Monocytes/Macrophages Cooperate with Progenitor Cells during Neovascularization and Tissue Repair

Conversion of Cell Columns into Fibrovascular Bundles

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The potential of monocytes/macrophages (MC/Mph) to contribute to neovascularization has recently become a topic of intense scrutiny. Here, we characterized the behavior of MC/Mph in cellular infiltrates, with emphasis on their spatial organization and localization in newly formed microvessels. To this end, we studied MC/Mph migration and assembly in basic fibroblast growth factor-supplemented Matrigel plugs placed in transgenic Tie2- β -galactosidase mice for up to 4 weeks. In these plugs, along with Nile Red-positive adipocytes, we found MC/Mph distributed in cell cords, also containing various mature and progenitor tissue cells; and functional Tie2-positive or -negative microvessels embedded in bundles of fibrillar collagen surrounded by F4/80-positive MC/Mph. At earlier stages of infiltration, we found tubular destruction of the matrix (tunnels) and MC/Mph-lined capillary-like structures occasionally containing erythrocytes, indicating their propensity for endothelial *trans*-differentiation. We also analyzed *in vitro* the MCP-1-induced chemotactic migration of fluorescently labeled peritoneal MC/Mph incorporated in Matrigel-containing fluorescent protease substrates. Many of these MC/Mph produced MMP-12- and TIMP-1-dependent tunnels coupled with acquisition of a lumen. In conclusion, long-term implantation of Matrigel plugs qualifies as a novel experimental model of tissue regeneration, in which neovascularization intimately couples with fibrosis and organogenesis and in which cells of MC/Mph phenotype play a key structural role. (*Am J Pathol* 2006, 168:529–541; DOI: 10.2353/ajpath.2006.050255)

Neovascularization in adult animals is influenced by various inflammatory cells, in particular by monocytes/macrophages (MC/Mph).^{1–5} These cells often precede, temporally and spatially, the formation of new sprouts,^{6–8} thus placing MC/Mph in a significant position to alter the microenvironment⁹ and to affect subsequent endothelial cell (EC) migration, proliferation, and vessel formation. Mph-specific metalloelastase (MMP-12) has been detected adjacent to developing capillaries during wound healing in mice,¹⁰ and Mph-derived metalloproteases regulate capillary branching during ischemia-reperfusion.¹¹ Mph also create a permissive environment for neurogenesis and vasculogenesis in the developing brain.¹²

The relationship between MC/Mph and ECs originates in embryogenesis. Embryonic angiogenesis is initiated by the hemangioblast, a precursor of both hematopoietic and endothelial systems.¹³ CD45⁺ progenitor cells precede the advancement of ECs into avascular zones in developing mouse embryos,¹⁴ and Mph have been observed at the tips of proliferating capillaries in neonatal mouse retina.¹⁵ The ability to acquire endothelial properties was repeatedly noted in MC/Mph placed in angiogenic conditions.^{16–20} Moreover, cells with a hybrid phenotype, positive for both the monocytic marker CD45 and endothelial VE-cadherin, were isolated from tumors.²¹ These findings argue for the ability of MC to contribute to neovascularization, given the appropriate conditions and settings. Nevertheless, the specific steps of this conversion *in vivo* are yet unknown. Many examples of vascular mimicry were recently described, including tumor cells,²² epithelial cells,²³ mesothelial cells,²⁴ or cytotropho-

Supported by the National Institutes of Health (grant HL-65983).

Accepted for publication September 29, 2005.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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blasts,²⁵ all functioning in an endothelial capacity in various physiological or pathological settings. The potential of selected subpopulations of circulating or bone marrow-derived mononuclear cells to (*trans*-)differentiate into cells of various lineages^{26,27} led recently to a hot debate of the plasticity of stem cells, along with the very notion of stemness.²⁸

Bone marrow-derived circulating cells are also increasingly recognized as potential contributors to adult vasculogenesis and to cellular repair of larger vessels and other tissues. The contributing cells include endothelial progenitor cells (EPCs),^{29–31} smooth muscle progenitor cells,³² cardiomyoblast progenitors,³³ adipocyte progenitors,^{34–36} and fibroblast progenitors (also known as fibrocytes³⁷). However, the identities and the relationships between different cells involved in neovessel formation are not yet elucidated.

Although in the mouse models both CD34⁺ hematopoietic progenitors and CD14⁺ MC seem to contribute to vasculogenesis,^{19,38} their relative roles are still a matter of intense research. Progenitor cells are frequently detected, but their integration into neovascular structures is variable and often only limited.^{1,39} This led to the suggestion that EPCs would contribute to vasculogenesis mostly with signaling (software), rather than with building blocks (hardware).⁴⁰ The question then remains, which other cell types cooperate and contribute to the formation of new vessels.

We have previously demonstrated the presence of cells expressing the leukocyte markers CD18⁴¹ and Thy-1⁹ lining blood conduits in the hearts of a transgenic model of inflammatory cardiomyopathy induced by the constitutive expression of MCP-1.⁹ We have also described the formation of solid cords by MC/Mph,⁴² consecutive to local degradation of extracellular matrix, with creation of corridors of lower density that we called "tunnels."^{3,9}

In the cited study,⁴² we found that migrating THP-1 cells induced *in vitro* the lasting degradation of Matrigel and produced cell columns, a process amplified by monocyte chemoattractant protein-1 (MCP-1). We also reported the co-localization with THP-1 cells of erythrocytes and micron-sized plastic beads. Endothelium-free tunnels containing MC/MPH, neutrophils, or erythrocytes were also observed *in vivo*, in Matrigel-filled chambers lined by a semipermeable membrane, implanted subcutaneously in mice. In free subcutaneous Matrigel plugs, we found MC/MPH-based columns harboring isolated Tie-2⁺ cells (a marker of endothelial progenitor phenotype), as well as fibroblasts, dendritic cells, and adipocytes. Many of these cell columns displayed conspicuous branching. Our data demonstrated the formation of branched MC/MPH cell columns *in vitro* and *in vivo*, a previously unrecognized pattern of penetration of extracellular matrices by inflammatory cells. However, the fate of these cell cords, as well as the functional implications of their presence, remained unexplored.

