A subpopulation of peritoneal macrophages form capillary-like lumens and branching patterns *in vitro*

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Abstract

Objective: We have previously shown that monocytes/macrophages (MC/Mph) influence neovascularization by extracellular matrix degradation, and by direct incorporation into growing microvessels. To date, neither the phenotype of these cells, nor the stages of their capillary-like conversion were sufficiently characterized. **Methods:** We isolated mouse peritoneal Mph from transgenic mice expressing fluorescent proteins either ubiquitously, or specifically in the myelocytic lineage. These Mph were embedded in Matrigel which contained fluorescent protease substrates, exposed to an MCP-1 chemotactic gradient, and then examined by confocal microscopy after various intervals. **Results:** Within 3 hrs after gel embedding, we detected TIMP-1 and MMP-12 dependent proteolysis of the matrix surrounding Mph, mostly in the direction of high concentrations of MCP-1. After 2 days, Mph developed intracellular vacuoles containing degradation product. At 5 days these vacuoles were enlarged and/or fused to generate trans-cellular lumens in approximately 10% of cells or more (depending on animal's genetic background). At this stage, Mph became tubular, and occasionally organized in three-dimensional structures resembling branched microvessels. **Conclusion:** Isolated mouse peritoneal Mph penetrate Matrigel and form tunnels *via* a metalloprotease-driven proteolysis and phagocytosis. Following a morphological adjustment driven by occurrence, enlargement and/or fusion process of intracellular vacuoles, similar to that described in bona fide endothelium, a subpopulation of these cells end up by lining a capillary-like lumen *in vitro*. Thus we show that adult Mph, not only the more primitive 'endothelial progenitors', have functional properties until now considered defining of the endothelial phenotype.

Keywords: monocytes/macrophages • endothelial • lumen formation • intracellular vacuole • functional adaptation

Introduction

Circulating or bone marrow-derived mononuclear cells were previously shown to acquire endothelial

properties *in vitro* [1, 2] and *in vivo* [3]. In addition, inflammatory cells induced in the peritoneal cavity and which express the leukocyte marker CD18 [4], could also display endothelial markers when exposed to blood flow [5, 6]. Moreover, macrophages (Mph) were recently found to incorporate in the developing lymphatic microvessels [7] and to regulate, through secretion of metalloproteases, the branching of blood capillaries [8].

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Evidence for direct incorporation of mononuclear phagocytes in endothelial structures was also provided for tumors [9].

Recently, in Matrigel plugs implanted subcutaneously in mice for a week, we found F4/80 positive monocytes/macrophages (MC/Mph) organized as branched cell columns, and as capillary-like structures [10]. We also showed that after a month of implantation, MC/Mph may incorporate in the lumen of functional blood conduits, and in the lumen-forming cellular sheaths of fibro-vascular bundles containing microvessels [11]. However, the sequence of events leading to these cellular transformations is largely unknown [12, 13].

In the current study, we scrutinized the cellular mechanism of conversion of Mph to a tubular morphology *in vitro*. In this regard, we examined the matrix degradation by Mph and their intracellular processes leading to acquisition of a lumen. To this end, we used intrinsically labeled mouse peritoneal Mph and Matrigel, as cellular and extracellular matrix models, respectively. Three-dimensional reconstitutions of confocal microscopy images of live cells were used to simultaneously assess penetration of Matrigel by Mph *in vitro*, as well as their proteolytic activity, along with the associated cellular features. Our data show that indeed a subpopulation of peritoneal Mph adopt an endothelial pattern, and that the lumen generation is based on intracellular vacuole formation, tightly coupled with, although distinct from, endocytosis of ingested extracellular matrix.

Materials and methods

Animals

For isolation of thioglycollate-elicited peritoneal Mph, we used 4–6 weeks old C57BL/6-Tg(ACTbEGFP)10sb/J transgenic mice expressing an enhanced green fluorescent protein (eGFP) under the control of chicken beta-actin promoter and cytomegalovirus enhancer [14], as well as C57BL/6J control (non-labeled) mice, purchased from Jackson Laboratories. We derived peritoneal Mph from a transgenic mouse line expressing Yellow Fluorescent Protein

(YFP) under the *fms* (M-CSF) promoter, created as previously described [15]. All experiments were performed in accordance with the guidelines of the Committee for Animal Research of the Ohio State University.

