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### ISOLATION AND CHARACTERIZATION OF RAT GASTRIC MICROVASCULAR ENDOTHELIAL CELLS AS A MODEL FOR STUDYING GASTRIC ANGIOGENESIS *IN VITRO*

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We have previously characterized morphologic features of wounding-induced angiogenesis that occurs in response to acute and chronic gastric mucosal injury. As a means of investigating the molecular mechanisms underlying gastric angiogenesis, microvascular endothelial cells were isolated from stomachs of normal (non-injured) rats. The isolation procedure adapted and combined aspects of previous methods and employed positive selection using magnetic beads coated with monoclonal antibody specific for rat CD31 (PECAM-1), a cell surface marker restricted to platelets, monocytes, T lymphocytes and endothelial cells. The isolated microvascular endothelial cells expressed vascular endothelium-specific antigen and the endothelial-specific receptors, Tie2 and *flt-1* (VEGFR1). When plated on growth factor-reduced matrigel, the isolated microvascular endothelial cells formed capillary-like structures reflecting *in vitro* angiogenesis. These cells were also responsive to vascular endothelial growth factor, VEGF, further verifying their endothelial nature. The rat microvascular endothelial cells isolated by this procedure should be useful in delineating molecular mechanisms and regulation of the angiogenesis that is essential for the healing of acute and chronic gastric injury.

**Key words:** *gastric endothelial cells, isolation, Tie2, flt-1, VEGF, angiogenesis, PECAM-1*

#### INTRODUCTION

Angiogenesis — the formation of new capillary microvessels — is essential to the healing of both acute (e.g. mucosal erosions) and chronic (gastric and duodenal ulcers) gastrointestinal injury (1). The molecular mechanisms that underlie the angiogenic process are complex and have not been fully elucidated. This is, in part, due to the difficulty in delineating the exact aspects that are attributable to a given cell type, particularly endothelial cells, in the *in vivo*

situation where there is close interaction between several different cell types within the same tissue. To circumvent this problem, *in vitro* models of angiogenesis have been developed which have enabled the study, at the molecular level, of the processes by which isolated endothelial cells migrate, divide and form capillary-like structures closely resembling the angiogenesis that occurs in response to wounding *in vivo* (2).

The majority of *in vitro* angiogenesis studies have employed the use of endothelial cells derived from large vessels, such as aorta and umbilical vein, primarily because of the relative ease of isolating these cells (3, 4). However, extrapolation of results obtained using these cells, as having direct relevance to endothelial cells of the gastric microvasculature may lead to erroneous conclusions.

Here we describe the isolation of gastric microvascular endothelial cells from normal rat stomach that are suitable to our established model of *in vitro* angiogenesis (5). We further demonstrate that these cells express rat vascular endothelium-specific antigen (6) and endothelial cell-specific Tie2 (7) and *flt-1* (VEGFR1) (8) receptors.

## MATERIALS AND METHODS

### *Isolation of rat gastric microvascular endothelial cells (RGMEC)*

This study was approved by the subcommittee for animal studies of the Long Beach (California) Department of Veterans Affairs Medical Center. The procedure for isolating rat gastric microvascular endothelial cells (RGMEC) was a modified adaptation of two previously described methods (9, 10). For each isolation, the stomachs of four fasted rats were harvested under anesthesia by laparotomy. The stomachs were opened along the greater curvature to expose the mucosal surface and were washed in sterile Hank's buffered saline solution (HBSS) supplemented with antibiotic/antimycotic solution (Life Technologies, Gaithersburg, MD, USA). The stomachs were then sequentially incubated 4 times in 5 mM EDTA/HBSS at 37°C for 15 min with gentle surface scraping, to remove epithelial cells, in between incubations. The mucosa was carefully stripped from the muscle layer, cut into pieces approximately 5 mm in size, and incubated at 37°C for 1 hour in sterile RPMI 1640 medium (Life Technologies) containing 1 mg/ml collagenase A (Sigma Chemicals, St. Louis, MO, USA) and 2 mg/ml dispase (Life Technologies). Alternatively, the mucosal pieces were incubated at 37°C for 1 hour in sterile HBSS containing 1 mg/ml type II collagenase (Sigma Chemicals) and 0.1% BSA followed by incubation at 37°C for 15 min. in sterile HBSS containing 1 mM EDTA/0.5% trypsin (Life Technologies). The collagenase/dispase or trypsin solution was removed following brief centrifugation and the mucosal pieces containing single cells were resuspended in cold endothelial medium (Life Technologies) supplemented with 30% FBS (Atlanta Biologicals, Atlanta, GA, USA) and antibiotic/antimycotic solution (Life Technologies). The suspension was sequentially passed through sterile 500, 300 and 105 µm polypropylene meshes (Spectra, Laguna Hills, CA, USA), centrifuged at 1,000 rpm for 5 min and resuspended in 1 ml cold endothelial medium supplemented with 30% FBS and antibiotic/antimycotic solution. Fifty µl of a 30 mg/ml suspension of magnetic tosyl-activated Dynabeads M-450 (Dyna, Lake Success, NY, USA), which had been sequentially coated with

