50±1.88 mm², which was not significantly different from the mean neointimal area of aorto-iliac grafts distal to a proximal graft (5.93±0.61 mm², p=0.15).

Smooth Muscle Cell Proliferation

The thymidine labeling index (Table 1 and Figure 7) of proximal, high-shear aorto-aortic grafts was significantly less than that of the distal, low-shear aorto-iliac grafts (p<0.05). As previously noted, the majority of the proliferating SMCs were located near the lumen of the graft as opposed to deep within the neointima (data not shown).

Neointimal Smooth Muscle Cell Content

Table 1 shows that the overall fraction of the neointima occupied by SMCs was the same in both proximal and distal grafts (~20%). However, the distal, low-flow grafts were significantly more cellular than the proximal, high-flow grafts (p<0.05) in the 30-μm-wide zone of greatest SMC proliferation ad-
FIGURE 5. Line plot of distribution of neointimal area along the length of grafts. Values are mean neointimal area (±SD) of all available cross sections at the indicated graft region.

jacent to the lumen. Differences in cellularity were not evident at any deeper level in the neointima.

Overall SMC volume in proximal, high-flow grafts was only half that in distal, low-flow grafts (p<0.01). This approximates the relative difference seen in neointimal area between proximal and distal grafts; thus, it appears that the difference in neointimal thickening between high- and low-flow grafts is largely explained by a difference in SMC content and associated matrix.

Discussion

Shear Stress Magnitude Regulates Neointimal Thickening

Shear stress is most appropriately defined as a vector quantity with dual properties of magnitude and direction. Conceivably, changes in either or both of these properties could be responsible for the observed effects of flow on vascular wall behavior.

The current study demonstrates that SMC proliferation and neointimal thickening in porous PTFE grafts is reduced by elevated shear stress within the physiological range. Proximal, high-shear grafts had significantly less neointimal thickening than did distal, low-shear grafts. The contour of the velocity waveform was similar in both grafts, and therefore the directional behavior of the shear vector throughout the cardiac cycle was similar in both types of grafts. However, the magnitude of mean shear stress in the proximal grafts was double that in the distal grafts.

Using a similar baboon model of bilateral aortoiliac grafts, we inserted a distal arteriovenous fistula to augment flow on one side and found that the resulting high-shear stress (well above the usual physiological range) inhibited neointimal thickening in these grafts. However, this fistula flow was quite different from that of most peripheral vessels and grafts in the present study in that both the magnitude and the directional behavior of the shear vector were significantly altered. Mean shear magnitude was elevated to clearly supraphysiological values (—80 dynes/cm²) in the graft proximal to the fistula. The shear vector produced by the fistula was also unidirectional throughout the entire cardiac cycle due to significant forward diastolic flow. This contrasts with most peripheral vessels, in which shear stress oscillates in direction as velocity falls to zero or reverses during diastole.

From the results of these two flow studies, we conclude that the most important determinant of neointimal hyperplasia is the magnitude of the shear stress vector, not its oscillation in direction. The difference in neointimal area between proximal and
distal grafts in the present model indicates that a modest elevation of shear stress magnitude to \(\geq 20\) dynes/cm\(^2\) can inhibit the thickening process. Using a model of human carotid bifurcation atherosclerosis, Ku et al\(^5\) suggested that both the variation in direction and the magnitude of the shear stress vector may be important in governing the location and degree of atherosclerotic intimal thickening in the carotid artery. They observed pronounced intimal thickening in the outer wall of the carotid bulb, where shear stress not only was of low magnitude but also oscillated in direction during the cardiac cycle. Minimal intimal thickening was present in the inner wall of the proximal internal carotid artery (the flow divider), where shear stress was not only high in magnitude but also unidirectional throughout the cardiac cycle. Because both the magnitude and direction of shear forces vary greatly at the carotid bifurcation, it was not possible to determine the relative importance of the two properties.