We proposed³ that these cell columns could further evolve into more advanced capillary-like structures. Here we sought to test this hypothesis by using Matrigel, the EC basement membrane-like material,⁴³ and MCP-1 as a

migratory stimulus.⁴⁴ *In vivo*, we studied the cellular mechanisms by which a vasculogenic factor stimulates tissue organization in Matrigel plugs in mice. Our current results show that subcutaneous Matrigel containing basic fibroblast growth factor (bFGF) undergoes a process of complex cellular organization that comprises robust adipogenesis and vasculogenesis. We detected Mph forming a lumen and containing vascular cells, present in collagen bundles covered by Mph, likely to be derived from the MC/Mph based cell columns previously described.⁴² *In vitro*, we found that cells with a well-defined Mph phenotype could develop a lumen and thus initiate capillary-like structures.

Our study indicates that the paradigms of neovascularization based on the contribution of a single cell type (parent endothelium or EPCs) might not be sufficient to explain all of the observed circumstances of neovascularization. Instead, we propose a model based on the role of infiltrating mononuclear cells, able to assist the other precursor cells present in the infiltrate to convert into the needed components of a newly formed tissue. This helps to understand better the often noted but poorly explained coordination between neovascularization and the other elements of tissue formation, regeneration, or repair.

Materials and Methods

Animals

FVB/N-Tg182Sato/J transgenic mice expressing β -galactosidase gene under the endothelial Tie2 promoter (Tie2- β Gal),⁴⁵ either young (6 to 8 weeks) or old (18 months), were used for Matrigel plug and Matrigel chamber assays. For obtaining thioglycolate-elicited peritoneal Mph, we used young C57BL/6-Tg(ACTbEGFP)10sb/J transgenic mice expressing an enhanced green fluorescent protein (eGFP) under control of the chicken β -actin promoter with a cytomegalovirus enhancer element⁴⁶ or C57BL/6J control mice (Jackson Laboratories, Bar Harbor, ME). We also used a transgenic mouse line expressing yellow fluorescent protein (YFP) under the *fms* (M-CSF) promoter, as previously described.⁴⁷ All experiments were performed in accordance with the guidelines of the Committee for Animal Research of the Ohio State University.

Matrigel Plug Assay

Briefly, 500 μ l/sample cold Matrigel (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10 μ g/ml of human recombinant basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN) was injected subcutaneously into the abdomen of Tie2- β Gal mice.⁴⁸ Alternatively, we used VEGF-165 and SDF-1 α (used at 10 ng/ml; both from R&D Systems, Minneapolis, MN), and non-supplemented Matrigel plugs served as controls. After 7 days (group 1) or 4 weeks (group 2) mice were anesthetized and euthanized by CO₂ inhalation, and the plugs retrieved and fixed in 2% formaldehyde and 2.5% glutaraldehyde in phosphate-buffered saline (PBS).

The Matrigel plugs were incubated overnight in X-gal solution (Amersham, Piscataway, NJ) for visualization of β -galactosidase activity at pH > 7.0, to avoid the detection of endogenous β -galactosidase.⁴⁹ The filters were then embedded in paraffin, and 4- μ m sections were cut and used for hematoxylin and eosin staining, Masson Trichrome, or for immunohistochemistry. The sections were incubated with the anti-mouse Mph antibody F4/80⁵⁰ (MCAP497; Serotec Raleigh, NC) or anti-mouse smooth muscle α -actin (IA4; DAKO, Glostrup, Denmark) and further processed with an ABC kit from Vector Laboratories (Burlingame, CA).

The connectivity of the microvascular structures found in Matrigel plugs with the general circulation was directly assessed using infused India ink (a suspension of carbon particles, catalog no. s223, from Polyscientific R&D Corp., Bayshore, NY) as tracer of the vascular lumen. India ink (2 ml) was injected directly into the beating hearts of anesthetized mice 5 minutes before sacrifice and collection of the Matrigel plugs.⁵¹

To find the nature of vacuoles detected in many sections within large cells developing in Matrigel, we used the lipid staining with Nile Red.⁵² To this end, Nile Red (Molecular Probes, Eugene, OR) was diluted in dimethyl sulfoxide to obtain a stock concentration of 1 mg/ml. The stock was diluted in PBS to a final concentration of 10 μ g/ml. Cryosections were stained at room temperature for 10 minutes, followed by three washes with PBS. The samples were then coverslipped and mounted for microscopy using FluoroMount (Southern Biotechnology Associates, Birmingham, AL).

In Vivo Matrigel Chamber Assay

Plastic chambers, modified after Dvorak and colleagues,⁵³ with one sidewall made of Nuclepore filters of 8- μ m-pore size, were filled with Matrigel, with or without 10 μ g/ml human bFGF (R&D System). The chambers were implanted subcutaneously into Tie2- β Gal or wild-type control mice, with the filter facing toward the abdominal wall. Chambers were extracted from anesthetized mice and Matrigel was recovered from the chambers while still attached to the Nuclepore filters, and then fixed and processed as described above.

Isolation of Mph

Mice were injected intraperitoneally with 1.5 ml of 2.9% thioglycolate broth (Sigma, St. Louis, MO) 3 days before the experiment. Peritoneal Mph were obtained by lavage of the peritoneal cavity with 3 $\times$ 5 ml cold PBS and purified by adherence on plastic for 1 hour at 37°C. The cells were labeled while attached, with 1 mmol/L of either Cell Tracker Green (CTG) or Cell Tracker Red (CTR) (Molecular Probes) for 20 minutes at 37°C, according to the manufacturer's instructions, and then detached in cold PBS for 5 minutes.

Chemotaxis Assay

The migratory ability of Mph in Matrigel was directly assessed by a *trans*-filter migration assay using 8- μ m-pore filter inserts covered with Matrigel (Transwell; BD Biosciences, Franklin Lakes, NJ), as described.⁴² The cell-loaded inserts were incubated for 24 hours in the presence of stimulators (MCP-1 and VEGF, at 100 ng/ml and 10 ng/ml, respectively, in the lower compartment). To assess the intra- and pericellular degradation of the matrix, we used folimycin (50 nmol/L, Sigma), a vacuolar ATPase inhibitor also known to regulate pericellular proteolysis.⁵⁴

In Vitro Tunneling Assay

Matrigel was supplemented with DQ Red bovine serum albumin (BSA, Molecular Probes, Eugene, OR) at 20 μ g/ml. DQ-labeled proteins are fluorogenic substrates for proteases, used previously for the study of photolytic activity during co-migration and intercellular cooperation of tumor cells.⁵⁵ Matrigel-embedded Mph were exposed to the MCP-1 gradient by placing side by side 100 μ l of Matrigel containing fluorescent substrate and 10⁶/ml labeled cells, in lateral contact with another droplet of 100 μ l of Matrigel containing DQ proteins and MCP-1 (0.1 μ g/ml), both confined in a 1-cm diameter plastic ring that was glued to a coverslip. To inhibit metalloprotease activity, TIMP-1 (50 to 100 ng/ml; human recombinant; R&D Systems) or MMP-12-neutralizing polyclonal antibody (50 μ g/ml; a gift from Dr. S. Shapiro, Harvard University, Boston, MA) was added both to the medium and to the Matrigel matrix.

