Article

Mast Cells Interact with Endothelial Cells to Accelerate In Vitro Angiogenesis

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Abstract: Angiogenesis is a complex process that involves interactions between endothelial cells and various other cell types as well as the tissue microenvironment. Several previous studies have demonstrated that mast cells accumulate at angiogenic sites. In spite of the evidence suggesting a relationship between mast cells and angiogenesis, the association of mast cells and endothelial cells remains poorly understood. The present study aims to investigate the relationship between mast cells and endothelial cells during in vitro angiogenesis. When endothelial cells were co-cultured with mast cells, angiogenesis was stimulated. Furthermore, there was direct intercellular communication via gap junctions between the two cell types. In addition, the presence of mast cells stimulated endothelial cells to release angiogenic factors. Moreover, conditioned medium from the co-cultures also stimulated in vitro angiogenesis. The results from this investigation demonstrate that mast cells have both direct and indirect proangiogenic effects and provide new insights into the role of mast cells in angiogenesis.

Keywords: mast cells; angiogenesis; endothelial cells; tube formation assay; co-culture; gap junction

1. Introduction

Angiogenesis depends on endothelial cell proliferation and migration. Blood vessel formation is also dependent on the interaction of endothelial cells with other cell types as well as with the tissue microenvironment [1-3]. Therefore, it is essential to understand the interaction of endothelial cells with other cell types and how this interaction can activate endothelial cells during blood vessel formation. Throughout angiogenesis, the various cell types communicate via a complex network of intercellular signaling pathways, mediated by cytokines, receptors and surface adhesion molecules [2,4-6].

Cell to cell communication, is critical in the control and coordination of vascular function [7-9]. The gap junction is the main mediator of cell to cell communication. It is formed by connexins [10,11], a family of transmembrane proteins that interact to form connexons that act as channels which directly connect the cytoplasm of adjacent cells, allowing the passage of current and small signaling molecules [8,12]. Several studies have suggested that gap junctions and connexins may play an important role during physiological or pathological processes associated with angiogenesis [9,13,14]. Furthermore, gap junctions have been identified at areas of contact between immune cells and endothelial cells. Gap junctions have also been implicated in immune cell trans-endothelial migration and in the contact between eosinophils and tissue resident cells during inflammatory reactions [15-17].

Recently the interaction between immune cells such as macrophages, lymphocytes, neutrophils, and endothelial cells during angiogenesis has been described [1,18,19]. However, little is known about the interaction between mast cells and endothelial cells during angiogenesis. Mast cells are

46 immune cells that are derived from myeloid lineage cells in the bone marrow [20-22]. They are found
47 associated with connective tissue throughout the body, particularly in locations that are in close
48 contact with the external environment [23-25]. Mast cells have been shown to be associated with
49 blood vessels and they accumulate near sites of new capillary formation, such as in atherosclerosis,
50 hemangioma development, and tumorigenesis [26-30]. Notably, mast cell accumulation is correlated
51 with increased neovascularization, tumor aggressiveness, and metastatic spread [31-34]. Previous
52 studies have focused on the role of mast cells in enhancing angiogenesis through the release of pro-
53 angiogenic factors [35-38]. However, the involvement of mast cells in angiogenesis is complex and
54 not completely understood. The present study was undertaken to investigate the interaction of mast
55 cells and endothelial cells in angiogenesis. During *in vitro* angiogenesis, when mast cells were co-
56 cultured with endothelial cells, an association between mast cells and endothelial cells was observed.
57 Furthermore, this association induced the release of pro-angiogenic factors which accelerated
58 angiogenesis.

59 **2. Results**

60 *2.1. Mast cells accelerate in vitro angiogenesis*

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62   To evaluate the ability of mast cells to induce angiogenesis, the mouse endothelial cell line
63 SVEC4-10 and the murine mast cell line P815 were co-cultured using an in vitro angiogenesis assay
64 system. Tube formation was evaluated after 5 hours of co-culture by actin staining as well as by
65 scanning electron microscopy. Tube formation and sprouting by endothelial cells was increased in
66 the presence of mast cells (Figure 1A-D). A quantitative analysis of the co-culture showed that the
67 percentage of tubes and loops was higher, 85±12% and 842±108% respectively, when SVEC4-10 cells
68 were cultured together with the mast cells (Figure 1E and F). Also, the area covered by cells was
69 significantly higher in the co-cultures, 233±33%, (Figure 1G), indicating an increase in cell spreading.

