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Rapid communication

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ISOLATION OF MORPHOLOGICALLY AND FUNCTIONALLY INTACT GASTRIC MUCOSAL MICROVESSELS RAPID COMMUNICATION

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Gastric mucosal microvessels were isolated after arterial perfusion of the rat stomach with magnetized iron oxide suspension. After homogenization of scrapped gastric mucosa, microvessels were initially separated with a high power magnet and further separated and purified by using a nylon sieve. Aliquots of purified microvessels were assessed for viability, histologic appearance, ultrastructure and generation of prostacyclin. Microvessels were plated on Matrigel and cultured in DMEM with high glucose and 10% FBS for 1, 3 or 5 days. After 1, 3 and 5 days of culturing, endothelial viability was assessed with Fast green exclusion, and the basal and stimulated (with calcium ionophore) generation of prostacyclin was determined by assaying aliquots of the incubating medium for 6-keto $PGF_{1\alpha}$. At 1 and 3 hrs after isolation, microvessels demonstrated intact morphologic structures as reflected by transmission EM and $92 \pm 4\%$ of viable endothelial cells. The microvessels plated on Matrigel maintained good viability for at least 5 days and generated prostacyclin at the baseline and following ionophore stimulation. These data demonstrate that isolated microvessels cultured under optimal conditions are fully viable and functional.

K e y w o r d s: *gastric microvessels, isolation, endothelial cells, prostacyclin, ultrastructure*

INTRODUCTION

Blood flow through gastric mucosal microvessels – capillaries and collecting venules – plays a crucial role in supplying mucosa with oxygen and

nutrients and for removal of toxic metabolic products (1). Moreover, endothelial cells of microvessels synthesize various anti coagulants, prostanoids (e. g. prostacyclin), and are the targets for action of numerous hormones, growth factors and pharmacological compounds. Regulation of homeostasis is another central role of the endothelium (2–6).

Endothelial cells lining gastric mucosal microvessels are, in addition to the surface epithelium, major targets of acute of injury by ethanol and NSAIDs (7–10). Injury of the microvessels produced by these damaging agents leads to microvascular stasis and ischemia, which may result in and/or contribute to development of focal deep necrosis, such as erosions or ulcerations (7–10). The participation of endothelial cells in various biological phenomena in the gastrointestinal tract, however, has been difficult to explore in the absence of a valid culture model. There is evidence indicating that endothelial cells from different organs exhibit tissue – specific differences and that gastrointestinal endothelial cells behave differently from their counterparts in other vascular beds (11, 12). For example, microvascular endothelial cells of the blood-brain barrier differ in their responses from those of endothelial cells from the lungs, kidney, skin, heart or omentum (13, 14). Our current knowledge about the structure and function of human endothelial cells stems primarily from studies of large vessel endothelial cells, while all major physiological and pathological events take place mainly at the level of the tissue microvasculature. Furthermore, recent work has shown several differences between the endothelium isolated from large vessels and cultured microvascular endothelium (15–18). Immunohistochemical identification of cellular antigen expression in situ supports this view. Functional and pharmacological studies based on large vessels may therefore not be valid for the microvascular endothelium. Furthermore, endothelial cell lines, such as human umbilical vein endothelial cells (HUVEC) may have dedifferentiated during prolonged cultures and originate from large vessels not related to gastrointestinal tract. Therefore, information derived from these cell lines may not be relevant to gastrointestinal endothelial cells.

Our present paper describes a procedure for isolation and culture of gastric mucosal microvessels from normal rat stomach, which seems to be a suitable *in-vitro* model for physiological and pharmacological studies.

METHODS

Isolation of gastric mucosal microvessels

Gastric mucosal microvessels were isolated from the gastric mucosa after arterial perfusion of the stomach with oxygenated PBS followed by injection of magnetized iron oxide suspension. After homogenization of the scraped mucosa in 10 ml of Tris-HCl buffer, microvessels were initially

separated with a magnet and further separated and purified by using nylon sieves, as described previously for kidney microvessels (19). Purified gastric microvessels were then suspended in nutrient oxygenated media, identically as described in the original method (19). The preparation of microvessels was standardized by determining their dry weight per 1 ml suspension and measuring their protein content with Lowry's method (20).