Matrigel-embedded cultures were maintained at 37°C and 5% CO₂ and examined after 3 hours and then daily up to 7 days using a Zeiss LSM510 multiphoton confocal inverted microscope. The average proteolysis was graded *in situ* by confocal examination of each cell in multiple optical planes, on a scale of scores ranging from 0 (absent) to 4 (maximal), and counting 10 to 14 cells per sample by a single observer. The experiments were repeated three times. The results were compared nonparametrically by χ^2 test, with $P < 0.05$ being considered statistically significant.

We also analyzed cell polarity in Matrigel by using high-resolution histology. We followed the standard procedure of sample preparation for electron microscopy, except that semithin (0.5 μ m) sections were examined with a Nikon Eclipse 800 microscope in transmission mode. This approach provided better preservation of morphological detail and a broader sampling area. Briefly, the gels were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.3 overnight, postfixed in 1% OsO₄ for 1 hour, dehydrated in graded alcohols, and embedded in resin (Spurr; Ted Pella, Inc., Redding, CA). Semithin sections were cut and stained with toluidine blue.

Figure 1. MC/Mph migration leads to tunnel formation in Matrigel. **A:** Matrigel retrieved after 1 week from a subcutaneously implanted, Nuclepore filter-limited chamber. Note the cells accumulated underneath the filter (**arrowheads**) and the tunnels left behind the front of migrating cells (**arrow**). **B:** Most of the cells present in the tunnels are F4/80⁺ MC/Mph (brown, **arrows**). **C:** Mph-lined, capillary-like structures (**arrow**) detected at 1 week in Matrigel plugs in Tie2-βGal mice (no Tie2-βGal-positive cells present). Note the branching (**arrowheads**) and the F4/80⁺ cells in the lumen. **Inset:** Enlargement of the F4/80⁺ cell marked with **dashed lines**, showing a lumen-like vacuole and a low-density tunnel in Matrigel (**arrowhead**). **D:** Negative control for the F4/80 immunostaining (omission of the primary antibody). The cell columns line a collagen bundle cut longitudinally, visible in the background of this phase contrast micrograph (**arrow**, compare with Figure 3A). In this field, a Tie2-βGal-positive cell (light blue) is wrapped by another cell (**arrowhead**, compare with Figure 4, D and E). Counterstaining with hematoxylin. Original magnifications, ×120.

Results

In Vivo Observations

MC/Mph Form Tunnels and Cell Columns in Matrigel

We have previously shown that MC/Mph migration in solid tissues induces formation of long-lasting tracks of lower density that we called “tunnels.”³ Further, we demonstrated that tunneling of extracellular matrix leads to formation of cell columns that *in vivo* contain MC/Mph and various types of progenitor cells.⁴² Here, we examined how the formation of these cell columns contributes to the development of new, patent blood conduits. To this aim we used mice expressing LacZ under the endothelial Tie2 promoter as hosts for subcutaneous Matrigel implants.⁵⁶ To control the orientation (otherwise random) and amount of cellular infiltration (otherwise excessive) of the plugs, we used implanted chambers filled with bFGF-supplemented Matrigel and lined on one side by a polycarbonate filter with pores of 8 μm in diameter.⁴²

The chambers were collected after 1 week and analyzed for structural features of the gel and for cellular content. Among the cells that crossed the filter, we identified a population that accumulated just beneath it but did not enter the gel (Figure 1A). Only a subpopulation of cells migrated at intermediate levels, in pockets surrounded by acellular or sparsely populated zones (Figure 1A). These cells were primarily F4/80⁺ MC/Mph (Figure 1B). Structurally, the gel near the filter displayed a network of empty spaces (tunnels), of approximately a sin-

Figure 2. Cellular organization and vascularization of subcutaneous Matrigel plugs in Tie2-βGal mice. Matrigel plugs containing bFGF (**A**, **C**, **D**) or VEGF-165 and SDF-1α (**B**) retrieved after 4 weeks from Tie2-βGal mice, sectioned, and stained for LacZ (light blue) and counterstained with H&E (**A**, **B**, **D**) or for the Mph marker F4/80 (brown) (**C**). **A:** Massive adipogenesis throughout the whole plug. **B:** Adipocytes and blood vessels are missing in the plug, and the overall infiltration is reduced. The lower portion in **A** and **B** is the attached tissue. **C:** Adipocyte cluster containing microvessels positive (**arrow**) and negative (**arrowhead**, containing erythrocytes) for Tie2-βGal. **D:** Section running obliquely through microvessels that are Tie2-βGal-positive (**arrow**) or -negative (**arrowhead**, containing pale erythrocytes). Note the oriented distribution of rows of adipocytes (**asterisks**), flanked by F4/80⁺ Mph. Hematoxylin counterstaining. Original magnifications: ×40 (**A**, **B**); ×120 (**C**, **D**).

gle-cell diameter. These features were associated only with the infiltrating cells and were restricted to the rear of the leading front of cell invasion (Figure 1, A and B).

We also injected subcutaneously Matrigel plugs that were retrieved after 1 week. At this time point, the cell density was much higher than in the corresponding chambers (not shown). Because of fixation, extraction, and paraffin-embedding, and lacking the mechanical support of the filter,⁴² the tunnels were no more directly seen as empty spaces in gel. However, we found cell columns displaying a F4/80⁺ MC/Mph lining. These structures were distantly similar to capillaries, due to the arrangement of the cells and the presence of a lumen and of ramifications (Figure 1C). At this time, these structures did not contain Tie2-βGal⁺ cells or erythrocytes, although they contained within the lumen F4/80⁺ mononuclear cells (Figure 1C). Branches with circular cross-section emerged from these structures (Figure 1C), making unlikely that they are mere flat sheets of cells. Occasionally, F4/80⁺ cells displayed lumen-like vacuoles and were associated with the tunnels (Figure 1C; compare with Figure 4).

