Temporal gradients in shear, but not spatial gradients, stimulate ERK1/2 activation in human endothelial cells

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White, Charles R., Hazel Y. Stevens, Mark Haidekker, and John A. Frangos. Temporal gradients in shear, but not spatial gradients, stimulate ERK1/2 activation in human endothelial cells. Am J Physiol Heart Circ Physiol 289: H2350–H2355, 2005; doi:10.1152/ajpheart.01229.2004.—We have previously demonstrated temporal gradients in shear stress stimulate endothelial cell proliferation, whereas spatial gradients do not. In the present study, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway was investigated as a possible mediator for the promitogenic effect of temporal gradients. The sudden expansion flow chamber (SEFC) model was used to differentiate the effect of temporal gradients in shear from that of spatial gradients on ERK1/2 activation in human umbilical vein endothelial cells (HUVEC). ERK1/2 activation in the SEFC was not significantly different from control when HUVEC were exposed to spatial gradients alone. When a single temporal impulse was superimposed on spatial gradients, ERK1/2 activation was stimulated 330% (relative to spatial alone) within the region of spatial gradients. Inhibition of the ERK1/2 pathway with U-0126 abolished all effects of temporal gradients. To further separate temporal and spatial gradients, a conventional parallel plate flow chamber was utilized. Acute exposure to oscillations in flow at a frequency of 1 Hz stimulated ERK1/2 activation 620 ± 88% relative to control, whereas a single impulse of flow increased ERK1/2 activation 166 ± 19%. Flow without the temporal component did not significantly activate ERK1/2. These results suggest that the ERK1/2 pathway directly mediates the promitogenic effects of temporal gradients in shear stress.

shear stress gradient; proliferation; recirculating flow; atherosclerosis

ATHEROSCLEROSIS remains a leading cause of morbidity and mortality in the Western world. It is a chronic systemic disease attributed to many well-identified risk factors (i.e., diabetes mellitus, hyperlipidemia, hypercholesteremia, hypertension, and cigarette smoking). Yet the formation of atherosclerotic lesions do not occur in a random fashion. The coronary arteries, the major branches of the aortic arch, and the abdominal aorta are particularly susceptible sites. Given the focal nature of plaque formation within these regions, it has long been suggested that certain characteristics of fluid shear stress unique to these regions may potentiate the early stages of atherogenesis independent of other risk factors (12). Detailed analyses of fluid mechanics in atherosclerosis-susceptible regions of the vasculature have identified unique patterns of disturbed flow characterized by regions of flow separation, recirculation, reattachment, and perhaps most importantly, significant temporal and spatial gradients of shear stress (12, 20). Temporal shear stress gradients are defined as the increase or decrease of shear stress over a small period of time at the same location, whereas spatial shear stress gradients are defined as the difference of shear stress between two close points of a cell at the same point in time. To date, the role of temporal and spatial gradients of shear stress in the pathogenesis of atherosclerosis remains controversial. Some studies link atherogenesis to the large temporal gradients in shear due to the change of shear direction (20, 22), whereas others relate this to different spatial distributions of mean wall shear stress (15).

An established in vitro model used to simulate in vivo spatial patterns of flow separation, recirculation, and reattachment is the sudden expansion flow chamber (SEFC) (14, 27). By creating a sudden asymmetric expansion in the flow path of perfusing media across a cultured endothelial monolayer, the SEFC generates a large spatial gradient in shear stress over a relatively small region of a cultured endothelial monolayer. This high gradient is caused by flow separation. Near to the expansion step, flow recirculates in an eddy, whereas further downstream, the flow reforms to the regular parabolic profile. In between, there is a point of flow reattachment where shear stress is zero (stagnation point). When the flow of perfusing media is held constant, a stable recirculating flow pattern is generated over the same spatial region of a cultured monolayer (a spatial gradient in shear stress). With the sudden onset of flow, recirculating flow undergoes a distinct developmental phase of several hundred milliseconds in which the reattachment point moves out from the expansion step (9, 14). If flow is pulsatile, the reattachment point migrates back and forth across the cultured monolayer with each oscillatory cycle of flow. Cells directly beneath the migrating reattachment point experience a dramatic and rapid change in shear stress as the stagnation point passes above (a temporal gradient in shear stress). Therefore, both maximal temporal and spatial gradients can overlap each other in this in vitro model system. Detailed numerical analyses of the SEFC have shown that a negligible temporal change can be achieved in this model if the onset of flow is slowly ramped up over time (14). Given that temporal gradients have been shown to induce atherogenic phenotypes (1–3), this technique has proven to be a powerful tool to elucidate the mechanochemical coupling between fluid shear stress and the localization of atherosclerotic lesions.