Others have correlated the presence of highly varying shear stresses during the cardiac cycle with diminished intimal thickening. Morinaga et al\(^18\) quantified the variation in shear stress during the cardiac cycle in canine vein grafts, in which flow and shear variation were separately manipulated by placing an arteriovenous fistula either proximal or distal to the graft. They reported greater intimal thickening in vein grafts experiencing low shear-stress variation but relatively high flows (distal fistula) than in grafts with high variation but low flow (proximal fistula). Their results suggest that the degree of shear stress variation is more important than the time-averaged magnitude of shear stress. However, it is not clear whether both groups of vein grafts were subjected to the same intraluminal pressure or tangential wall stress, which may also affect intimal thickening.\(^19\) Since waveforms for the two groups were not provided, it is also not known whether the shear stress in the high-variation group was unidirectional or oscillatory.

Friedman's group\(^20\) studied velocity profiles and intimal thickening in a human aortic bifurcation. They found that pulse shear (a measure of magnitude variation over time) was the most important determinant of intimal thickening (it was negatively correlated with thickness); however, the mean and peak values of shear stress were also important. These factors probably change in a similar manner in vessels with normal distal peripheral resistance (no proximal or distal arteriovenous fistula), and therefore the relative contribution of each to wall thickening is difficult to determine. Other groups, using a variety of models, have also emphasized the magnitude of shear as the important hemodynamic parameter influencing intimal thickening.\(^7-10,21\)

In summary, while most studies have shown that high magnitudes of shear stress are associated with less intimal thickening, the effects of shear variation or direction over time seem less consistent. Unidirectional shear forces have been linked with both less intimal thickening\(^5-16\) and more intimal thickening.\(^18\) Our studies suggest that the magnitude of shear stress rather than its direction over time is the most important regulator of intimal thickening.

Smooth Muscle Cell Proliferation Is Increased in Grafts With Low Shear Stress

The extent of neointimal thickening can be altered by a change in SMC content, the amount of extracellular matrix SMCs produce, or both. Our data indicate that the greater thickening in the low-shear grafts is primarily a result of increased SMC proliferation confined to a narrow region near the lumen. There does not appear to be a flow-induced change in the amount of matrix each SMC produces. Although the difference in SMC proliferation between high- and low-flow grafts is significant, the absolute proliferative rate in both is low (<0.25%/day). Most of the proliferating SMCs are located in the region immediately subjacent to the endothelium, which is also the only region in which low-flow grafts have increased SMC content. Therefore, the greatest effects of flow on SMC proliferation are found in the region closest the lumen, where shear stress is likely to have its greatest effects.

The overall proportion of the neointima occupied by SMCs is the same in both proximal, high-shear and distal, low-shear grafts (~20%), indicating that there are not large flow-mediated differences in the amount of extracellular matrix production by each SMC. This four to one ratio of extracellular matrix to cells was also observed in the intimas of injured rat carotid arteries once the SMCs had reached quiescence.\(^22,23\) Thus, it appears that resting SMCs under a variety of circumstances are surrounded by a large and predictable volume of extracellular matrix.

Finally, the relative difference in SMC volume between proximal and distal grafts is nearly equal to the relative difference in neointimal areas. Taken together, all of these findings indicate that the ultimate differences in neointimal thickening can be
explained by shear-mediated differences in SMC proliferation at the blood–tissue interface. Once these SMCs are formed, they then go on to produce a fairly constant and predictable amount of extracellular matrix that contributes to neointimal volume. **Cellular Response to Shear Stress**

Why do changes in shear result in modification of blood vessel or graft architecture? Perhaps an optimal shear environment for luminal cells is necessary for normal cellular function. The concept of cellular "tensegrity" as advanced by Ingber and Jamieson and Ingber and Folkman may be relevant to the effects of flow on wall structure. They have shown that endothelial cell phenotype in vitro is altered by varying the number of contact points between the cells and the underlying matrix substrate. A high number of these contacts were associated with proliferation, while fewer contacts induced tubule formation, a phenotype associated with cellular quiescence. Cell–matrix contacts along with cell–cell contacts impart a "resting cell tension" to the individual endothelial cell. Alterations in this resting level of tension (through a change in shear stress, for example) might trigger a change in phenotype from the growth to the differentiated mode or vice versa.