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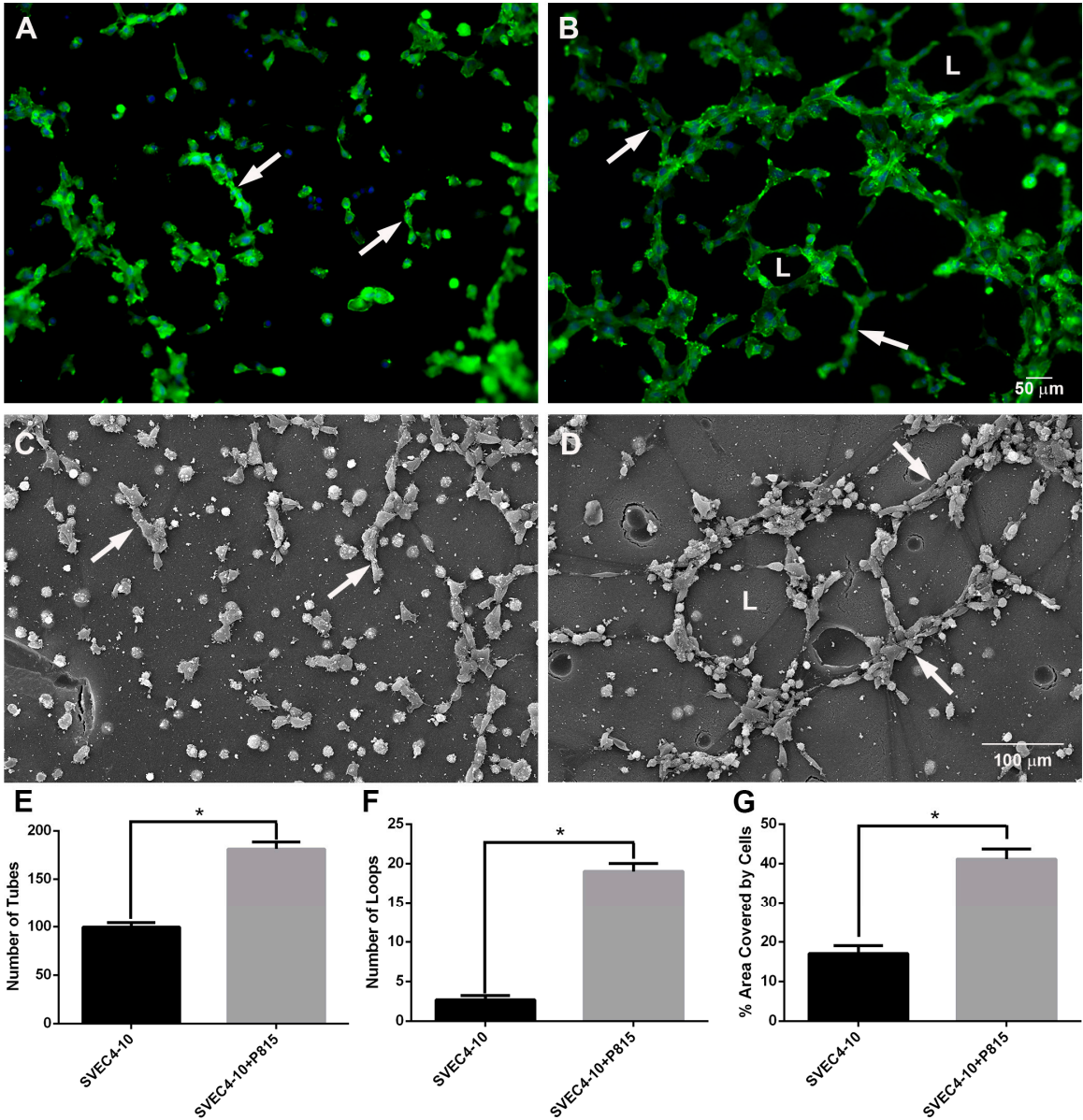
Figure 1. Mast cells induce tube and loop formation. (**A,C**) SVEC4-10 cells; (**B,D**) SVEC4-10 cells co-cultured with P815 mast cells. Tubes (arrows) and loops (L) were increased in the co-cultures after 5 hours. (**A,B**) Cells were stained with phalloidin conjugated to Alexa 488; the nuclei were counterstained with DAPI. (**C,D**) Scanning Electron Microscopy. (**E,F,G**) The graphs show the average number of tubes (**E**) and loops (**F**) and the area covered by cells (**G**) after 5 hours of culture. The structures were quantified using Wimasis WimTube. Data are presented as mean ± SD from five independent experiments. *$p \leq 0.05$.

Since it is known that macrophages can also enhance angiogenesis [39-41], endothelial cells were co-cultured with macrophages in order to verify the results seen with mast cells. Co-culture of endothelial cells with mast cells (Figure 2A) was more effective in inducing tube and loop formation than co-culture of SVEC4-10 cells with macrophages (Figure 2B). The percentage of tubes increased 43±9% (Figure 2C) and the percentage of loops increased 462±88% (Figure 2D) in the co-cultures of endothelial cells with mast cell when compared with the co-cultures of endothelial cells with macrophages. The results of the tube formation assays showed that mast cells are more effective in promoting in vitro angiogenesis than macrophages.
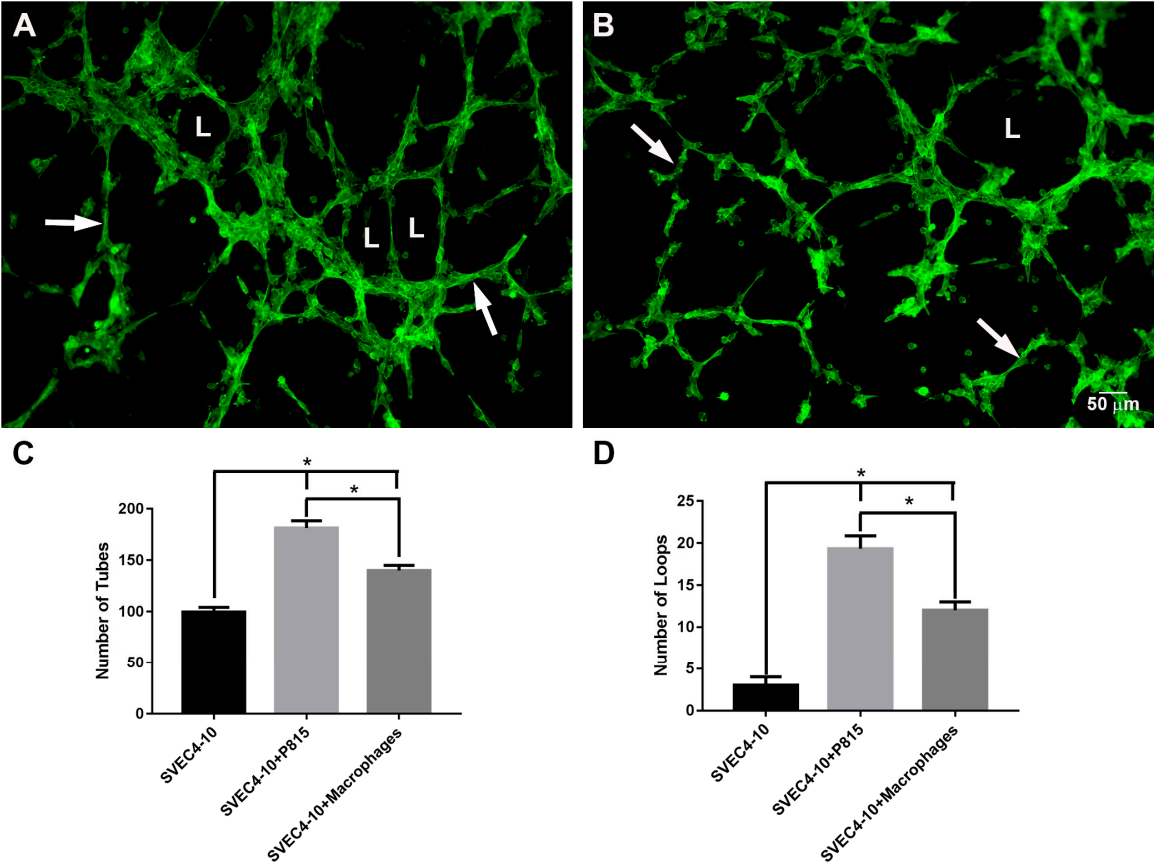
Figure 2. Mast cells are more effective in inducing tube formation than macrophages. **(A)** SVEC4-10 cells co-cultured with P815 mast cells; (**B**) SVEC4-10 cells co-cultured with macrophages. A significant increase in the number of tubes (arrows) and loops (L) was observed during co-culture of endothelial cells with mast cells when compared with co-cultures of endothelial cells with macrophages. (**A,B**) Cells were stained with phalloidin conjugated to Alexa 488. (**C,D**) The graphs show the average number of tubes and loops after 5 hours of culture. The structures were quantified using Wimasis WimTube. Data are presented as mean ± SD from five independent experiments. *p≤ 0.05.