Isolation of Mph

Mice were injected i.p. with 1.5 ml of 2.9% thioglycollate broth (Sigma, St Louis, MO) three days before the experiment. Peritoneal Mph were obtained by lavage of the peritoneal cavity with 3 x 5 ml cold phosphate buffered saline (PBS), purified by adherence on plastic for 1 h at 37°C, and then detached in cold PBS for 5 min. The cells were labeled while attached, with 1 mM of either Cell Tracker Green (CTG) or Cell Tracker Red (CTR) (Molecular Probes) for 20 min at 37°C, according to manufacturer's instructions. eGFP labeled Mph were used without further staining. YFP-Mph were elicited similarly, but were used without a purification step.

In vitro tunneling assay

Matrigel (BD Biosciences) was supplemented with DQ Red bovine serum albumin (BSA) or DQ Green Collagen IV (Molecular Probes) at 20 µg/ml. DQ labeled proteins are fluorogenic substrates for proteases, used previously for the study of proteolytic activity during co-migration and intercellular cooperation of tumor cells [16]. DQ Red BSA was preferred because produced lower background fluorescence. A MCP-1 gradient was created across Matrigel-embedded Mph by placing 100 µl of Matrigel containing fluorescent substrate along with 10⁶/ml labeled cells, in contact with another droplet of 100 µl of Matrigel containing DQ proteins and MCP-1 (0.1 µg/ml) in a 1 cm diameter plastic ring, glued to a coverslip. To inhibit metalloproteases activity, we used TIMP-1 (human recombinant, R&D Systems) at 50–1000 ng/ml, and anti-MMP-12 polyclonal antibody (a kind donation by Dr. S. Shapiro, Harvard University, Boston, MA), at a dilution of 50 µg/ml.

The incubations were maintained in the tissue culture incubator at 37°C and 5% CO₂, and examined after 3 h, and then daily up to 7 days using a Zeiss LSM510 multiphoton confocal inverted microscope. In selected experiments, the cells embedded in the gel were counterstained with Hoechst 3342 (Molecular Probes), just before the final confocal analysis.

Results

Mph produce asymmetrical degradation of the matrix

We incorporated fluorescently labeled peritoneal Mph in Matrigel containing DQ Red BSA, a fluorescent substrate for proteases, and we stimulated their migration by formation of a local chemotactic gradient of MCP-1. In reconstituted 3-dimensional confocal images, we noticed that near Matrigel surface, Mph produced holes of about cell's diameter. Based on the orientation of cells relative to the surface, we concluded that the proteolysis product was more abundant at the leading edge of the cell, in the direction of higher MCP-1 concentrations (Fig. 1). When we displayed the distribution of fluorescence intensity in individual frames of serial optical sections, we found in each frame a mark of cell's passage; this consisted of a circular region of lower optical density, with the diameter comparable to that of a Mph, usually found at the rear of the cell and representing a tubular shape (a 'tunnel'; data not shown).

Pericellular and intracellular distribution of proteolytic products

Proteolysis was detected around the Matrigel-embedded Mph after as early as 3 h. The proteolysis product was usually found aggregated in small clumps, either at the cell surface, or within the cell (Fig. 2A). To characterize the molecular factors involved in matrix degradation and tunnel formation, we included in the gel a polyclonal antibody against MMP-12 (Mph metalloelastase), which others and we found previously to be associated with MC/Mph migration [17–19]. We also used TIMP-1, an inhibitor of this and other metalloproteases [20]. Both inhibitors markedly reduced the surface proteolysis, as revealed by the amount of fluorescent product (Figs. 2 B–C).

While at short incubation times the proteolysis product was taken up in small vesicles, after 2 days these vesicles became prominent, occupying an increasingly more significant volume of the cytoplasm (Fig. 2D). The vesicles contained a fluorescent degradation product more clearly delineated at their center (Fig. 2D, 4B) than the structures

Fig. 1 Migration of peritoneal Mph in Matrigel. Lateral view of a three-dimensional reconstitution from optical sections through a CTG-labeled (green) Mph, penetrating a Matrigel surface (5 days of incubation). Note the lacuna visible at the brighter Matrigel-dish interface (arrow), the proteolytic activity at the leading front of the cell (arrowhead), and the concave distribution of the cytoplasm.

involved in the uptake at 3h. This content consisted of well-defined, fluorescent granules of degraded DQ albumin with diameters of about 1 μm , surrounded by larger vacuoles. Occasionally, the vacuoles were fused and produced 'canaliculi' (when inside of the cells) or 'crevasses' (when fused with the plasma membrane), crossing the cells from one pole to the other (Fig. 3). Counterstaining with the nuclear stain Hoechst ruled out the possibility that these apparently empty spaces were occupied by the nucleus (Fig. 3, last panel).

