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# CHARACTERISTICS OF PORCINE CORONARY ARTERY ENDOTHELIAL CELLS IN CULTURE: COMPARISON WITH AORTIC ENDOTHELIUM

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**Summary:** In the present study we compared the characteristics of porcine coronary artery endothelial cells in culture with the characteristics of aortic endothelium. Morphologically, coronary artery endothelial cells were larger than aortic endothelial cells. Under basal culture conditions, both coronary artery and aortic endothelial cells secreted significant amounts of prostacyclin and endothelin-1. The outstanding finding is that coronary artery endothelial cells secreted larger amounts of endothelin-1 than aortic endothelial cells. Immunofluorescence staining revealed that individual endothelial cells synthesize both substances. This comparative study provides important evidence indicating that the characteristics of coronary artery endothelial cells differ from those of aortic endothelial cells. 

Vascular endothelial cells play a primary role in the initiation of atherogenesis, and many studies have been performed on cultured endothelial cells to date (1,2). The endothelial cells used in previous experiments, however, have been mostly collected from human umbilical vein and bovine or porcine aorta (3), and the findings have been extrapolated to coronary artery endothelial cells.

Recent studies indicate that endothelial cells from different tissues vary in cell function, e.g., in the production of various biological substances and their response to specific vasoactive agents (4,5,6). We presented evidence suggesting different characteristics of endothelial cells in coronary arteries and the aorta in a previous paper (7). Based on these findings, we think that it is essential to characterize coronary artery endothelial cells specifically in order to understand the pathogenesis of coronary arteriosclerosis.

To our knowledge, only a few studies have concerned the coronary artery endothelial cells. Burnet et al. reported a primary culture of coronary artery endothelial cells from a single pig

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(8), and Revtyak et al. (6) and Hieda et al. (9) subcultured coronary artery endothelial cells obtained from a single cow. Thus, our knowledge of the characteristics of coronary artery endothelial cells is very limited. Very recently we established a culture of porcine coronary artery endothelial cells, and in this paper we present data demonstrating an important finding that the characteristics of coronary artery and aortic endothelial cells differ.

## Materials and Methods

**Endothelial cell culture**: Fresh porcine heart and thoracic aorta removed within 3 hours after sacrifice were vigorously rinsed with 500 ml sterile RPMI1640 medium in a flask. Both right and left coronary arteries were excised from the heart. After removing the connective and adipose tissue around the aorta and coronary arteries, they were cut open (an approximately 10 cm length of aorta and 3-7 cm lengths of coronary artery) on a stainless steel dish using scissors and forceps. The luminal surface was gently applied to filter paper, which was then dipped in 1700 U/ml dispase solution for 20 minutes at 37℃. After digestion, the filter paper was removed, and the luminal surface of the arteries was rinsed with RPMI1640 containing 10% calf serum. The cell suspension thus obtained was centrifuged at 300× g for 6 min, and the pellet was resuspended in 4 ml of RPMI1640 supplemented with 20% fetal calf serum, to which $NaHCo_3$, 25 mM, penicillin, 25 U/ml, heparin, 180 mg/ml, and endothelial cell growth supplement, 3 mg/ml were added. The suspension was then seeded onto a 60 mm culture dish (Corning, USA). Culture was continued under 5% $Co_2$-95% air at 37℃, and the medium was changed every other day (10,11,12).

After 3-4 days, colonies were removed and seeded onto a 12-well culture plate (Corning, USA). Serial passages were accomplished by brief exposure to 0.25% trypsin with 0.01% EDTA. After resuspension in culture medium, the pellets were plated at approximately 1.0-1.7× $10^5$ cells/well. Cells between the second and the fifth passage were used. The cells were identified as endothelial cells by both their typical cobblestone appearance, and the detection of human platelet relating factor VIII by immunofluorescence staining (13).

**Cell size:** The 30-80% confluent cells subcultured at the fifth passage on a Lab-tek chamber slide (Nunc, Denmark) were fixed with 95% methanol for one hour and then stained with hematoxylin-eosin. The size of 26 to 73 cells from each animal was measured using Olympus SP500 color image analyzer equipped with a microscope (Olympus, Japan).