Using the SEFC model, we (14, 28) have previously demonstrated temporal gradients in shear stress stimulate endothelial cell proliferation, whereas spatial gradients affect endothelial proliferation no differently from steady uniform shear stress. Given that the promitogenic extracellular signal-regu-
labeled kinases 1 and 2 (ERK1/2) pathway is a known shear responsive kinase and is rapidly and specifically activated by temporal gradients in fluid shear stress (2, 3, 25), it represents a likely mediator for the promitogenic effects of recirculating flow that we have previously reported. ERK1/2 is a member of the mitogen-activated protein kinase (MAPK) conserved cascade of kinases that stimulate the phosphorylation of transcription factors and other targets in response to extracellular signals such as growth factors, cytokines, and fluid shear stress (29). The ERK1/2 pathway is thought to be primarily involved in the regulation of cell proliferation and differentiation (19), whereas other members of the MAPK family may also be important in the regulation of cell apoptosis and inflammation (16, 23). ERK1/2 is believed to mediate cell proliferation through the phosphorylation of other downstream proteins, one of which is retinoblastoma protein (Rb), which controls the cell cycle transition from G1 phase to S phase (19, 24).

The present studies examine the effect of temporal and spatial gradients on ERK1/2 activation and the resultant proliferation in the recirculating flow model system. The SEFC model was used to differentiate the effect of temporal gradients in shear from that of spatial gradients on ERK1/2 activation in primary human umbilical vein endothelial cells (HUVEC). The spatial distribution of ERK1/2-activated cells in the endothelial monolayer were identified via en face immunohistochemical staining for phospho-ERK1/2. Downstream from ERK1/2 activation, rates of HUVEC proliferation were determined with staining for phospho-ERK1/2. Downstream from ERK1/2 activation factors and other targets in response to extracellular signals such as growth factors, cytokines, and fluid shear stress (29). The ERK1/2 pathway is thought to be primarily involved in the regulation of cell proliferation and differentiation (19), whereas other members of the MAPK family may also be important in the regulation of cell apoptosis and inflammation (16, 23). ERK1/2 is believed to mediate cell proliferation through the phosphorylation of other downstream proteins, one of which is retinoblastoma protein (Rb), which controls the cell cycle transition from G1 phase to S phase (19, 24).

METHODS

Cell culture. Primary HUVEC isolation was performed as previously described (10). Cells were seeded onto glass microscope slides and grown to confluence within 3 days in medium 199 (Irvine Scientific). All cell cultures were maintained in a humidified 5% CO2-95% air incubator at 37°C. Confluent cell cultures used for the ERK1/2 inhibition assay were pretreated with 3 μM U-0126 (a specific inhibitor of the ERK1/2 enzyme) (CalBiochem) or vehicle (DMSO) for 1 h immediately before flow experiments.

Flow experiments. Dulbecco’s modified Eagle’s medium (Irvine Scientific) supplemented with 2% fetal bovine serum (Hyclone), 0.5 U/ml penicillin, and 0.05 mg/ml streptomycin was used as the perfusing medium for all experimental procedures. All flow chambers and accompanying apparatus were maintained at 37°C throughout the experiment. Time-matched sham controls (slides mounted on flow chambers without flow) and static controls (undisturbed slides in Petri dishes) were performed for all experimental groups.