Endothelial cell viability

Endothelial viability was assessed using the Fast green exclusion test, as described in our previous paper (21). The principle of this method is based on the fact that viable cells are able to exclude the dye while dead cells lose this ability. Coded cytospin slides were evaluated and counted under a Nikon Optiphot light microscope (magnification 200 ×) by two investigators unaware of the code. A minimum of 10 fields and 200 cells were counted per slide. The viability was expressed as the percentage of viable cells per total counted cells in ten microscopic fields.

Transmission EM

Samples of the microvessels were taken at the end of each experiment, fixed and processed routinely for transmission electromicroscopy (EM) similarly as in our previous study of isolated human gastric glands (21). Coded specimens were viewed under a Phillips 400 transmission EM scope. Three specimens from each group were evaluated.

Culturing microvessels on Matrigel

Standardized aliquots of microvessels were plated on tissue culture plates evenly coated with Matrigel (Becton Dickinson) 0.1 ml/well, similarly as described in our previous study of human endothelial cells (22), and cultured in DMEM medium containing 10% FBS, antibiotics: antimycotics in atmosphere of 5% and 95% air at 37°C in a humidified incubator.

Prostacyclin generation

After washing with fresh medium, microvessel cultures were incubated for 1 hour with either medium alone or with 5 μM A23187 (calcium ionophore) for 30 min at 37°C. The samples were centrifuged at $7,000 \times g$ for 30 sec and the supernatants stored in a deep freezer at $-70°C$ until the day of assay.

The 50 μl aliquots of the incubation medium were used for assay of 6-keto-PGF$_{1\alpha}$ using ^{125}I-6-keto-PGF$_{1\alpha}$ RIA (New England Nuclear, Boston, MA). This method includes separation with Vac — Elut and Bond — Elut C$_{18}$ extraction columns (Analytichem International, Harbor City, CA) and extraction with methanol, cyclohexan and ethyl acetate as described in detail in our previous study (23).

Statistical Analysis

Values are expressed as the mean $\pm$ S.E.M. Student's t-test and Pearson's correlation coefficient were used to analyze the data for cell viability and prostacyclin generation. One way ANOVA and Bonferroni correction were used to compare data between more than two groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

A purified preparation of rat gastric microvessels containing predominantly capillary microvessels and collecting venules was obtained (*Figure 1*). At 1 and 3 hours after isolation, microvessels demonstrated intact morphological structures reflected by transmission electronmicroscopy (EM) (*Figure 2*) and viability of $92 \pm 4\%$ of endothelial cell.

When plated on matrigel, endothelial cells of microvessels demonstrated viability of $92 \pm 3\%$, $90 \pm 4\%$ and $94 \pm 5\%$, respectively, at 1, 3 ad 5 days. Five days after plating on matrigel, microvessels had a normal appearance structurally and demonstrated capillary sprouting (*Figure 3*).

At each study time (1, 3 and 5 days) plated microvessels generated prostacyclin at the baseline. Addition of calcium ionophore at concentration 5 µM significantly increased prostacyclin generation (*Figure 4*).
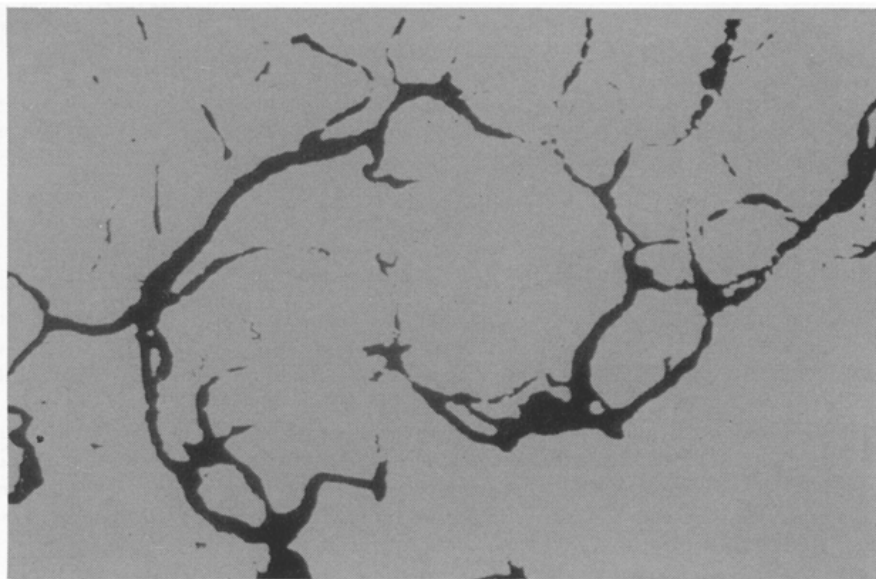