rabbit anti-mouse IgG (Chemicon, Temecula, CA, USA) and mouse anti-rat PECAM-1 (Chemicon), was added to the cell suspension and the mixture was incubated at 4°C for 15 min with gentle agitation. The volume was increased to 10 ml by further addition of cold endothelial medium and the endothelial cells bound to the magnetic beads were separated from the unbound non-endothelial cells by aspiration under a magnetic field produced by a MPC-1 magnet (Dyna). Another 10 ml of fresh endothelial medium was added to the cell-bound beads and the magnetic separation was repeated an additional 3 times. Finally, the cell-bound beads were resuspended in 5 mls of growth medium [endothelial medium supplemented with 30% FBS, 100 µg/ml heparin sulfate, endothelial cell growth supplement (Sigma Chemicals), and antibiotics/antimycotics], plated in a 60 mm collagen-coated dish and grown at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The isolation process using the anti-PECAM-1 coated magnetic beads was repeated once more following the first passage of the isolated gastric microvascular endothelial cells. The isolated RGMEC cells were routinely grown in endothelial medium supplemented with 20% FBS, 100 µg/ml heparin sulfate, endothelial cell growth supplement, and antibiotics/antimycotics.

### *In vitro angiogenesis*

The *in vitro* angiogenesis assay was performed essentially as previously described (5). Briefly, cells were grown in 60 mm tissue culture dishes until ~80% confluent. The growth medium was replaced with serum-free endothelial medium, supplemented with 100 µg/ml heparin and antibiotics, and the cells were incubated for an additional 24 hours. The cells were then trypsinized, counted, and resuspended in serum-free endothelial medium (at  $4 \times 10^4$  cells/ml). The wells of 24-well tissue culture plates were evenly coated with 0.1 ml/well of growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) which was allowed to solidify at 37°C for 30 minutes, according to the manufacturer's instructions, prior to plating cells. The cell suspension was then plated at 1 ml/well onto the surface of the Matrigel and incubated at 37°C. Four to 8 hours later, the cells were photographed using a Nikon inverted phase contrast photomicroscope (Nikon USA, Garden City, NY) with a video image analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA).

### *Immunostaining for vascular endothelial antigen and Tie2 receptors*

RGMEC cells and normal rat gastric epithelial RGM1 cells (negative controls) were cultured in six-well plates on collagen- or matrigel-coated glass coverslips. After reaching ~70% confluence or undergoing *in vitro* angiogenesis, the monolayers or capillary-like structures adherent to the coverslips were washed twice in phosphate-buffered saline (PBS), and fixed in methanol for 10 min at -20°C. The coverslips were then washed for 5 min three times in PBS and blocked for 5 min in Super Block (Scytek Laboratories, Logan, Utah, USA). The coverslips were again washed three times in PBS and incubated with either mouse monoclonal anti-rat vascular endothelium-specific antigen (MRC-OX 43) (Harlan Sera-Lab, Crawley Down, UK) (1:100 dilution) or rabbit polyclonal anti-Tie-2 receptor (Santa Cruz Biotechnology, Santa Cruz, California, USA) (1:100 dilution) overnight in a humid chamber at 4°C. Normal (pre-immune) mouse IgG and normal (pre-immune) rabbit IgG served as controls. The coverslips were washed for 5 min three times in PBS, and incubated with secondary fluorescein isothiocyanate-conjugated goat anti-mouse (1:100 dilution) or goat anti-rabbit (1:50) antibody (Sigma Chemical) in a humid chamber for 50 min at room temperature. Coverslips were mounted in Citifluor Mounting Media (Ted Pella Inc, Redding, California, USA). Cells were photographed using a Nikon Optiphot microscope with 1600 ASA photographic film (Kodak).