The fact that shear stress in normal arteries seems to be fairly constant in a variety of species supports the concept of a biologically optimal range of shear stress. Zarins et al reported a value of 15–16 dynes/cm² in the monkey iliac artery and 12 dynes/cm² in the aorta. Large canine arteries experience a shear stress of 15–20 dynes/cm², and in smaller arteries, values of 14–22 dynes/cm² are reported. If an optimal range for shear stress does exist, then a perturbation in the normal shear environment might be expected to generate a compensatory response. Several authors have correlated the degree of luminal enlargement in arterial tributaries feeding arteriovenous fistulas to that required to reestablish a normal shear stress value. Additionally, Langille and O'Donnell have demonstrated that, in rabbits, an adaptive decrease in carotid artery luminal diameter occurs when flow is chronically reduced. This adaptation could initially be overcome with vasodilators but not at later times, suggesting an early vasoconstrictive response made permanent by a later change in wall structure. The reduction in luminal diameter was shown to be endothelium dependent, as it did not occur in arteries denuded of endothelium before flow reduction. In our model, the prosthetic graft wall is relatively rigid and therefore incapable of vasoactive changes in luminal diameter in response to changes in flow. However, luminal diameter can be altered by regulation of neointimal thickness. Our results indicate that porous grafts placed into sustained low-shear environments respond by reducing luminal diameter through neointimal thickening, which increases shear stress.

Since the endothelial cell is in contact with the flowing blood, it most likely transduces the physical stimulus of shear stress to a biologic effect. Endothelial coverage of the porous PTFE grafts in our model was verified by SEM, is typically complete within 2 weeks of implantation, and precedes SMC proliferation and neointimal thickening. Endothelial cells are clearly sensitive to changes in shear stress. Responses include alterations in morphological appearance, cytoskeletal organization, prostacyclin production, pinoctosis, potassium ion channel activation, and tissue plasminogen activator transcription and secretion. Cultured endothelial cells have also been shown to decrease production and release of endothelin-1 when exposed to 25 dynes/cm² of shear stress. Hsieh and colleagues have also shown that the expression of both platelet-derived growth factor A and B mRNA is sensitive to changes in shear stress. In their experiments, they demonstrated higher levels of platelet-derived growth factor transcripts with shear stresses of 10–20 dynes/cm² than with shear stresses of 30–40 dynes/cm². These levels correspond with the calculated shear stress in the distal and proximal grafts of our model, respectively, and further support the concept that hemodynamic forces can regulate growth factor production.

If endothelial cells truly sense low shear stress and mediate neointimal thickening, they should be capable of stimulating SMCs to grow. The factors actually regulating SMC growth in prosthetic grafts and injured arteries have not been defined. In the past, attention has been focused on the mitogenic potential of platelets in the intimal thickening process, especially platelet-derived growth factor. Yet, in our endothelialized grafts, SMCs proliferate at rates above baseline, even though platelets are not present. We observed similar degrees of neointimal thickening in aorto–iliac grafts with or without a proximal aorto–aortic graft. This indicates that growth factor activation by the proximal graft was not responsible for the increased thickening in the distal, low-shear grafts. The mitogenic signal for this late cellular proliferation may thus be arising from the cells within the neointima. Since SMCs proliferate in low-shear grafts only in the layers close to the lumen, the endothelial cell may be the source of SMC mitogens.

Potential mitogens for SMCs known to be produced by endothelial cells include platelet-derived growth factor, basic fibroblast growth factor, and endothelin-1. Previous work in our laboratory has demonstrated the presence of platelet-derived growth factor mRNA in the neointima of porous PTFE grafts. Ex vivo perfusates from these grafts possess mitogenic activity for cultured fibroblasts, which is inhibitable by antibodies to platelet-derived growth factor. We have recently demonstrated the presence of platelet-derived growth factor A chain protein in PTFE graft neointima and have localized it immunohistochemically to the periluminal region. Positive immunostaining for a platelet-derived growth factor receptor protein as well as basic fibro-
blast growth factor has also been observed in graft neointima (Kraiss et al, unpublished results). Apart from endothelin\textsuperscript{36} and platelet-derived growth factor,\textsuperscript{37} it remains unknown whether the expression of these growth factors is responsive to changes in shear stress. The actual mechanism by which the endothelial cell senses and responds to shear stress is also unknown.

In conclusion, we have presented evidence that neointimal SMC proliferation and subsequent thickening in porous PTFE grafts are altered by changes in mean shear-stress magnitude that remain in the physiological range. This may reflect regulation of neointimal SMC proliferation by endothelial cells in response to changes in shear stress.

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