Mph form trans-cellular lumens and branching in Matrigel

To rule out the possibility that the lumen was an artifact of non-homogenous distribution of the tracer in the cytoplasm (as we used in our previous work), here we carried out the experiments with Mph obtained from mice with ubiquitous expression of eGFP. We performed three-dimensional reconstitutions of optical sections obtained by confocal microscopy through Mph maintained

Fig. 2 Pericellular proteolysis and formation of intracellular vacuoles in Matrigel-incubated MPh. **A-C.** Confocal image of DQ Red BSA proteolysis (red) by a CTG-labeled Mph (green) in Matrigel, after 3 h. **A.** Surface degradation of the substrate, and intracellular uptake of the proteolysis product. **B-C.** Less proteolysis and uptake occurred when TIMP-1 (**B**) or the anti-MMP12 antibody (**C**) were present in the gel during incubation, although the emergence of a canalicular system is visible. **D.** Vacuoles of various sizes (arrowheads) develop in Mph maintained in Matrigel for 2 days, suggesting vacuole enlargement and/or fusion. Note the central granules of degraded albumin. In these median sections the pericellular proteolysis product, mostly confined at the leading edge, is less obvious.

Fig. 3 The canalicular system of Matrigel-incubated Mph, in serial optical sections (confocal microscopy). CTR-labeled Mph (red) were maintained in Matrigel for 2 days, in the presence of the fluorescent substrate DQ Green collagen IV (green). A branched crevasse (arrowhead) is apparent in contact with plasma membrane and crosses the cell from one pole to the other. Last panel: A CTG-labeled Mph (green) maintained in the presence of DQ Red BSA (red), and counterstained with Hoechst (blue), displays granules of proteolysis product (arrow) and the nucleus, which is located aside from the crevasse (arrowhead).

Fig. 4 Lumen formation in eGFP-expressing Mph. Virtual sections through a three-dimensional confocal reconstitution of an eGFP-Mph (green) exposed to MCP-1 gradient. **A.** Frontal section, displaying a proteolytic rim (red, arrowheads). **B.** Middle section through the same cell; note the constant diameter of the cylindrical structure, and the cytoplasm folded around the proteolysis product localized in, but distinct from the lumen (arrowhead). **C.** Longitudinal virtual section through the same cell, displaying the cylindrical disposition of the cytoplasm, as well as the filling of the lumen with proteolysis product (the reference crossing blue and green lines were produced by the software). **D.** Lateral view of another cell, indicating a rear trail of proteolysis (arrowhead), still confined to the cell's center.

in Matrigel for 5 or more days. At this time, large vacuoles were present within the cytoplasm of most cells. In a subpopulation of Mph that represented about 10% of the cells, the cellular geometry was cylindrical, with the length usually larger (Fig. 4C) but sometimes smaller (Fig. 4D) than the cell diameter. The matrix surrounding these cells appeared degraded asymmetrically, *i.e.* the proteolysis product accumulated at the cell pole facing higher MCP-1 concentration (Fig. 4A, compare with 4B), and also inside the lumen (Fig. 4C) or expanded beyond the physical limits of the cell (Fig. 4D). Occasionally, the cells displayed a lateral junctional cleft, similar to the one found in endothelial cells covering the lumen of a capillary (Fig. 4B).

To verify that the cells described here were indeed Mph, we also used in our experiments cells expressing Yellow Fluorescent Protein under the Mph-specific *fms* promoter [15] (YFP-Mph). Within these Mph we also detected the formation of large intracellular vacuoles, strongly resembling the trans-cellular lumens, after as early as three hours of incubation in Matrigel (Fig. 5A). At the same time, the intracellular accumulation of proteolysis product was stronger than in eGFP-labeled cells. We occasionally detected a space adjacent to the cells of approximately a cell's volume, containing degraded extracellular matrix, (Fig. 5A), reminiscent of a tunnel.

After five days, we found cells in different stages of lumen formation. Many cells displayed a cup-like, concave morphology, without the full extension of the lumen across the cell; in other instances, the distribution of the whole cell body, and even of the nucleus itself, was found to be ring-like (data not shown). The complete lumen occurred in about 10% of the examined population in the eGFP-expressing mice, and in almost 50% of the YFP transgenics (a difference supposedly due to the mice backgrounds). We also identified vacuoles/lumen-containing Mph positioned in three-dimensional, branched structures (Fig. 5B).