### *Expression of Flt-1 mRNA by RT/PCR*

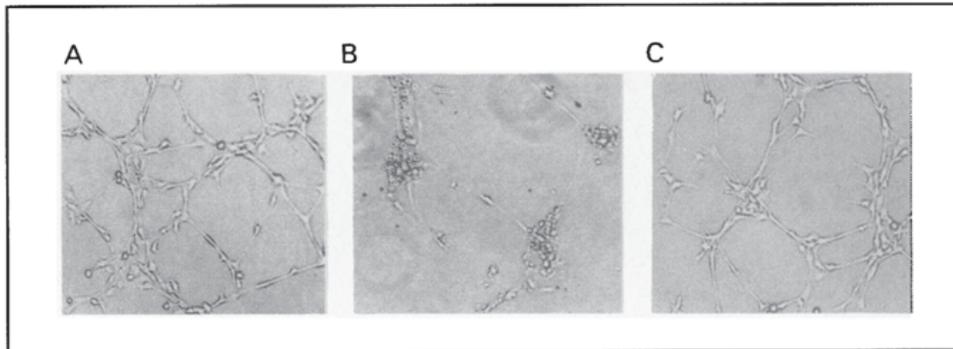
Total RNA was obtained from the isolated RGMEC cells, grown to confluence in 100 mm dishes, using the guanidinium isothiocyanate-phenol-chloroform method (11). Reverse transcription was carried out as previously described (2). The resulting cDNA was used as a template in the subsequent PCR reaction. The PCR specific primer set used for rat *flt-1* was 5'-CTGGAAGCTACAGCTGCAAG-3' (forward) and 5'-AGGCGCAGGGACACCTCTAG-3' (reverse). The PCR for  $\beta$ -actin was used as a positive control for both the reverse transcription and PCR reactions. The specific primer set for rat  $\beta$ -actin (Clontech Laboratories, Inc., Palo Alto, CA) was 5'-TTGTAACCAACTGGGACGATATGG-3' (forward) and 5'-GATCTTGATCTTCATGGTGCTAGG-3' (reverse). The PCR reaction was performed in 50  $\mu$ l of buffer containing 10 mmol/L Tris-HCl [pH 8.3], 2 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 0.2 mmol/L each of deoxyribonucleoside triphosphates, 0.25  $\mu$ g of each primer, and 2 units of *Taq* DNA polymerase. The amplification was performed for 35 cycles of 1 minute at 94°C for denaturing, 1 minute at 58°C for annealing and 2 minutes at 72°C for extension.

## RESULTS

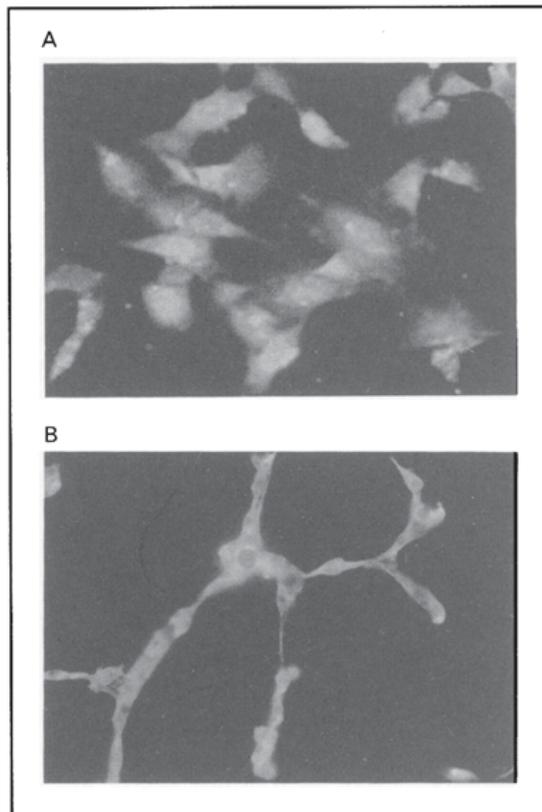
Following initial isolation of the RGMEC cells using the PECAM coated paramagnetic beads, cell colonies consisting of 10 or more cells appeared within 5 to 7 days. Once cells reached confluence (approximately 14 to 18 days), the paramagnetic bead isolation process was repeated. Cell growth was initially slow, with doubling times exceeding 20 hrs, even in the presence of 30% fetal calf serum. However, following passage 5, doubling time was reduced to 16 hrs and the cells took on a typical endothelial cell appearance, more closely resembling human dermal microvascular endothelial cells (HMVEC) than human umbilical vein cells (HuVEC). These cells were also able to grow at a comparable rate in medium containing 2.5–5% fetal calf serum provided the medium was also supplemented with 1 ng/ml vascular endothelial growth factor (VEGF) and 1  $\mu$ g/ml hydrocortisone — both known to enhance microvascular endothelial cell growth in the presence of low serum (12, 13).