**Growth activity:** Cell growth activity was determined according to the method described by Hall et al. (14). The 90% confluent cells at the fifth passage were subcultured in a 100 mm culture dish ( cell density: $10^4$ cells/$cm^2$ ). After 24 hours, the cells were trypsinized and the precipitated cells were fixed with 70% cold ethanol for one hour. They were then incubated with anti-proliferating cell nuclear antigen (PCNA) antibody (DAKO, Denmark) at room temperature, and further incubated with FITC-conjugated anti-mouse IgG antibody (DAKO, Denmark) for 30 minutes at room temperature. The cells were subsequently resuspended in 1 ml of 0.1 M phosphate-buffered saline (PBS), and the number of PCNA-positive cells was counted by flow cytometry. Growth activity is expressed as percentage of PCNA-positive cells.

**Determination of prostacyclin and endothelin-1:** Both coronary artery and aortic endothelial cells at the fifth passage reached confluence at 6-12 days. The confluent coronary artery endothelial cells and aortic endothelial cells cultured in a 12-well culture plate were rinsed twice with serum-free RPMI1640, and the plate was then incubated with 2 ml/well

RPMI1640 containing 20% fetal calf serum (without antibiotics or growth factor) at 37℃ for varying periods (15, 30, 60 minutes). At the end of the incubation period, aliquots of culture medium were removed and stored frozen at -80℃ until assayed. The amounts of prostacyclin in the medium, were measured with the commercially available 6 keto PGF1$\alpha$ kit manufactured by Cayman Chemicals, Ann Arbor, MI. Endothelin-1 released into the medium was measured directly by enzyme immunoassay (EIA) (15).

**Identification of endothelial cell synthesizing prostacyclin and endothelin-1:** We performed immunofluorescence staining in an attempt to identify cells synthesizing either prostacyclin and endothelin-1 or both. Coronary artery endothelial cells subcultured in a Lab-tek chamber slide were fixed with 95% methanol for one hour and double-stained with anti-6 keto PGF1$\alpha$ rabbit antibody (Advanced Magnetics Inc., Cambridge, MA) and anti-endothelin-1 mouse monoclonal antibody (Kokusai Shiyaku, Kobe, Japan). Rhodamine conjugated anti-rabbit IgG swine antibody (DAKO, Denmark) and FITC conjugated anti-mouse IgG rabbit antibody (DAKO, Denmark), respectively, were used as the second antibody. The stained preparations were observed under a laser scanning fluorescence microscope (Olympus, Japan) to assess the distribution of prostacyclin and endothelin-1.

**Chemicals:** RPMI1640 medium, fetal calf serum, and calf serum were purchased from GIBCO Laboratories, N.Y., and dispase was from Godo Shusei Co., Ltd., Tokyo. Heparin was obtained from Sigma Chemical Co., St. Louis, Mo., endothelial cell growth supplement from Collaborative Research, Inc., Bedford, MA, penicillin from Ban-yu Pharmaceutical Co., Tokyo, streptomycin from Meiji Seika, Tokyo, amphotericin-B (Fungizon) from Bristol-Myers Squibb, Tokyo, vancomycin hydrochloride from Shionogi Co., Ltd., Osaka, Japan, and trypsin from Nakarai-Tesque, Kyoto, Japan.

**Statistical analysis:** Values are expressed as means ± S.D. Statistical analysis of the data was performed using Student's $t$ test or paired $t$ test. A $P<0.05$ level was considered to be significant for all tests.

## Results

### Cell size

Both coronary artery and aortic endothelial cells in primary culture became confluent in 7-10 days. Morphological examination revealed that coronary artery endothelial cells in primary culture tended to be larger than aortic endothelial cells. In succeeding passages, the endothelial cells from coronary arteries become larger than the endothelial cells from the aorta, and at the fifth passage the coronary artery endothelial cells were significantly larger than the aortic endothelial cells (Table 1). A typical picture of cells at the fifth passage is shown in Fig. 1.