The SEFC was a modification of a chamber previously described (28). The ratio between the sudden-expansion step and the inflow channel was 2:1. HUVEC monolayers were subjected to 1 h of flow for ERK1/2 activation studies and 4 h of flow for HUVEC proliferation studies. One of two methods for the onset of flow was used: 1) ramped onset (a smooth ramped increase from 0 to 3.5 ml/s within 15 s), or 2) sudden onset (the initiation of fully established flow at 3.5 ml/s within 300 ms). The flow rate of 3.5 ml/s was calculated to produce a shear stress of 10 dyn/cm2 in the region of reestablished flow downstream from the reattachment point. The continuous flow of media through the SEFC was maintained with a constant hydrostatic pressure head flow loop apparatus (10). Ramped flow was manually controlled through a screw-type pinch valve (Flow-Rite, PV-9). Immediately after the completion of each specific flow profile, slides were removed from the SEFC and stained for ERK1/2 activation or HUVEC proliferation (see En face staining for ERK1/2 active cells).

The geometry of the conventional PPFC was identical to the SEFC but lacked the sudden expansion of the flow path. In the PPFC, confluent HUVEC monolayers on glass slides were subjected to one of the following three laminar flow profiles (see Fig. 3A): 1) impulse (3-s impulse of 10 dyn/cm2 followed by 10 min of no flow), 2) ramped transient (a smooth 15-s ramped increase from 0 to 10 dyn/cm2, sustained for 3 s, followed by a 15-s ramped decrease and 10 min of no flow), and 3) 1 Hz pulsatile (a repeated sequence of 0.5-s impulses of 10 dyn/cm2 at a frequency of 1 Hz sustained for 10 min). Perfusing medium was driven by a computer-controlled syringe pump (pump 22, Harvard Apparatus with a controlling PC). Immediately after the completion of each specific flow profile, slides were removed from the PPFC and stained for ERK1/2-activated cells (see En face staining for ERK1/2 active cells).

En face staining for ERK1/2 active cells. Immediately after exposure to flow, slides were fixed in 4% paraformaldehyde for 30 mins at 4°C and then methanol for 10 min at −20°C. Slides were washed with 0.1% of Tween 20 with Tris-buffered saline (TBST) and blocked at 22°C for 30 min in TBST with 5% goat serum. Slides were then probed at 4°C overnight with a primary anti-phospho-ERK1/2 rabbit monoclonal antibody (Cell Signaling Technology) at a 1:250 titer. Slides were then washed in TBST and incubated for 1 h at 22°C with a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Cell Signaling Technology) at a 1:250 titer. To visualize the distribution patterns of phospho-ERK1/2-positive cells across the HUVEC monolayer, slides were subsequently incubated at 22°C for 10 min with TrueBlue Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). In cells where ERK1/2 was activated, TrueBlue Peroxidase develops a distinct dark blue precipitate that is clearly visible distributed throughout the cell. This distinct precipitate was readily identifiable against the background staining in nonactivated cells. Phospho-ERK1/2-positive cells were counted by eye within adjacent ×100 high-power fields (HPF) of view along the centerline of each slide. In the SEFC, each HPF was divided into 0.86-mm sections extending 9.46-mm downstream from the expansion point. In the PPFC, 20 HPF were counted for each slide.

Immunofluorescent staining. Proliferating HUVEC were identified by using a commercially available in situ monoclonal antibody kit for the detection of bromodeoxyuridine (BrdU) incorporation into cellular DNA during DNA synthesis (Boehringer Mannheim). Immediately after exposure to flow in the SEFC or the PPFC, slides were quickly removed from the chamber and incubated at 37°C in medium 199-BrdU (10 fmol/l BrdU) for 22 h. Slides were fixed in 70% ethanol (in 50 mM glycine buffer, pH 2.0) and immunostained for BrdU incorporation. BrdU-positive cells were visualized under a fluorescence microscope (Nikon, Diaphot TMD). Proliferating cells were counted by eye within adjacent ×100 high-power fields (HPF) along the centerline of each slide. In the SEFC, each HPF was divided into 0.86-mm sections extending 9.46 mm downstream from the expansion point. In the PPFC, 20 HPF were counted for each slide.

Statistics. All experimental values are given as means ± SE. All reported values of n refer to the number of separate and independent experiments from multiple primary HUVEC cultures. Significant differences between means were calculated using a Student’s t-test. The Wilcoxon test was used to test for a significant departure of the median from sham control. Statistical significance was taken at the P < 0.05 level.