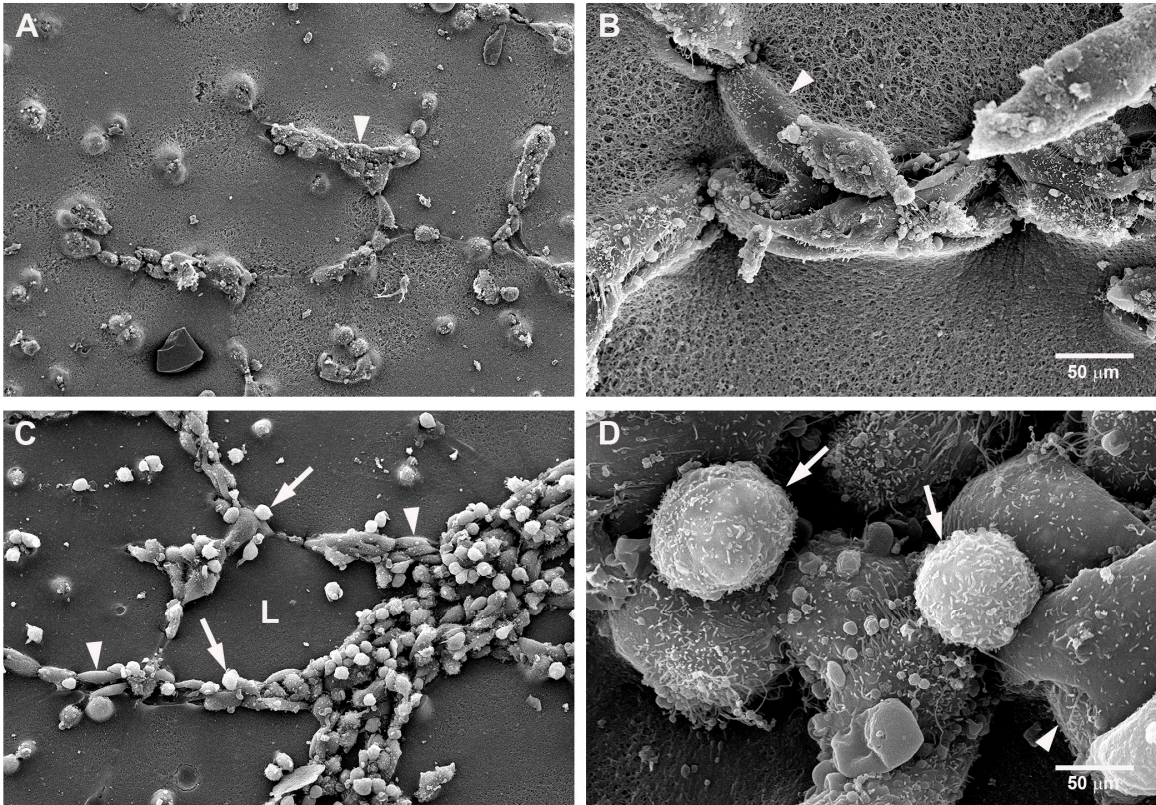*2.2. Mast cells and endothelial cells are associated*