When RGMEC cells were plated without serum on growth factor-reduced matrigel, in our established model of *in vitro* angiogenesis (5), they formed capillary-like structures essentially identical to those formed by HMVEC, HuVEC and aortic endothelial cells (*Fig. 1 A*). The *in vitro* angiogenesis was complete within 4–8 hours. By 24–32 hrs, most of these capillary-like structures regressed and the cells appeared apoptotic (*Fig. 1 B*). Inclusion of 10 ng/ml VEGF in the plating medium prevented regression of the capillary-like structures (*Fig. 1 C*) consistent with the role of VEGF as an anti-apoptotic endothelial cell survival factor (14).

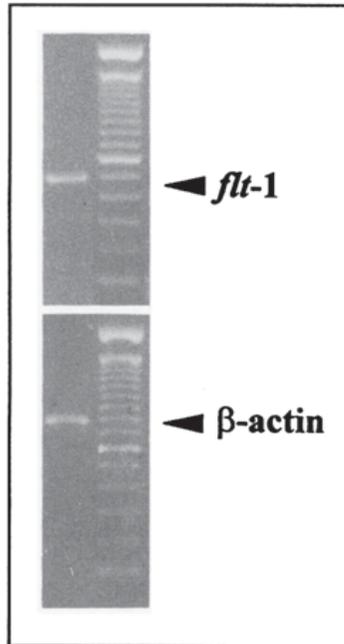
RGMEC cells were also found to stain positively for rat vascular endothelium-specific antigen (6) (*Fig. 2 A*) and for the endothelial cell-specific Tie2 receptor (7) (*Fig. 2 B*). Furthermore, these cells also expressed endothelial cell-specific *flt-1* (VEGF receptor 1) receptor (8) (*Fig. 3*).



*Fig. 1.* *In vitro* angiogenesis of RGMEC cells. Serum-starved RGMEC cells were plated without serum on growth factor-reduced matrigel and incubated. A) Within 4–8 hrs, the RGMEC cells had migrated and joined through the process of anastomosis to form extensive capillary-like structures. Cells were photographed at 8 hrs. B) Within 24–32 hrs, most of the capillary-like structures had regressed and the cells appeared apoptotic. Cells were photographed at 28 hrs. C) In the presence of 10 ng/ml VEGF, regression/apoptosis was prevented even after 32 hrs of incubation. Cells were photographed at 28 hrs. Photographs were at 200 $\times$  magnification.



*Fig. 2.* RGMEC cells express two independent markers of vascular endothelial cells. A) RGMEC cells immunostained for rat vascular endothelium-specific antigen (b). Magnification 1,000 $\times$ . B) RGMEC cells plated on growth factor-reduced matrigel and immunostained for endothelial-specific Tie2 receptor. Magnification 400 $\times$ . Staining was specific since normal rat gastric epithelial cells (RGM1) did not positively stain for either marker (data not shown).



*Fig. 3.* RGMEC cells express *flt-1*. RT/PCR was performed using total RNA from isolated RGMEC cells. (Upper panel) Expected 483 bp PCR product of the rat *flt-1* transcript. (Bottom panel) RT/PCR was performed for  $\beta$ -actin as a control for reverse transcription and PCR reactions. Size standard ladders are shown to the left.

#### DISCUSSION

We have previously demonstrated that angiogenesis is crucial to the healing of both acute and chronic gastric mucosal injury (2, 15). We have also demonstrated that endogenous VEGF is required for the angiogenic response to ethanol-induced injury, as well as for the healing of erosions; and, that the common NSAID, indomethacin, strongly inhibits gastric angiogenesis resulting in delayed healing (15). The molecular processes underlying gastric angiogenesis are, however, difficult to assess and/or dissect in the *in vivo* situation because the number of different cells, and the interactions between them, preclude relating a particular process to a specific cell type with any degree of certainty. We have previously used an *in vitro* angiogenesis assay to demonstrate that mitogen activated protein kinase (MAPK) of endothelial cells is a chief target of the inhibition of angiogenesis by both non-selective NSAIDs and selective COX-2 inhibitors (5). Although this finding was found to apply to three different types of endothelial cells, our study did not include endothelial cells of gastric origin.

Several studies indicate that there are not only phenotypic differences between endothelial cells of large vessels and those of the microvascular endothelium but also that there are tissue-specific differences between microvascular endothelial cells derived from different organs (16–19). We have

adapted aspects of two previous methods (9, 10) as a means of efficiently isolating rat gastric microvascular endothelial cells. We have demonstrated that these cells express rat vascular endothelium-specific antigen and both Tie2 and *flt-1* receptors, receptors specific to endothelial cells (7, 8). We have further shown that these cells form capillary-like structures on growth factor reduced matrigel in the absence of serum and that they are responsive to the endothelial growth factor VEGF. These cells will likely be useful for investigating the molecular mechanisms underlying the injury-triggered angiogenesis crucial to gastric healing and its impairment by mediators such as NSAIDs.

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