RESULTS

Mathematical modeling of flow recirculation in the SEFC. For the fully established flow in a 2:1 SEFC, the calculated reattachment point was found at 2.3 mm downstream from the sudden-expansion point. The computer simulations revealed that the location of the highest temporal gradient...
The highest temporal gradient for the ramped flow was (16 dyn·cm⁻²·s⁻¹) and occurred at the same location. During the dynamic onset phase the reattachment point moves from 0.6 mm (at Reynolds number = 10) to its final position at 2.3 mm. This movement is the primary source of the temporal gradient in shear stress. The maximum temporal gradient generated with the sudden onset of flow in the PPFC was calculated to be 300 dyn·cm⁻²·s⁻¹ (28).

Effect of flow onset in SEFC on ERK1/2 activation and HUVEC proliferation. The region extending 2.6 mm downstream from the point of expansion was taken to fully contain the spatial pattern of flow separation, recirculation, and reattachment. The region of reestablished unidirectional flow was taken to fully contain between 3.4 and 8.6 mm downstream from the expansion point.

All ERK1/2-positive cells were counted in each 0.86-mm section along the centerline of the slide and expressed as ERK1/2-positive cells per HPF. Within the region of recirculating flow, sudden onset of flow stimulated a significant (P < 0.05) peak 330% increase in ERK1/2-positive cells relative to the corresponding region of ramped onset (1.72–2.58 mm from expansion) (Fig. 1). This region closely correlates with the calculated location of flow reattachment and maximal temporal gradient for the 2:1 SEFC (14). Between the two onset profiles, ERK1/2-positive cells were not significantly different at corresponding locations within the region of unidirectional flow downstream from the reattachment point. Within the region of recirculation and in the absence of temporal gradients (ramped onset), spatial gradients in shear stress affected ERK1/2 activation no differently from steady shear stress. When cells were pretreated with U-0126 and then exposed to the sudden onset of flow, BrdU-positive nuclei were identified along the centerline of the slide (data not shown).

All BrdU-positive cells were counted in each 0.86-mm section along the centerline of each slide and expressed as BrdU-positive nuclei per HPF. When cells were pretreated with U-0126 and then exposed to the sudden onset of flow, BrdU incorporation into HUVEC nuclei was not significantly greater than zero across the entire centerline of the slide (Fig. 2). In untreated cells exposed to sudden onset of flow, the mean BrdU-positive nuclei per HPF within the region of unidirectional flow was 9 ± 2. Within the region of recirculating flow, sudden onset of flow stimulated a 411% increase in HUVEC proliferation (relative to the region of unidirectional flow). Maximal BrdU incorporation was between 1.72 and 3.44 mm from expansion and averaged 37 ± 3 BrdU-positive nuclei per HPF. This region correlates with the calculated location of flow reattachment and maximal temporal gradient for the 2:1 SEFC (14). Rates of HUVEC proliferation at all other points within the region of recirculation were not different from rates observed for unidirectional flow.

Effect of flow in the PPFC on ERK1/2 activation and HUVEC proliferation. ERK1/2-positive cells were expressed as percent increase in ERK1/2-positive cells above sham controls (Fig. 3B). When HUVEC were exposed to continuous pulsatile flow at a frequency of 1 Hz sustained for 10 min, ERK1/2-positive cells were significantly increased above sham by 620 ± 88% (n = 12). When HUVEC were exposed to a single 3-s impulse of flow, ERK1/2-positive cells were significantly increased by 166 ± 19% (n = 24). The degree of ERK1/2 activation in response to oscillatory flow was significantly greater than the activation obtained in response to a single impulse of flow. A single ramped transient of flow only increased ERK1/2 activation by 32 ± 20% (n = 8). This increase was not a significant departure of the median from sham control. ERK1/2 activation could not be detected in HUVEC pretreated with U-0126 in sham controls or with exposure to flow (data not shown).

HUVEC proliferation was expressed as percent increase in BrdU-positive nuclei above sham controls (Fig. 3C). No significant differences were observed in the level of proliferation between sham controls and static controls (data not shown). When HUVEC were exposed to continuous pulsatile flow at the frequency of 1 Hz sustained for 10 min, proliferation

Fig. 1. Effect of ramped and sudden onset of flow on extracellular signal-regulated kinase (ERK1/2) activation in the sudden expansion flow chamber (SEFC). Spatial distribution of ERK1/2-positive cells exposed to 1 h of recirculating flow in the SEFC is shown. ERK1/2-positive cells were counted in each 0.86-mm section along the centerline of the chamber. Initial onset of flow was either 15 s ramped onset (black line) or sudden onset (gray line). Arrow, calculated point of flow reattachment. HPF, high-powered field. *Significant difference between corresponding regions of ramped onset and sudden onset. All values are express as means ± SE.