Since the presence of mast cells accelerated tube and loop formation by endothelial cells, the association between these two cell types was further investigated. SVEC4-10 cells were labeled with CellTracker™ Green CMFDA and P815 mast cells were labeled CellTracker™ Red CMTPX. After 5 hours of co-culture, P815 mast cells were in close association with SVEC4-10 cells (Figure 3) especially in the loops. The presence of mast cells in close association with endothelial cells during the co-cultivation suggests that the mast cells play a critical role in vitro angiogenesis.
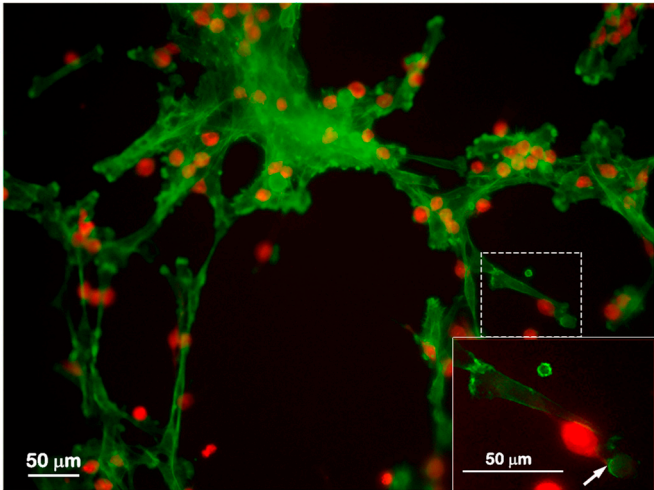
Figure 3. Mast cells are in close approximation to endothelial cells. SVEC4-10 cells co-cultured with P815 mast cells. SVEC4-10 cells were labeled with CellTracker™ Green CMFDA and P815 mast cells were labeled with CellTracker™ Red CMTPX. After 5 hours of co-culture, SVEC4-10 cells (green) and P815 mast cells (red) are associated. Mast cells are in contact with endothelial cells (arrow). Inset: High magnification of the area delimited by dotted line.

This association between mast cells and endothelial cells was confirmed by scanning electron microscopy (Figure 4). In the co-culture, most of the mast cells were in contact with endothelial cells. They could also be seen in the loops, which are the final step in tube formation (Figure 4C and D). The morphological response of the two cell types to culture on Geltrex® was different. SVEC4-10 cells spread on the Geltrex® while the P815 mast cells remained rounded (Figure 4C and D). Mast cells also remained rounded when cultured on Geltrex® even in the absence of endothelial cells (data not shown).

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Figure 4. Mast cells and endothelial cells associate during the tube formation assay. **(A,B)** SVEC4-10 cells; **(C,D)** SVEC4-10 cells co-cultured with P815 mast cells. **(A,B)** In cell cultures without mast cells, the endothelial cells form only incomplete loops (arrowheads). **(C,D)** During co-culture SVEC4-10 endothelial cells (arrowheads) and mast cells (arrows) are associated especially in the loops (L). The endothelial cells are spread on the substrate (arrowheads) while P815 mast cells remain rounded (arrows). Cells were cultured for 5 hours prior to assay. Scanning electron microscopy.

2.3. Gap junctions connect endothelial cells and mast cells

The presence of gap junctions between endothelial cells and mast cells was investigated by immunostaining the co-cultures for connexin 43. The results showed that both mast cells and endothelial cells expressed this protein and that connexin 43 was present throughout the cells including cellular projections as well as the area of contact between mast cells and endothelial cells (Figure 5).

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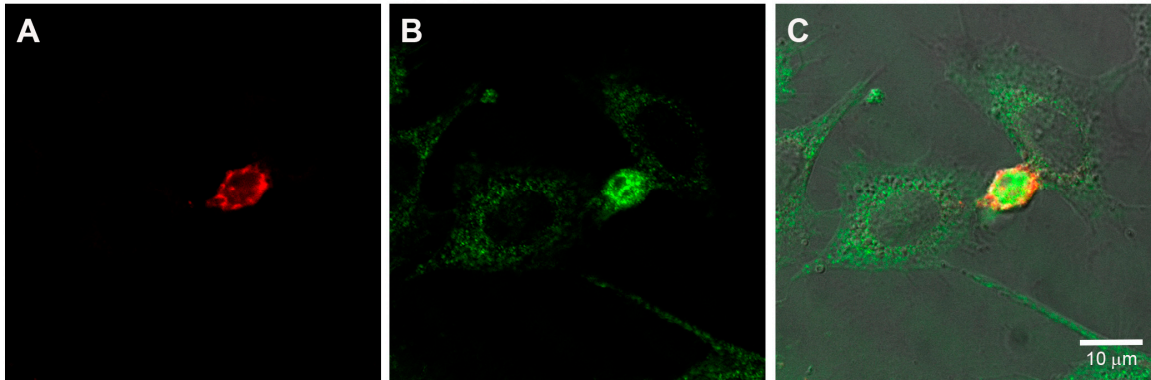
135   **Figure 5.** Connexin 43 is expressed in P815 mast cells and endothelial cells. (**A**) P815 mast cells were
136   labeled with CellTracker™ Red CMTPX. (**B**) After 5 hours of co-culture with SVEC4-10 cells, the
137   cultures were immunostained with antibody against connexin 43 (green). (**C**) Merge of A and B.

138   The association between mast cells and endothelial cells was further investigated by
139   transmission electron microscopy. Mast cells and endothelial cells were co-cultured on Geltrex® for 5
140   hours and then processed for transmission electron microscopy. An intercellular space (7-15Å)
141   between the endothelial cells and mast cells filled with lanthanum, which is indicative of gap
142   junctions, was evident (Figure 6). As suggested by the immunostaining, the results of the
143   transmission electron microscopy indicate a direct intercellular communication, via gap junctions,
144   between mast cells and endothelial cells during angiogenesis.