Discussion

By using two transgenic mouse lines intrinsically expressing fluorescent proteins (either in all cells, or specifically in the myeloid cell lineage), we demonstrate that the adult peritoneal Mph contain a subpopulation of cell capable of lumen and branching formation. We consider that younger MC are no more present in this population, due to (1) the long incubation times (up to 5 days), that make it unlikely that MC would survive (their lifespan is limited of about 24h before they either become Mph, or undergo apoptosis) and (2) the fact that in Matrigel we usually detected isolated,

individual cells, and not cell clusters which could derive from proliferating progenitors.

At shorter incubation times, the pericellular proteolysis was asymmetrical, as indicated by the localized accumulation of proteolysis product at the leading front of the cells. This was shown for migration-associated proteolysis in other cellular systems as well [16]. Over time, this product became more uniformly distributed around the cells, but still remained increased in the direction of cell advancement, associated with tunnel-like destruction of the matrix. Moreover, we observed the stepwise development of a system of intracellular vacuoles, culminating with the formation of a trans-cellular lumen. This mechanism is similar to the one described for endothelial cells, where $\alpha_2\beta_1$ integrins, cdc42/Rac1 and membrane-type metalloproteases (MT1-MMP) were mechanistically involved [21, 22]. More specifically, intracellular vacuole formation and coalescence was required for lumen formation in several models of morphogenesis, which we believe applies to adult mononuclear phagocytes as well. In addition, a series of studies have observed vacuoles *in vivo* during

angiogenic events. These vacuoles were formed through an integrin-dependent pinocytotic mechanism in either collagen or fibrin matrices [22]. Formation of the microvascular lumen *in vivo* could be detected only recently, after the use of live imaging in zebra fishes, and was shown to proceed *via* generation and fusion of intracellular vacuoles, spanning more than one cell either longitudinally, or laterally [23].

Thus, we found a subpopulation of Mph that adopted a cylindrical morphology by formation of a large lumen, comparable to the one usually seen only in capillaries. In some cases a lateral junctional cleft was also visible, indicating wrapping of the cell's cytoplasm around the tunnel, in addition to the formation of the lumen. These data add up to our previously published evidence using high-definition histology [11], and rule out the possibility that the cells would have a native toroidal shape. Existence of a subpopulation of murine peripheral blood MC [24] and peritoneal Mph [25] with a ring-shaped nucleus was previously reported, although the proportion of these cells was much lower than those seen here (for instance, 0.95% for MC [24]).

Moreover, we observed by optical sectioning of both eGFP and YFP-expressing Mph that the whole cells acquired this tubular shape.

We also could discern the steps of Mph transformation that led to lumen formation, namely the occurrence of trans-cellular canaliculi. At this stage, few cells displayed signs of apoptosis such as blebbing or cytoplasmic and nuclear fragmentation. This did occur increasingly at later time points, but we did not consider them in this analysis. Vacuolation and apoptosis were considered to be components of lumen formation in endothelial capillaries [26] and genes of apoptosis were upregulated in endothelial cultures in conditions of tube formation [21]. In our Matrigel assays, the surviving Mph seemed to display an increased robustness, in line with the known activating effect of MCP-1 on Mph [27].

To our knowledge, this is the first time when formation of a well-defined lumen was detected in isolated cells, which were not part of a pre-existent cell column. Since lumen formation *in vitro* and *in vivo* in endothelial cells requires cell polarity [28], we propose that this is produced in Mph by the gradient of chemoattractant (here MCP-1), which stimulates proteolysis at the leading edge of the cell.

Matrigel, used as a model of extracellular matrix in these studies, is known to contain angiogenic growth factors such as VEGF and bFGF. Although our preliminary experiments with the growth factor reduced form did not show any difference vs. regular Matrigel in terms of lumen formation (data not shown), the biochemical stimulation of Mph in these experiments was likely to be more complex than the mere MCP-1 gradient.

In conclusion, the formation of lumens in Mph confirms the propensity of this cell type to integrate among endothelial cells in the process of endothelial repair, as suggested for large vessels [3, 29], but also for *de novo* formation of capillaries and for lymphatic vessels [23]. Our findings that adult Mph may form lumens and branching structures add a new dimension to the vascular plasticity of these cells. We do not believe that these observations should be necessarily interpreted as ‘trans-differentiation’ of MC/Mph into endothelial cells (and definitely not in this *in vitro* model). Instead, we believe these data bring further arguments for their *functional* plasticity [30], and for the intricacy of the mononuclear phagocytes with endothelial cells, within the age-honored concept of ‘reticulo-endothelial system’ [31].

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