Figure 3. Lipid accumulation and formation of MC/Mph-lined fibrovascular bundles in bFGF-supplemented Matrigel plugs (4 weeks old). **A:** Nile Red-stained lipid droplets (arrows) in a Matrigel cryosection. **B** and **C:** Structures containing collagen in the Matrigel plug (Masson Trichrome staining). **B:** Cell column containing fibrillar collagen (arrow, light blue) and an adipocyte (asterisk). Note the bundle branching (arrowhead) and other adipocytes that align with the main bundle (asterisks). **C:** Cells present inside the collagen bundles (arrows); the upper structure displays a lumen. **D–F:** Anti-F4/80⁺ immunostaining of the Matrigel plug. **D:** F4/80⁺ MC/Mph distributed in a capillary-like pattern (arrows). Note the Tie2-βGal⁺ cell aggregate without a lumen (arrowhead, light blue). **E:** Tie2-βGal⁺ cell column inside a collagen bundle lined by F4/80⁺ Mph (arrow). Other Mph-wrapped collagen bundles are nearby (arrowheads). **F:** Erythrocytes present in a Tie2-βGal⁺ microvessel, within a collagen bundle lined by F4/80⁺ cells (arrow). **G:** Erythrocytes inside a collagen bundle (arrow) (H&E staining). **H** and **I:** Anti-smooth muscle α-actin immunostaining. **H:** α-Actin-expressing cell within a cell-lined collagen bundle. **I:** α-Actin-positive cells surrounding a Tie2-βGal⁺ cell aggregate (light blue) without a lumen (arrow). Note the presence of a nearby actin-positive single cell (arrowhead). **B–I:** Hematoxylin counterstaining. Original magnifications: ×80 (**A**); ×120 (**B**, **D**); ×200 (**C**, **E–I**).

MC/Mph Surround Microvessel-Containing Collagen Bundles

Other Matrigel plugs were retrieved after 4 weeks. When supplemented with bFGF, these plugs presented massive cellular infiltration, lipid accumulation (Figure 2A and Figure 3A, for the presence of Nile red-positive lipid vesicles), and vasculogenesis (Figures 2 and 3). In contrast, VEGF and SDF-1, alone (not shown) or in combination (Figure 2B), as well as the nonsupplemented control plugs (not shown), did not stimulate cell infiltration, lipid accumulation, or angiogenesis.

Given that the Matrigel plugs were retrieved from Tie2-βGal-expressing mice, we expected that the blood-containing conduits to be LacZ-positive. However, in these long-term plugs we often found functional blood conduits that were either Tie2-positive or Tie2-negative (Figure 2D). The same labeling pattern for Tie2 was found in the nearby tissue. Proximally to the skin, mature adipocytes (each containing a single large lipid droplet) occurred in clusters, associated with Tie2-positive and Tie2-negative blood conduits (Figure 2C). Within the plugs the adipocytes were often aligned in rows (Figure 3A) running in parallel with the microvessels (Figure 2D) and with collagen bundles intermeshed with cells (Figure 3B). Occasionally, the adipocytes were found incorporated into these bundles (Figure 3B).

Figure 4. Cellular phenotypes at the distal margin of a Matrigel plug 4 weeks after implantation. **A:** F4/80⁺ cell with vacuole (arrow) and one displaying a fibroblastic morphology (arrowhead), in a field containing immature adipocytes (asterisks). **B:** F4/80⁺ MC/Mph displaying vacuoles (arrows) or lumen (arrowhead), and immature adipocytes (asterisks). **C:** F4/80⁺ Mph presenting a large vacuole (arrow) comparable to the size of a Tie2-βGal⁺ capillary lumen (arrowhead, light blue). **D:** Intimate apposition between an F4/80⁺ Mph (arrow) and a Tie2-βGal⁺ cell without lumen (arrowhead, light blue). **Inset:** Tie2-βGal⁺ cell within an F4/80⁺ lumen. **E:** F4/80⁺ Mph wrapping cells that are Tie2-βGal-positive (arrowhead, light blue). **F:** Erythrocytes present in a lumen lined by F4/80⁺ MC/Mph (arrow and inset). Also, note the presence of an erythrocyte in an F4/80⁺ structure (arrowhead). Hematoxylin counterstaining. Original magnifications: ×120 (**A**, **B**, **F**); ×200 (**C**, **D**, **E**).

Surprisingly, many of the collagen bundles were wrapped by cells, producing in cross-section the pattern of a microvascular field (Figure 3C). The bundle-covering cells, disposed in a circular distribution reminiscent of capillaries, were F4/80⁺ MC/Mph (Figure 3, D–F; Figure 4D). Their morphology was similar to those already presented in oblique sectioning in the 1 week-old plugs (Figure 1C). At the core of some collagen bundles surrounded by Mph rings, we detected tubular structures (Figure 3, C, F, G), as well as *bona fide* capillaries containing erythrocytes or other cells (Figure 3, F and G; and Figure 5A). Some were Tie2-βGal⁺ cell columns without a well-formed lumen (Figure 3, D, E, I), but we also found many Tie2-βGal⁺ blood-containing microvessels. The heterogeneous distribution of Tie2-βGal was similar to that presented in Figure 1D. Remarkably, we never observed so far Tie2-βGal-positive and -negative microvessels within the same collagen bundle.

Inside the bundles, we detected few cells expressing α-smooth muscle actin (Figure 3H). Outside the bundles, although mature microvessels resembling arterioles and venules were labeled for smooth muscle actin, the frequency of isolated cells positive for this marker was low

Figure 5. Connectivity of the fibrovascular bundles developing in Matrigel plugs with the main blood circulation. **A:** Erythrocyte-filled microvessel (arrow) found at the core of a fibrovascular bundle. **B:** Erythrocytes (arrow) and dense India ink (carbon particles, arrowheads) aggregates present in a fibrovascular bundle at the interface of Matrigel plug (top right) with the tissue (left side of the image). **C:** India ink-filled microvessel in a fibrovascular bundle found deeper in the Matrigel plug. **D:** Uptake of carbon particles in a phagocytic cell (arrowhead), close to an India ink-filled space inside a fibrovascular bundle (arrow). Original magnifications: $\times 200$ (A, B, D); $\times 120$ (C).