Fig. 1. Photomicrograph of mucosal microvessels isolated from the rat stomach. Microvessels were suspended in oxygenated nutrient medium DMEM with high glucose and 10% FCS. Histologic assessmet 3 hrs after isolation demonstrates intact morphologic structures. Viability assessment with the fast green exclusion test demonstrated endothelial cell viability of $92 \pm 4\%$.
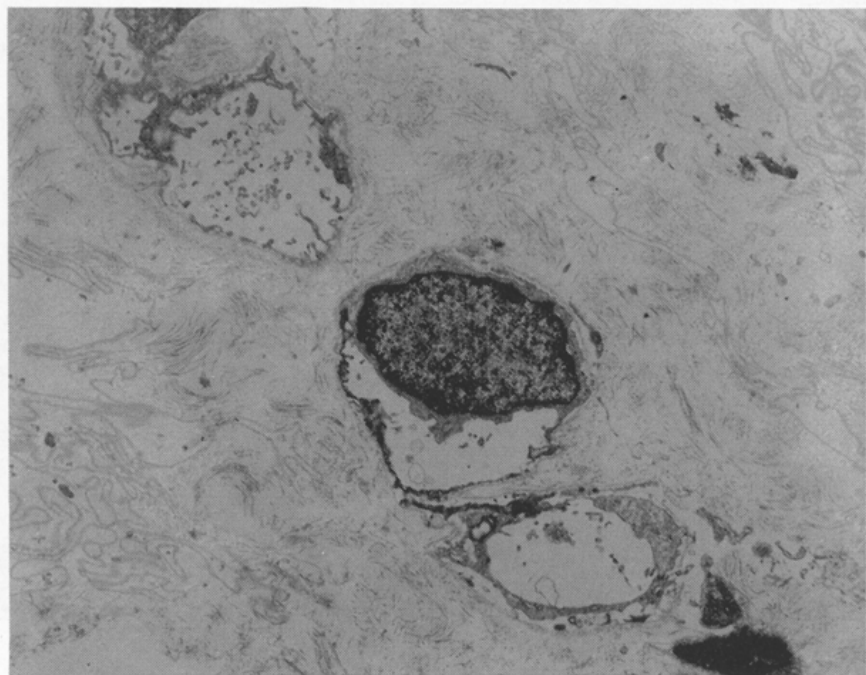
Fig. 2. Transmission electron micrograph of gastric microvessels isolated from the rat stomach, microvessels were suspended in oxygenated nutrient medium. Three hours after isolation, the basement membrane and endothelium have normal appearance (magn ~ 5,000 ×).
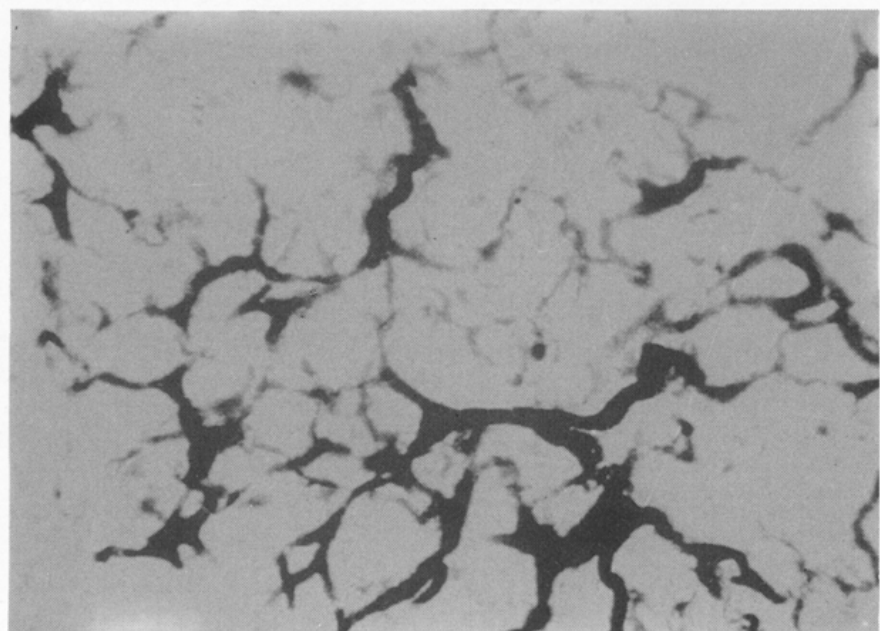
Fig. 3. Photomicrograph of mucosal microvessels isolated from the rat stomach, 5 days after plating on Matrigel inserts and culturing in DMEM with high glucose and 10% FCS. Structurally, microvessels have a normal appearance and demonstrated some capillary sprouting. Viability assessment with fast green exclusion demonstrated 94 ± 5 viable endothelial cell.
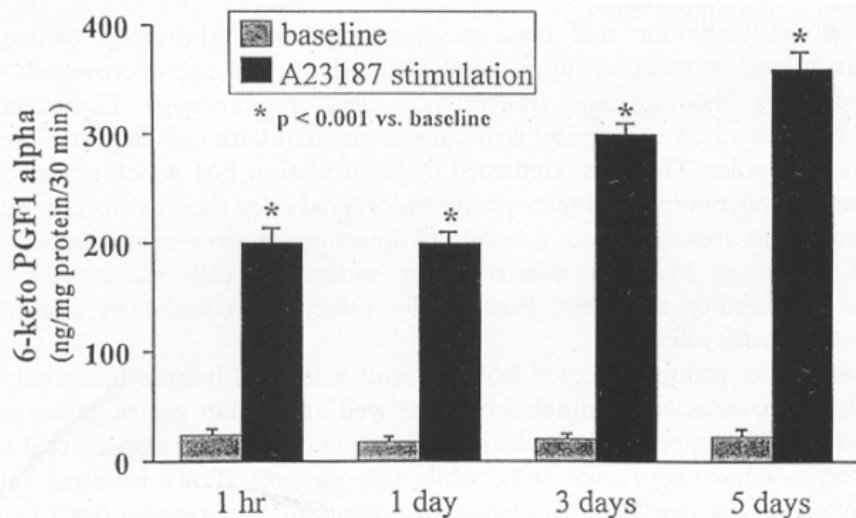
Fig. 4. Generation of 6-keto PGF1α by gastric microvessels at 1 hr after isolation and 1, 3 and 5 days after plating on Matrigel inserts. Microvessel preparations were incubated in a shaking bath at 37°C in medium alone for 30 min. The medium was replaced with the medium containing calcium ionophore A23187,5 µM and incubation continued for the next 30 min. The 100 µm aliquots of medium were frozen and stored at −70°C. Assay of 6-keto PGF$_{1a}$ (stable product of prostacyclin hydrolysis) was performed identically as in our previous study of isolated gastric surface epithelial cells using the RIA Kit. Results represent the mean±SE of six determinations in two separate experiments. The data demonstrate that isolated microvessels retain ability to generate 6-keto PGF$_{1a}$ and are able to significantly increase its generation upon A23187 stimulation. Since one of the important functions of endothelial cells is synthesis of prostacyclin, these data clearly indicate that the cultured microvessels are not only viable by morphologic criteria, but also by functional assesment.

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DISCUSSION

A novel technique has been developed in our laboratory to isolate selectively gastric mucosal mircovessels. The identity of the microvessels was confirmed by histology and transmission electronmicroscopy. Light microscopy demonstrated anatomical structures consistent with capillary vessels and collecting venules. This was confirmed by transmission EM which revealed no evidence of morphological injury to the endothelial cells; their cytoplasm nuclei and basement membrane as a result of injection of iron oxide suspension. When plated on Matrigel, microvascular endothelial cells maintained high degree of viability and were functionally intact as reflected by ability to generate prostacyclin.

Some other groups reported isolation and culture of human intestinal and colonic microvascular endothelial cells, as well as, human gastric endothelial cell (24–26). Their preparations clearly differ from ours in this respect, that they cultured isolated endothelial cells, while our method allows isolating intact microvessels. The model of isolated gastric mucosal microvessels described in this study appears to be suitable for investigating *in vitro* effect of a variety of biologically active substances: hormones, calcium, growth factors, phospholipids, prostanoids, as well injurious and protective agents. This model can be used for a short-term *in vitro* study (1–12 hrs) by utilizing microvessel suspension, or for longer-term studies utilizing culture on Matrigel.

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