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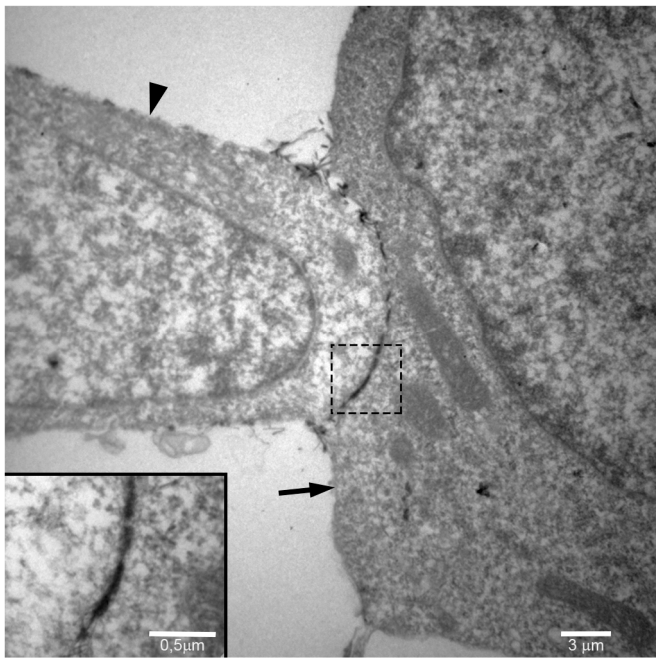
146   **Figure 6.** Gap junctions are present between mast cells and endothelial cells during in vitro
147   angiogenesis. Transmission electron microscopy demonstrates that the space between the mast cell
148   (arrow) and the endothelial cell (arrowhead) is filled with lanthanum, indicative of a gap junction.
149   Inset: High magnification of the area delimited by dotted line.

150   *2.4. Angiogenic factors are released by endothelial cells in the presence of mast cells*

151   Since co-culture of mast cells with endothelial cells induced tube formation, it was of interest to
152   investigate which angiogenic factors were released during the co-culture. The supernatants from the
153   co-cultures of SVEC4-10 cells with P815 mast cells or from cultures of SVEC4-10 cells alone were
154   analyzed using a proteome profiler array. In the presence of mast cells, SVEC4-10 cells released 33
155   more angiogenic factors than the SVEC4-10 cells alone (Table 1). These results provide evidence that
156   the presence of mast cells stimulates the release of angiogenic factors.

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163   **Table 1.** Angiogenic factors released in the presence or absence of P815 mast cells.

Angiogenic Factor	SVEC4-10 cells	SVEC4-10 +P815 cells	Angiogenic Factor	SVEC4-10 cells	SVEC4-10 +P815 cells
ADAMTS1	-	+	MMP-8	-	+
Amfiregulin	-	+	MMP-9	-	+
Angiogenin	+	+	IGFBP-9	-	+
Coagulation III	-	+	Osteopontin	-	+
CXCL16	-	+	PDGF-AA	-	+
DPPIV/CD26	-	+	PDGF	-	+
Endostatin	+	+	Pentraxin	-	+
Endotelin 1	-	+	PF 4	+	+
FGF-1	-	+	PlGF-2	-	+
FGF-2	-	+	Prolactin	-	+
Hepatopoietin A	-	+	Proliferin	-	+
IGFBP-1	-	+	SDF-1 CXCL12	-	+
IGFBP-2	-	+	Serpin E1	-	+
IGFBP-3	+	+	Serpin F1	-	+
IL-1	-	+	Trombospondin-2	-	+
Leptin	-	+	TIMP-1	-	+
MCP-1	+	+	TIMP-4	-	+
MIP-1	-	+	VEGF VPF	-	+
MMP-3	-	+	VEGF-B VRF	-	+

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165   *2.5. Conditioned Medium from the co-cultures increases tube formation*

166   Since co-culture of endothelial cells with mast cells increased tube and loop formation, it was of
167   interest to determine if the physical presence of mast cells was necessary to stimulate in vitro
168   angiogenesis. Therefore, endothelial cells were cultured with conditioned media from various culture
169   conditions (Figure 7). When SVEC4-10 cells were cultured on Geltrex® in the presence of conditioned
170   medium from SVEC4-10 cells, there was a significant increase in tube, but not loop formation.
171   However, there was an even more significant increase in both tube and loop formation when SVEC4-
172   10 cells were cultured on Geltrex® in the presence of conditioned medium from the co-cultures of
173   endothelial cells and mast cells. Furthermore, when SVEC4-10 cells were cultured on Geltrex® in the
174   presence of conditioned medium from P815 mast cells, there was no increase in tube or loop
175   formation. These results indicate that mast cells themselves and/or the angiogenic mediators released
176   by endothelial cells in the presence of mast cells both stimulate angiogenesis.