(Figure 3I). We also detected tight aggregates (or columns) of Tie2- β Gal⁺ cells without a lumen but surrounded by an α -actin-positive sheath (Figure 3I).

MC/Mph in Recently Infiltrated Regions of Matrigel Plugs Display Lumens

Analysis of 4-week-old Matrigel plugs in the deepest places reached by the cellular front (and thus, the most recently colonized) revealed that in these regions the adipocytes were immature (Figure 4, A and B). This as-

sumption is based on the presence of multiple small lipid droplets, instead of the single lipid droplet typical for the mature adipocyte.⁵⁷ The other cell types present in these fields also displayed peculiar features, absent in cells from the regions of Matrigel plug where the adipocytes were fully developed: 1) The majority of F4/80⁺ cells were isolated, not assembled in cords or rings, and many contained vacuoles suggestive of a *trans*-cellular lumen (Figure 4, A–F). 2) Some of the F4/80⁺ cells had a fibroblastic aspect (elongated and with long, undulated processes) (Figure 4A). 3) Tie2- β Gal⁺ cells without lumen were present in an intimate apposition with, or encircled by, F4/80⁺ Mph (Figure 4, D and E). However, we did not find any morphological evidence of phagocytosis (internalization of the cells of interest in F4/80⁺ cells) or apoptosis (fragmented nuclei, apoptotic bodies). Most often, the lumen was created by contribution of multiple MC/Mph (Figure 4, E and F), further ruling out that the erythrocytes encircled by MC/Mph are phagocytosed.

The most remarkable finding, in our appreciation, is the presence of erythrocytes in spaces lined by F4/80⁺ MC/Mph (Figure 4F). The labeling was heterogeneous, with some of the participating cells forming the lumen being F4/80-negative (Figure 4F). Similar observations regarding MC/Mph-forming lumens (Figure 2C) and immature adipocytes⁴² were made in the 1-week-old Matrigel plugs.

Fibrovascular Conduits Are Connected to the Main Circulation

To verify that the blood conduits detected within fibrovascular bundles are connected to the animal's circulatory system, we perfused the mice, before collection of Matrigel plugs, with India ink (a suspension of carbon particles visible in histological specimens).⁵⁸ In 4-week-

Figure 6. Coupling of matrix degradation with lumen formation in MC/Mph. **A–C:** High-resolution histology of peritoneal Mph embedded in Matrigel and exposed to a MCP-1 gradient for 24 hours. **A:** Ruffled zone and secretion granules at one pole of the cell (arrow). **B:** Large intracellular vacuole (arrow); arrowhead, the nucleus. **C:** Tunnel (arrow) half-covered by an Mph cytoplasmic fold that contains an array of dense intracellular granules (arrowhead). **D–F:** Virtual sectioning through a three-dimensional reconstruction of a pair of CTG-labeled Mph, embedded in Matrigel containing DQ Red BSA, for 24 hours. **D:** Proteolysis was observed closest to the source of MCP-1 (arrow). **E:** Distribution of CTG fluorescence in Matrigel-embedded Mph: the right-hand cell displays a vacuole or a lumen (arrow). **F:** Overlay of the red (DQ BSA) and green (CTG) fluorescence channels. Note the presence of degradation product in the empty space (arrowhead). **G–J:** DQ Red BSA proteolysis and Mph morphology after 5 days in Matrigel. **G:** Lateral view of a three-dimensional reconstruction of optical sections (see also Movie 1, Supplemental data, at <http://ajp.amjpatbol.org>). The dashed lines indicate where the planes in **H** and **I** were taken. **H:** Virtual section through the image in **G**. **I:** Frontal section of the matrix modification in **G**, showing proteolytic degradation (red) of a size similar to a single cell diameter (the tunnel). **J:** Mph displaying a lumen and containing large amounts of intracellular proteolysis product, both on the inner face of the lumen (arrow) and within plasmalemmal vesicles (arrowhead). Scale bars: 10 μ m (D–F); 10 μ m (G–I); 5 μ m (J). Original magnifications: $\times 200$ (A–C).

old plugs, we found fully developed fibrovascular bundles, many having at their core erythrocyte-containing microvessels (Figure 5A). In these fibrovascular bundles we also found abundant India ink perfusion with central localization within bundles (Figure 5, B and D). At the interface of the plug with the tissue, we found emerging fibrovascular bundles that contained both erythrocytes and ink (Figure 5B). Deeper into the gel, the lumen-bearing structures were less well developed, but still displayed clear labeling with carbon particles (Figure 5, C and D). The finding of particles internalized in cells with Mph features placed in tunnels (Figure 5D) rules out the possibility that the carbon particles were relocated from the nearby tissue during sectioning.

In Vitro Observations

Matrix Degradation by Mph Is Coupled with Trans-Cellular Vacuole (Lumen) Formation

The presence of lumen-bearing MC/Mph raised questions on the mechanism of their formation and on their role in microvessel generation. In light of the previously noted potential of MC/Mph to acquire EC properties,⁵⁹ we were particularly interested in replicating this phenomenon *in vitro* and understanding its mechanism. We conducted experiments with purified mouse peritoneal Mph embedded in Matrigel exposed to an MCP-1 chemotactic gradient, as described in Materials and Methods.

First, we examined the cellular morphology by high-resolution histology on resin-embedded samples and semithin sectioning. In these specimens, we found polarized cells with ruffled zones and secretion granules at the cellular poles facing the dissolute gel (Figure 6A). In a subpopulation of cells, we also detected large, empty intracellular vacuoles (Figure 6B). We also found cytoplasmic folds containing a dense array of intracellular granules, which extended and halfway covered a nearby tunnel (Figure 6C).