Fig. 2. Effect of sudden onset of flow on bromodeoxyuridine (BrdU) incorporation with and without inhibition of ERK1/2 in SEFC. BrdU incorporation into cell nuclei was taken as an index of human umbilical vein endothelial cells (HUVEC) proliferation. Initial onset of flow was sudden and then held steady for 4 h in SEFC. Confluent cell cultures were pretreated with 3 μM U-0126 (black line) or vehicle (gray line) for 1 h immediately before flow experiments. BrdU-positive cells were counted in each 0.86-mm section along the centerline of the chamber. ERK1/2 inhibition effectively abolished HUVEC proliferation in both sham controls (data not shown) and cells exposed to flow. Arrow, calculated point of flow reattachment. All values are expressed as means ± SE.
Increased endothelial turnover in regions of recirculating flow has long been implicated in the process of atherogenesis (5, 7). A number of studies have demonstrated enhanced macromolecular permeability of aortic endothelial cells during mitosis (4, 21). Because the vascular endothelium serves as a dynamic interface between circulating blood elements and the interstitial tissues, disruption of its permeability characteristics may permit the localized influx of circulating low-density lipoproteins and other proinflammatory macromolecules into the artery wall (17). Previous studies of recirculating flow have suggested that the induction of promitogenic phenotypes in the sudden asymmetric expansion model were a result of spatial gradients of shear stress (7, 26, 27). More recently, we have shown that temporal gradients in shear stress that overlap spatial gradients during the sudden onset of flow or with pulsatile flow induce the mitogenic response within regions of recirculating flow (14, 28). Spatial gradients were found to affect endothelial proliferation no differently from steady uniform shear stress (14, 28). In the present study, we investigated the ERK1/2 pathway as a potential mediator for the promitogenic effects of temporal gradients within regions of recirculating flow.

Through the utilization of two different flow systems (SEFC and PPFC), cells were exposed individually to pulsatile and impulse flow, and without temporal or spatial gradients as well as combinations of the two. Consistent with our previous findings, when the onset of flow in the SEFC was sudden, both ERK1/2 activation (Fig. 1) and endothelial proliferation (Fig. 2) were significantly stimulated at the site of flow reattachment. Furthermore, when the onset of flow was slowly ramped up, ERK1/2 activation remained unstimulated within the same spatial region. Both onset flow profiles generate the same spatial gradient in shear stress (which is maximal at the site of flow reattachment). Thus further supporting previous studies suggesting that spatial gradients in shear stress affect endothelial proliferation no differently from steady uniform shear stress (28). In good agreement to patterns of ERK1/2 activation observed in the SEFC, in the PPFC where flow is uniform without spatial gradients, elevated ERK1/2 activation was only observed when HUVEC were exposed to temporal gradients (Fig. 3B).

ERK1/2 activation is regulated by the upstream enzyme MAPK/ERK kinase 1 and 2 (MEK1/2). ERK1/2 activation can be specifically blocked by the MEK1/2 inhibitor U-0126. In the current study, U-0126 was able to block all detectable ERK1/2 activation in both sham controls and by temporal gradients in shear. Previous studies using Western blot analysis quantification of ERK1/2 phosphorylation found that U-0126 was able to block the activation of ERK1/2 incurred by temporal gradients in shear, but not basal, ERK1/2 phosphorylation (18). Immunohistochemical staining for the spatial distribution of phosho-ERK1/2 is at least an order of magnitude less sensitive than Western blot analysis. It is likely that the spatial distribution of phospho-ERK1/2 is at least an order of magnitude less sensitive than Western blot detection. It is likely that basal ERK1/2 activation went undetected by the methodology employed in this study. This possibility is supported by the finding that BrdU incorporation was still observed 24 h after U-0126 treatment. However, BrdU incorporation in U-0126 treated controls was still significantly less than that observed for untreated controls.