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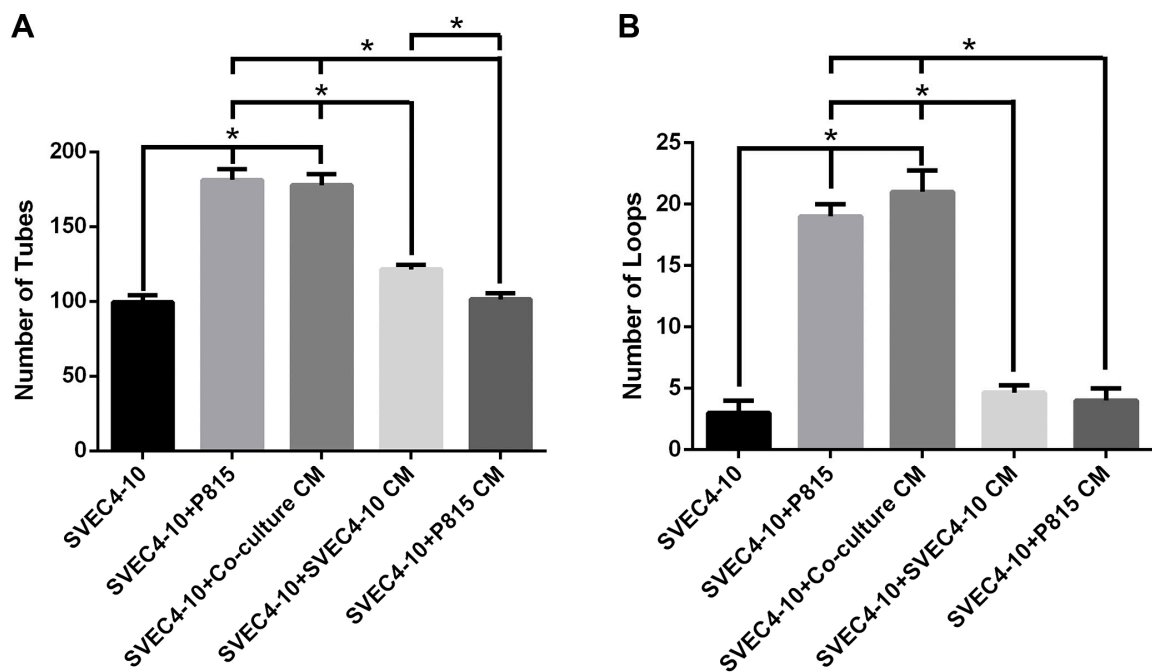
Figure 7. Conditioned medium from the co-culture increases in vitro angiogenesis. The conditioned medium from the co-culture of SVEC4-10 cells and P815 mast cells demonstrated the same ability as co-culture with mast cells to increase in vitro angiogenesis. (**A,B**) The conditioned media from SVEC4-10 induced a slight increase in tubes (A) but not in loops. The graphs show the average number of tubes and loops after 5 hours of culture. The structures were quantified using Wimasis WimTube. Data are presented as mean ± SD from five independent experiments. Conditioned Media (CM) *p≤ 0.05.

3. Discussion

The present study provides evidence of a direct interaction between endothelial cells and mast cells that results in the release of angiogenic factors from endothelial cells which then accelerate in vitro angiogenesis. Furthermore, conditioned medium from the co-culture of endothelial cells and mast cells contains the factors necessary to stimulate angiogenesis in vitro. These results demonstrate a critical role for mast cells in angiogenesis.

As shown in the present study, during in vitro angiogenesis, the mast cells were in close proximity to the endothelial cells where they were largely associated with loops. This is consistent with the localization of mast cells in vivo, where mast cells are normally found adjacent to the blood vessels [42,43]. Angiogenic factors induce endothelial cells to sprout and form branches that split in two, increasing the area of the new vessels. This branching also facilitates fusion of the endothelial cells in a process called anastomosis [44-46]. In the developing hindbrains of zebrafish, macrophages appear to form bridges between endothelial cells aiding in cell fusion and promoting anastomosis between endothelial cells [47]. The present study provides evidence that mast cells could also act as bridges giving physical support and establishing contact between endothelial cells during vascular anastomosis.

The current investigation also demonstrates that mast cells and endothelial cells can form gap junctions. Ultrastructural analysis indicated a direct intercellular communication, via gap junctions, between mast cells and endothelial cells during angiogenesis. Direct intercellular communication via gap junctions is critical in the control and coordination of vascular function [8,48]. Gap junctions are intercellular channels that directly connect the cytoplasm of adjacent cells, allowing the passage of current and small signaling molecules [11,49]. A gap junction is composed of two connexons integrated into the plasma membranes of the connected cells forming a channel [50,51]. Each connexon is composed of 6 connexins. The connexins mediate specific cell-to-cell signaling pathways,

210   and the molecular selectivity as well as subcellular localization differs among the connexins [8,52]. In
211   addition, Cx43 seems to be essential for coordination of endothelial cell proliferation and migration
212   in the vasculature [8,53,54]. Vliagoftis and colleagues demonstrated that mast cells express Cx43 and
213   Cx32, and that Cx43 is associated with the plasma membrane [55,56]. Recent studies have shown that
214   mast cells can also interact directly with fibroblasts, forming gap junctions between mast cells and
215   fibroblasts. This allows direct communication between the two cell types, stimulating fibroblast
216   proliferation, myofibroblast differentiation, and collagen lattice contraction [56-59]. The physical
217   association of mast cells and endothelial cells as well as the direct intercellular communication via
218   gap junctions observed in this study, provides another mechanism in which mast cells may affect
219   angiogenesis.

220   Mast cells produce and release a large number of mediators with diverse biological activities.
221   Previous studies have demonstrated that mast cell proteases are one of the main factors responsible
222   for stimulating angiogenesis [29,60]. However, when the activity of β-hexosaminidase and the
223   expression and activity of tryptase and chymase were analyzed in supernatants from co-cultures of
224   mast cells and endothelial cells, no β-hexosaminidase activity or tryptase or chymase expression or
225   activity was detected in the supernatants from these experiments (data not shown). This data
226   suggests that in co-culture with endothelial cells, the mast cells did not release mast cell proteases or
227   other preformed mediators.

228   In addition, the current investigation demonstrated that the presence of mast cells stimulated
229   the endothelial cells to release a wide range of angiogenic factors (Figure S1). The levels of the pro-
230   angiogenic cytokines angiogenin, endostatin, IGFBP-3 (Insulin-like Growth Factor Binding Protein -
231   3), and MCP-1 (Monocyte Chemoattractant protein-1) detected in cultures of SVEC4-10 cells alone,
232   increased when the SVEC4-10 cells were co-cultured with mast cells.