This pattern was suggestive of a tubular structure of the whole cell, ie, a *trans*-cellular lumen. To test further this possibility, we fluorescently labeled isolated peritoneal Mph and incorporated them into Matrigel containing DQ Red BSA (a fluorescent substrate for proteases). The cells were stimulated to migrate through the gel by a horizontal chemotactic gradient of MCP-1. We analyzed cells in this setting by confocal microscopy and three-dimensional reconstruction at different time intervals. At 24 hours, we found an active proteolytic activity surrounding the cells in the direction of migration (Figure 6, D and I). Interestingly, in a pair of adjacent cells, only that facing the source of the gradient displayed proteolytic activity (Figure 6, D and F). The cell facing higher MCP-1 concentrations and displaying abundant pericellular proteolysis product also presented a vacuole in cross-section, suggestive for a tubular shape (Figure 6E). The proteolysis product was present not only around cells but also within this luminal space (Figure 6, D and F). This association of cells in a tunnel was reminiscent of the

Figure 7. Factors controlling the chemotactic migration of peritoneal Mph and Matrigel proteolysis. Mph chemotaxis through a Matrigel-coated filter in response to MCP-1 (100 ng/ml), VEGF (10 ng/ml), or MCP-1 plus folinmycin (50 nmol/L). **P* < 0.05; NS, not significant.

intimate apposition of F4/80⁺ MC/Mph with Tie2-βGal⁺ EPCs (Figure 4, D and E).

We further confirmed the previous observations by reconstituting the three-dimensional morphology of fluorescently labeled MC/Mph in Matrigel after 5 days. The cytoplasm of many cells contained large vacuoles, but a subset of up to 10% of them displayed an evident cylindrical shape, with the length approximately threefold larger than the diameter (Figure 6, G–I, and Supplemental data, Movie 1, at <http://ajp.amjpathol.org>). The matrix surrounding these cells was again degraded asymmetrically, the proteolysis product being concentrated at the advancing cell edge, aligned with the increasing MCP-1 concentration (Figure 6, G and I). Occasionally, the red fluorescent proteolysis product was found aggregated inside the lumen (Figure 6H and Movie 1, at <http://ajp.amjpathol.org>, in accord with Figure 6D) or within intracellular vesicles (Figure 6J).

To exclude the possibility that the lumen was an artifact of nonhomogenous distribution of the fluorescent tracer, we repeated the experiments using Mph obtained from mice with ubiquitous expression of eGFP or expressing YFP under the Mph-specific *fms* promoter.⁴⁷ Both cell types had similar responses to the presence of MCP-1 gradient, including polarized proteolysis and lumen formation (not shown).

Molecular Control of Matrix Degradation and Lumen Formation in MC/Mph

To understand the factors that regulate extracellular matrix degradation by MC/Mph and lumen formation, we quantified the chemotactic migration of MC/Mph across a Matrigel layer placed on top of a porous polycarbonate filter. We found that although MCP-1 significantly stimulated gel penetration, VEGF (for which MC/Mph also have receptors⁶⁰) did not amplify MC/Mph migration across Matrigel (Figure 7A).

Because we found degradation products within cells as well (Figure 6J), we questioned whether inhibition of

Table 1. Semiquantitative Assessment of Proteolytic Activity of Mouse Peritoneal Mph in Matrigel towards DQ Red BSA in Response to a MCP-1 Gradient (Starting at 100 ng/ml)

Score	Control	+TIMP1	+Anti-MME
0	1	4	11
1	8	16	12
2	6	3	3
3	7	4	1
4	5	0	0

Three experiments were pooled for statistical analysis. Note that the number of cells with higher proteolysis scores was significantly decreased with the addition of inhibitors (for both, $P < 0.02$ versus control).

the lysosomal function would interfere with lumen formation and cell advancement. We included in the chemotactic experiments the lysosomotropic factor folimycin, an inhibitor of pericellular proteolysis via plasma membrane vacuolar-type ATPase.⁶¹ Folimycin strongly inhibited cellular migratory response in a 24-hour chemotaxis assay through the Matrigel-coated filter (Figure 7). Comparable results were obtained with the similar compound bafilomycin (not shown).

We also sought to confirm in our system the contribution of Mph-derived MMP-12 activity to tunneling, because we previously found that MMP-12 is produced during MC/Mph tunneling *in vivo*.^{9,41} We assessed semiquantitatively the degradation of DQ Red BSA, in the presence or absence of an anti-MMP-12 antibody or of TIMP-1, an inhibitor of MMP-12 and other metalloproteases.⁶² The surface proteolysis was significantly reduced in the presence of MMP-12 antibody and TIMP-1, as compared to the control (Table 1).

Discussion

We found that MC/Mph infiltrating Matrigel *in vitro*⁴² and *in vivo*^{9,41,42} organize in cell columns, and we proposed that this is the effect of the local dissolution of extracellular matrix in a tubular pattern (tunneling). We suggested that these cell cords could further evolve into capillary-like structures^{3,63} and control neovascularization,⁶⁴ depending on the presence among their components of cells with progenitor capabilities. The current study was designed to test this hypothesis.

To this end, we induced and analyzed the process of cellular infiltration of Matrigel plugs placed subcutaneously in mice for long intervals (up to 1 month), with emphasis on the role of MC/Mph. Matrigel plugs are frequently used to study neovascularization for brief intervals (up to 2 weeks) in adult animals. However, few studies addressed the details of cellular composition of the infiltrate and its fate,³⁵ or that of early microvascular structures.

For detection of MC/Mph *in vivo*, we chose the F4/80 antigen, one of the most accurate indicators of MC/Mph lineage, both in their circulating (MC) as well as tissue (Mph) stages.⁵⁰ Another advantage of this marker is its availability for detection with antibodies for paraffin his-

tochemistry, the best available approach for preserving the morphology of the soft extracellular matrix and that of the cells. For characterization of the EC lineage, we used transgenic mice expressing β -galactosidase under control of the Tie2 promoter (Tie2- β Gal⁺).⁴⁵ Tie2 is the angiopoietin tyrosine kinase receptor, whose expression is thought to be primarily restricted to adult ECs,⁴⁵ but is also a defining molecule of endothelial commitment in EPCs.^{20,65}

Our data show that the Matrigel plugs are the site of an intense neovascularization coupled to (and, we suggest, driven by) *de novo* formation of the adipose tissue, rather than sole consequence of angiogenic stimulation.⁶⁶ Although bFGF is an angiogenic factor in itself,^{67,68} the weaker potency in our model of either VEGF,⁶⁹ SDF-1,⁷⁰ or both to stimulate Matrigel colonization and formation of blood vessels, indicates that beyond angiogenesis there are other processes involved (such as secretion of angiogenic and/or differentiation factors by the other infiltrating cells). On this line, inhibition of adipocyte differentiation by transfection of preadipocytes with a peroxisome proliferator-activated receptor gamma (PPAR γ) dominant-negative construct did not only abrogate fat tissue formation, but also reduced angiogenesis.³⁴

Our data suggest that the ordered pattern noted for the adipocyte clusters, and of their blood supply, reflects the initial distribution of their progenitors. These progenitors would come into Matrigel along with other components of the initial cellular infiltrate, consisting predominately of MC/Mph. Their distribution is in the form of cell columns that we previously found to be present after 1 week,⁴² and here even after a month.