The ultimate response of an endothelial cell to any flow pattern is a balance between the magnitudes of the atherogenic/mitogenic signal (temporal gradient) and the anti-atherogenic/anti-mitogenic (steady shear) (1, 2, 12, 20). In the SEFC, the relative contribution of steady versus dynamic components varies with the location and the geometry of the chamber and increased by 171 ± 9% (n = 4). When HUVEC were exposed to a single impulse of flow, proliferation increased 168 ± 6% (n = 4). No significant differences were observed in the level of proliferation between a 1-Hz pulsatile and a single impulse. The rate of BrdU incorporation in HUVEC treated with U-0126 and exposed to pulsatile and impulse flow was not significantly different from U-0126-treated sham controls (−13 ± 13% and −3 ± 12%, respectively). Between sham controls treated with and without U-0126, rates of BrdU incorporation were not significantly different.

**DISCUSSION**

Increased endothelial turnover in regions of recirculating flow has long been implicated in the process of atherogenesis (5, 7). A number of studies have demonstrated enhanced macromolecular permeability of aortic endothelial cells during
the duration of flow (14). With the sudden onset of flow in the SEFC, the entire HUVEC monolayer is exposed to some degree of a temporal gradient. The peak temporal gradient occurs between 1.72 and 2.58 mm. Once flow is established, although certain regions are exposed to flow recirculation, a significant steady shear stress component is present throughout the majority of the chamber. If flow remains steady, over time, the steady component suppresses the proliferative response to the temporal component. At the reattachment point, the mean wall shear stress within that region is very low to zero. Without steady flow, the effect of the temporal gradient generated during the onset of flow is preserved in this region. In vivo, where blood flow is pulsatile, the reattachment point oscillates along the vascular wall continually exposing the underlying endothelial monolayer to strong temporal gradients in shear stress. Given that no significant component of steady shear can be developed within the migrating reattachment point, this may lead to a spatially broader atherogenic stimulus in vivo than seen in the SEFC. For the ERK1/2 study in the SEFC, flow was limited to 1 h. Whereas in studies of BrdU incorporation in the SEFC, flow was stable for 4 h. Time-course studies identified 1 h as a balance between the reported time course for maximal ERK1/2 activation in response to temporal gradients (3) and the time required for the suppression of ERK1/2 activation by steady shear (data not shown).

In the PFPC, we confirmed previous findings that a single impulse of flow stimulates HUVEC proliferation as effectively as sustained pulsatile flow (Fig. 3C) (28). In sharp contrast to that finding, ERK1/2 activation in response to pulsatile flow was stimulated 620% above sham control, whereas a single impulse of flow stimulated only 166% (Fig. 3B). Given that the elevation in ERK1/2 phosphorylation in response to pulsatile flow does not lead to a greater mitogenic response (in comparison to a single impulse), it is likely that ERK1/2 activation is far in excess of threshold levels required to stimulate a proliferative response. The transition from G1 phase to S phase of the cell cycle requires the phosphorylation of Rb at multiple sites. ERK1/2 is an upstream mediator of Rb phosphorylation (6, 8, 18). Hyperphosphorylation of Rb causes the release of the E2F transcription factor and commits the cell into the S phase (11). Once a cell has entered the S phase, continued ERK1/2 activity will not stimulate additional cell proliferation. Therefore, the increased phosphorylation of ERK1/2 in response to pulsatile flow may further indicate the exquisite sensitivity of ERK1/2 to temporal gradients in fluid shear stress. In further support of ERK1/2 as a critical signaling molecule mediating shear-dependent proliferation response, when cells were pretreated with U-0126, neither a single impulse of flow or sustained pulsatile flow resulted in increased rates of HUVEC proliferation.

The present study was designed to elucidate the upstream signal transduction pathway leading to endothelial proliferation in atherosclerosis-susceptible regions of the vasculature. The biophysical mechanism by which large temporal gradients in shear stress stimulate ERK1/2 activation remains to be determined. Rapid mechanochemical signal transduction during the sudden onset of flow similar to that observed in this study has previously been reported (13), where specific mitogenic G protein activation occurs within 1 s. When considering these findings, it is important to bear in mind that atherosclerosis is a protracted and multifactorial disease that involves many circulating blood elements, hemodynamic forces, and a complex cascade of molecular events within the endothelium and the arterial wall.

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