233   Endothelial cell spreading was accelerated in the co-cultures of mast cells and endothelial cells
234   as well as when the endothelial cells were cultured in conditioned medium from the co-cultures. This
235   may be due to the presence of various other proteins/cytokines that were detected in the supernatant
236   from the co-cultures that were not present in the culture media of endothelial cells alone. Some of
237   these proteins/cytokines are directly related to cell proliferation and migration such as SDF-1
238   (Stromal cell-Derived Factor 1), and Trombopondina-2 [61]. Therefore, the release of various
239   cytokines/proteins related to adhesion and migration when endothelial cells are in the presence of
240   mast cells may explain the increased spreading of endothelial cells, as well as the stimulation of tube
241   and loop formation during co-cultivation of endothelial cells with mast cells or with conditioned
242   medium from the co-cultures.

243   Taken together, the results of the current study demonstrate that mast cells have both direct and
244   indirect proangiogenic effects on endothelial cells. This investigation provides new insights into the
245   role of mast cells in angiogenesis.

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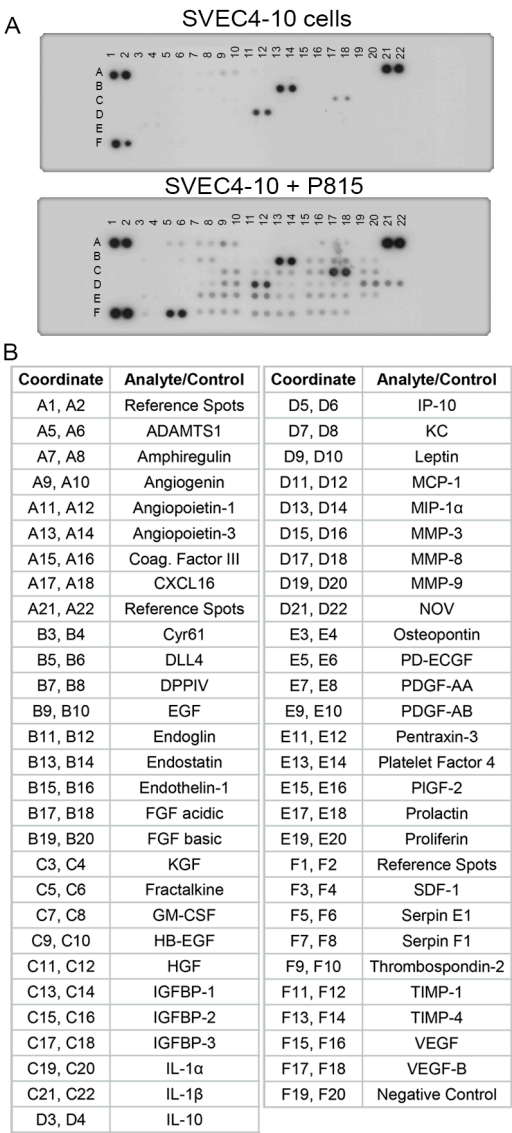
Figure S1. Differential release of angiogenic factors by SVEC4-10 cells in the presence or absence of mast cells. The SVEC4-10 cells were cultured for 5 hours at 37 °C on Geltrex® in the presence or absence of P815 mast cells. Culture supernatants were used to analyze the protein expression of pro- and anti-angiogenic factors using the Proteome Profiler™ Mouse Angiogenesis Array Kit. (**A**) Array membrane images are shown. (**B**) Table giving the mouse angiogenesis array coordinates with a description and location of each angiogenic factor in the membrane array.

4. Materials and Methods

4.1. Cells

The murine endothelial cell line SVEC4-10 (CRL-2181) was purchased from the American Type Culture Collection (ATCC; Manassas, VA).   The P815 mast cells (mouse lymphoblast-like mastocytoma cell line) was purchased from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% heat inactivated fetal bovine serum. The murine lung macrophage cell line AMJ2-C11 (ATCC® CRL-2456™) was cultivated in DMEM plus 5% heat inactivated fetal bovine serum.   All cell lines were cultured at 37 ºC in a humidified environment containing 5% CO_2 in air. All reagents used for cell culture were purchased from Thermo Fisher Scientific (Invitrogen, Thermo Fisher Scientific, Waltham, MA).

264 *4.2. In vitro angiogenesis - Tube Formation Assay*

265   10 μL of Geltrex® (Invitrogen, Thermo Fisher Scientific) was added to the wells of μ-slides
266 Angiogenesis® (IBIDI, Martinsried, Germany) and allowed to solidify at 37 °C for 30 min. After the
267 gel solidified, SVEC4-10 cells (1×10^4) were added in 50 μL of DMEM supplemented with 10% FBS.
268 For co-culture experiments, the cells were cultured at a ratio of 1 P815 mast cell or AMJ2-C11
269 macrophage to 2 SVEC4-10 cells.

270   In the experiment using conditioned media, the media were collected after tube formation assay
271 as described above and used in a new tube formation assay. The following conditioned media were
272 used in tube formation assays: Conditioned medium from the co-culture of mast cells and endothelial
273 cells; Conditioned medium from the culture of SVEC4-10 cells alone; Conditioned medium from the
274 culture of P815 mast cells alone.

275   The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in air for 5
276 hours and then analyzed. The parameters used for quantification [62-64] were:

277   Tubes: part of a tubular structure that goes from one branching point to another branching point
278 or to a loose end; Loops: enclosed (or almost enclosed) areas inside the tubular structure that fulfill
279 roundness conditions.

280   The images were acquired using a 10X objective and the measurements are expressed in pixels
281 (800x600) where 1 pixel equals $0.069 mm^2$. Tube formation was quantified using WimTube (Wimasis
282 Image Analysis, Munich, Germany).