The origin of the lipid droplets found in Matrigel, assessed both by regular histology and by direct labeling with the lipophilic stain Nile Red, was assigned in previous studies to the accumulation of adipocytes, likely to derive from progenitor preadipocytes, as an effect of bFGF stimulation.^{35,71} This was considered a demonstration that an abundant population of adipose precursor cells is present in connective tissues of adult animals and that they migrate to neovascularization sites (such as Matrigel plugs), for proliferation and maturation. The possibility that these cells are foam cell-like (lipid-loaded Mph) is unlikely, due to the absence of hyperlipidemia or of exogenous lipids in our model and to the dependence of their occurrence on bFGF, as shown by the control plugs placed in the same animals.

The columnar distribution of the adipocytes, and their co-localization within fibrovascular bundles described here, indicates their origin in the circulation, along with other inflammatory cells. In support, a very recent study indicates that the same CD14⁺CD34^{+(low)} monocytes behaving as EPCs (a subpopulation that could amount to up to 8.5% of all leukocytes) could generate in culture adipocytes, osteoclasts, or neuronal cells, arguing for their multipotency.⁷²

In the adipose-like tissue that develops in Matrigel plugs, we found large amounts of fibrillar collagen, apparently produced by fibroblasts derived from circulating fibrocytes.⁷³ Collagen was organized mostly in bundles of various diameters, the larger ones having a fairly ho-

mogenous cross-section. Unexpectedly, this collagen was often wrapped in MC/Mph sheaths, producing in cross-section the false aspect of a microvascular field. Longitudinal sections revealed these to correspond to F4/80⁺ MC/Mph disposed alongside the collagen bundles.

At the core of many (but not all) Mph-lined collagen bundles, we indeed found various stages of microcirculation development. For this reason we used for their naming the previously coined term of "fibrovascular bundles."⁷⁴ However, the smallest recognizable units, even before collagen was present in their proximity, were lumens containing erythrocytes and formed by few F4/80-positive cells. This cooperation of several cells to the formation of a lumen excludes the possibility that what we saw were in fact erythrocytes engulfed by MC/Mph. We could also exclude the likelihood of an overstaining artifact producing false-positives, because many F4/80⁻ cells were also present in the same microscopic fields. The omission of the primary antibody always eliminated the immunostaining. Moreover, these cells did not express Tie2, as expected for capillaries sprouting from the local microcirculation of the Tie2- β Gal⁺ mice bearing the plugs. Previously, F4/80⁺ cells presenting endothelial markers were described in the bone marrow, probably a population of EPCs analogous to that revealed by our study.⁷⁵

Next, inside the F4/80⁺ lumens composed of single or multiple cells we found isolated Tie2- β Gal⁺ cells without a lumen of their own. Based on the published evidence on the expression of Tie2 in EPCs,²⁰ we believe that these are indeed EPCs. We also detected Tie2- β Gal⁺ cellular cords encircled by MC/Mph, a stage described in the progression of Tie2- β Gal⁺ EPCs to functional capillaries.⁷⁶ The Tie2- β Gal⁺ cell columns are hallmarks of angiogenesis⁷⁷ and of EPC-driven vasculogenesis,⁷⁶ but their relationships with other cell types was not previously described. In our study collagen fibers at this stage were interposed between the wrapping MC/Mph and the EPCs.

Another remarkable finding was that the blood vessels present in these MC/Mph-wrapped collagen bundles (as well as those apparently free in the interbundle space) were either Tie2- β Gal-positive or Tie2- β Gal-negative. This heterogeneity in Tie2 expression, noted previously,⁷⁸ was also present in the nearby muscle and in the smallest erythrocyte-containing, free (not bundle-associated) capillaries in Matrigel. We tentatively assigned to these microvessels an arteriolar and venular nature, respectively, based on similar findings in the quiescent and angiogenic mesenteric fat and diaphragm.⁷⁹ We found most of the erythrocytes in Tie2- β Gal⁻ vessels, whereas the Tie2- β Gal⁺ cords either were collapsed or did not yet have a vascular lumen. In addition, so far we did not find Tie2- β Gal-positive and -negative microvessels within the same fibrovascular bundle, which suggests a separate origin of these structures.

The functionality of the bundle-associated microvessels was directly assessed by infusion in systemic circulation of a suspension of carbon particles (India ink). These particles were readily detected after 5 minutes of

perfusion at the center of many bundles, displaying various degrees of microvascular organization. These data supplement the concurrent finding of erythrocytes at the bundle's core. However, it is worth noting that erythrocytes were also present in collagen-free microvessels, intermixed with, or segregated from, the fibrovascular bundles. This pattern illustrates the complexity of the neovascularization process in which classical angiogenesis and cell column-derived fibrovascular bundles might co-exist.

The collagen bundles lined by MC/Mph contained smooth muscle precursors and/or myofibroblasts (isolated smooth muscle actin-positive cells) and F4/80⁻ cells. We consider the latter to be more mature fibrocytes as well as preadipocytes. In infiltrates of Matrigel plugs collected at 1 week, we previously found cells with features of F4/80⁺ preadipocytes.⁴²

In close resemblance of these MC/Mph-limited collagen bundles containing neovessels are the basement membrane-like structures containing collagen, laminin, Mph, and fibroblasts, which assist neurogenesis and vasculogenesis in the brain.¹² These structures have been called "fractones" for their branched distribution.⁸⁰

In the Matrigel fields presenting recent cellular infiltration (as those found at 1 week), MC/Mph displayed *trans*-cellular lumens. In 4-week-old plugs at the distal margins of the gel that contained immature adipocytes indicative of recent infiltration, F4/80⁺ cells also lined lumens containing erythrocytes. This pertains to the often mentioned but poorly documented propensity of adult MC/Mph to behave like endothelium, supposedly via *trans*-differentiation.^{17,24,81} Alternatively, this could be a case of vascular mimicry, similar to that undergone by cancer cells or other cell types,^{22,82} thought to be at the foundation of a more general process of functional adaptation.⁸³ However, we cannot exclude the possibility of cell fusion, another proposed mechanism that would explain the appearance of mixed phenotypes among different classes of cells.⁸⁴

We carefully examined Matrigel penetrated by MC/Mph both *in vivo* and *in vitro* for more evidence of lumen formation. *In vivo*, we limited the infiltrate in Matrigel to those cells able to cross the porous filter of a chamber inserted subcutaneously in mice. This approach confirmed that MC/Mph are indeed the cells that penetrate the gel and leave behind the marks of their passage as tubular, low-density domains (tunnels). Then we stimulated *in vitro* the migration of peritoneal MC/Mph in Matrigel containing a protease substrate that becomes fluorescent on proteolytic cleavage. Using confocal microscopy and high-resolution histology, we confirmed both formation of tunnels and the adoption of a cylindrical shape by many cells, consistent with a *trans*-cellular lumen. We also demonstrated that migration of MC/Mph in Matrigel is stimulated by MCP-1, but not by VEGF, and is metalloprotease-dependent. Consistent with others⁸⁵ and our previous immunohistochemical observations *in vivo*,^{9,41} we confirmed the role of MMP-12 in matrix degradation, although it is likely that other protease systems also contribute to MC/Mph migration in Matrigel. The strong inhibitory effect of folimycin (also known as

Figure 8. Diagram illustrating the proposed stages of conversion of MC/Mph-based cell columns into microvessels containing tissular units. Color code: brown, F4/80⁺ Mph and F4/80⁺ fibrocytes; blue, Tie2-βGal⁺ EPC/EC; light green, Tie2-βGal⁻ EPC/EC; yellow, (pre)adipocyte; orange, smooth muscle/pericyte precursor; pink, fibroblasts; red, erythrocytes; **black lines**, collagen. The numbers indicate figures in which these cells were described. Note: Because Tie2-βGal-positive and -negative microvessels were never found in the same fibrovascular bundle, separate EPC precursors of arteriolar (Tie2-βGal⁺) and venular (Tie2-βGal⁻) endothelium could be hypothesized.

concanamycin A), an inhibitor of vacuolar and plasma-membral type ATPase involved in EC angiogenic phenotype⁶¹ and in bone-drilling by osteoclasts,⁸⁶ indicates that intra- and pericellular procession of the gel is required for Matrigel penetration.

The polarized matrix dissolution and stepwise development of Mph-generated intracellular vacuoles, culminating with formation of *trans*-cellular lumen, is remarkably similar to lumen formation in ECs.⁸⁷ The asymmetrical pericellular proteolysis found in our model is reminiscent of that shown for migration-associated proteolysis in other cellular systems.⁵⁵ Analogous mechanisms of cell invasion have been described for leukocytes and tumor cells penetrating matrix,⁸⁸ or osteoclasts degrading and invading bone.⁸⁶ Moreover, Mph in our *in vivo* experimental model not only formed a lumen but also generated branching patterns, supporting the recent suggestion that Mph could control the branching of capillaries.¹¹

In conclusion we propose that the descendants of various progenitor cells do not differentiate separately but are co-localized first in MC/Mph-drilled tunnels as cell columns, then in collagen-limited bundles, and eventually in local tissue elements (linear arrays of adipocytes in our experiments, but also nerve cells, and so forth). These are, thus, naturally organized around, and derived from, the initial cell column, which would leave its mark on the tissue architecture. This cellular logic, derived from the spatio-temporal process of tissue infiltration, secures that the newly regenerated portion of tissue develops in parallel with the appropriate vascular supply. Moreover, a type of provisional, percolation-type microcirculation could be established locally to serve the needs of the infiltrating cells, even before true neovascularization is completed, as we suggested before.⁴² Thus, neovascularization can be also considered the consequence of tissue regeneration, not only its prerequisite. This would explain why bFGF functions *in vivo* as an angiogenic factor, whereas the sole addition of angiogenic stimulants

such as VEGF and SDF-1 did not induce vasculogenesis in our Matrigel plugs.

Our proposed model is presented in Figure 8. In essence, it consists in the conversion of a cell column produced by infiltrating MC/Mph, in either an arteriolar or venular microvessel, in the proximity of the new tissue elements destined to be fed. In this process the collagen-producing fibrocytes strengthen the cell column through formation of the collagen bundle delimited by MC/Mph. Thus, formation of a microvascular adventitia⁸⁰ would precede the development of the future neovessel. Collagen acts as a potent proangiogenic milieu in itself,⁸⁹ whereas matrix-associated fibroblasts lead the orientation of new blood vessels.⁹⁰

Our study clarifies the contribution of MC/Mph to vasculogenic regulation, which consists of: 1) patterning of the tissue space by local degradation of the extracellular matrix; 2) facilitating the penetration of progenitor (including vascular) cells via their tunneling activity, which determines a cohort-type cellular co-migration; and 3) assisting the conversion of cell columns containing progenitor cells in tissue structures, organized around the vascular elements. These principles might be applied to the understanding of developmental processes in embryological and adult tissues.

The clinical implications of our observations are multiple, expectedly covering many cases of neovascularization involving circulating progenitor cells. For example, a tunneling-based mechanism could be the basis of recanalization of thrombi, as we recently suggested.⁶³ Moreover, the vascularization of tumors is known to rely on the formation of a fibrin-based provisional matrix⁹¹ as well; in this instance, the only classical angiogenic mechanism convincingly demonstrated so far is vascular remodeling.⁹² However, the tumors harbor a large population of vascular leukocyte-derived microvessels, whose engraftment could take place via the tunneling-type mechanism described here. Thus, our results indicate that, besides the endothelium and its progenitors, the cells of the MC/

Mph (formerly reticulo-endothelial¹⁶) system should be included as targets of pro- and anti-angiogenic therapies.

Acknowledgments

We thank A. Bakaletz for help with the confocal microscopy; K. Wolken for electron microscopy; O. Butt for tissue procession; Dr. C. Barbacioru for statistical analysis; and Dr. S. Shapiro, Harvard University, Boston, MA, for the kind donation of MMP-12 neutralizing antiserum.

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