283 *4.3. CellTracker™ cell labelling*

284   SVEC4-10 cells were labeled with CellTracker™ Green CMFDA and P815 mast cells were labeled
285 with CellTracker™ Red CMTPX (Molecular Probes, ThermoFisher Scientific). The cells were
286 incubated for 30 min at 37 ºC in DMEM without serum, but containing 25 μg/mL of either
287 CellTracker™ green or red. After labeling, the cells were incubated for an additional 30 min at 37 ºC
288 in DMEM with 10% heat inactivated fetal bovine serum. The cells were used in the tube formation
289 assay as described above.

290 *4.4. Immunofluorescence*

291   To determine the presence of connexin 43 in endothelial cells and mast cells, the cells were fixed
292 with 2% formaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilized with 0.1% Triton
293 X-100 in PBS at room temperature, blocked with PBS containing 1% BSA, and incubated with rabbit
294 anti-mouse connexin 43 (1:50) (ATB T1046, Ameritech Biomedicines, Pflugerville, TX) for 1 hour.
295 Following incubation, cells were washed, and incubated with goat anti-rabbit IgG conjugated with
296 Alexa 488 (1:1000) (Molecular Probes, Thermo Fisher Scientific), washed and analyzed using a LEICA
297 TCS-NT SP5 laser scanning confocal microscope (Leica Microsystems; Heidelberg, Germany).

298 *4.5. Fluorescent Labeling*

299   Cells were fixed with 2% formaldehyde (Electron Microscopy Sciences), permeabilized with
300 0.3% Triton X-100 in PBS for 10 min, washed, and then incubated with 2.6U/ml Phalloidin-Alexa 488
301 (Molecular Probes, Thermo Fisher Scientific) for 30 min. Nuclei were counterstained with 4,6-
302 diamidino-2-phenylindole (DAPI; 1:500). The samples were washed in PBS and then observed with
303 an inverted fluorescence microscope (Nikon Eclipse TE2000-U, Nikon instruments Inc., NY, USA)
304 and the images acquired using a Nikon DS-1QM digital camera.

305 *4.6. Expression Profile of Angiogenesis Related Proteins*

306   The expression profile of angiogenesis related proteins was analyzed using the Proteome
307 Profiler™ Mouse Angiogenesis Antibody Array (R&D Systems Inc., Minneapolis, MN). After the
308 tube formation assay, the supernatants from SVEC4-10 cell cultures or co-cultures of SVEC4-10 cells
309 and P815 mast cells were collected and processed following the manufacturer´s instructions. Briefly,
310 the membrane containing immobilized antibodies to angiogenesis related proteins was incubated

311 with the supernatants. Bound protein was detected with a biotinylated antibody cocktail followed by
312 streptavidin conjugated to HRP. Membranes were washed and developed using the ECL™ Western
313 Blotting Detection Reagent, RPN2106 (GE Healthcare, Piscataway, NY).

4.7. Electron Microscopy

315 For electron microscopy assays, Geltrex® (100 µL) was placed on 13-mm-round coverslips coated
316 with Biobond (Electron Microscopy Sciences). After the gel solidified, SVEC4-10 cells (1×10^5) were
317 added in 500 µL of DMEM supplemented with 10% FBS. The cells were incubated at 37 °C in a
318 humidified atmosphere containing 5% CO_2 in air for 5 hours to allow for tube formation.

4.7.1. Scanning Electron Microscopy

320 For scanning electron microscopy, after tube formation, the samples were rinsed in warm PBS
321 (37 °C) and fixed in 2% glutaraldehyde (Electron Microscopy Sciences) in warm PBS for 2 hours at
322 room temperature. Samples were post fixed in 1% OsO_4 (Electron Microscopy Sciences) for 2 hours,
323 rinsed in Milli-Q water. The samples were dehydrated in a graded ethanol series and critically point-
324 dried with liquid CO_2 in a Tousimis Autosandri-810 critical point dryer (Tousimis Research Co.,
325 Rockville, MD), mounted on aluminum stubs with silver paint (EM Sciences), and coated with gold
326 in a BAL-TEC SCD 050 Sputter Coater (BAL-TEC AG, Balzers, Liechtenstein). Samples were
327 examined with a JEOL JSM-6610LV scanning electron microscope (JEOL, Ltd.; Tokyo, Japan)..

4.7.2. Transmission Electron Microscopy

329 For transmission electron microscopy, after tube formation, the samples were rinsed in warm
330 PBS (37 °C) and fixed in 2% glutaraldehyde (Electron Microscopy Sciences) 2% paraformaldehyde
331 (Electron Microscopy Sciences) in 0.1 M cacodylate buffer, pH 7.4. The samples were post fixed in 1%
332 OsO_4 (Electron Microscopy Sciences) in 0.1 M cacodylate buffer (pH 7.4) for 2h, rinsed in Milli-Q
333 water, dehydrated in a graded ethanol series. The Geltrex® with cells was removed from the
334 coverslips and embedded in EMBED 812 (Electron Microscopy Sciences). To demonstrate the
335 extracellular space and mark gap junctions, 1% lanthanum nitrate was added to the fixatives. Thin
336 sections were cut with a diamond knife, mounted on copper grids, and stained for 10 min each in
337 Reynolds's lead citrate and 0.5% aqueous uranyl acetate, and examined with a JEOL JEM-100CXII
338 (JEOL Ltd., Tokyo, Japan) transmission electron microscope,

4.8. Statistical Analysis

340 Values are expressed as the mean ± SD. Student's t-test was used to compare data. p-values of
341 ≤0.05 were considered significant. The data is expressed as mean ± SD from at least three independent
342 experiments.

343 **Supplentary materials:**

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