### Growth activity

The growth activity of both coronary artery and aortic endothelial cells was compared by determining the percentages of PCNA-positive cells. Cells at the fifth passage were used for this particular purpose. There were no differences between the cell growth activity of coronary artery and aortic endothelial cells (44.8 ± 12.4 vs. 42.1 ± 8.9%, 5 animals).

**TABLE 1.** Comparison of the size of cultured porcine coronary artery endothelial cells and aortic endothelial cells

| | Cell size ($\mu m^2$) | |
|---|---|---|
| Animal No. | C-EC | A-EC |
| 1 | 1021 | 640 |
| 2 | 828 | 558 |
| 3 | 968 | 819 |
| 4 | 1799 | 1164 |
| Mean±SD | 1154±438* | 795±269 |

C-EC, coronary artery endothelial cells; A-EC, aortic endothelial cells. The size of 26-73 cells was measured in each animal and the average size was calculated. Data represent the means± SD of four animals. * $P<0.05$, significant difference from aortic endothelial cells (Paired $t$-test).

## Prostacyclin and endothelin-1 secretion

**Time course experiments:** Secretion of prostacyclin and endothelin-1 into the medium was assessed in relation to time elapsed using cells obtained from a single pig. Cells at the second passage were used in the preliminary experiment. Endothelial cells from both the coronary artery and aorta secreted prostacyclin and endothelin-1 in a time-dependent manner,

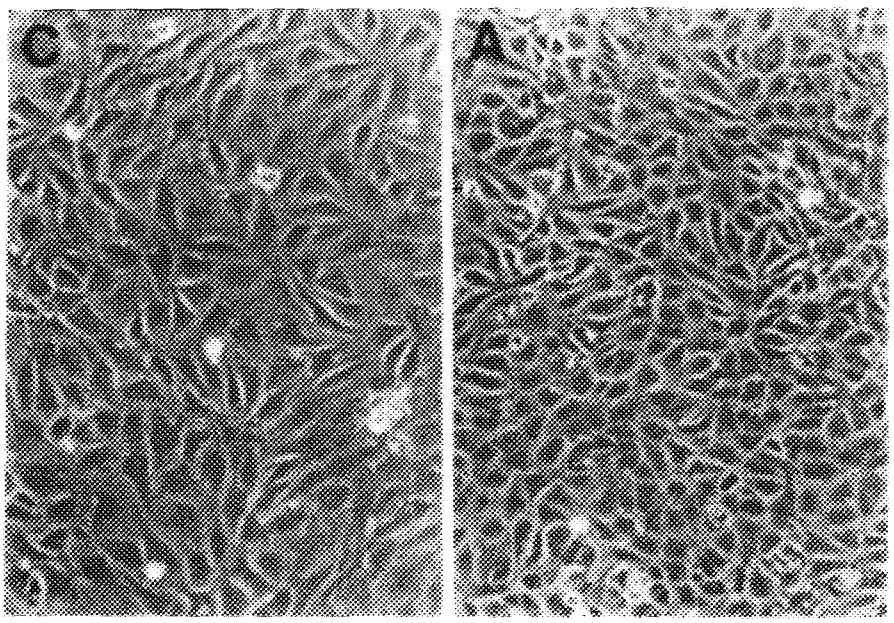


**Fig. 1.** Light photomicrographs of cultured porcine coronary artery endothelial cells (C) and aortic endothelial cells (A) at confluence (original magnification, × 66). Coronary artery endothelial cells and aortic endothelial cells obtained from a single animal were subcultured under the same condition. Cells from the fifth passage are shown.

as shown in Fig. 2. In further experiments, cells were cultured for 30 minutes and medium was removed to measure both prostacyclin and endothelin-1.

**Secretion of prostacyclin and endothelin-1 by cultured endothelial cells in serial passages:** Secretion of prostacyclin and endothelin-1 by both coronary artery and aortic endothelial cells in relation to serial passages is shown in Fig. 3. The amount of prostacyclin secreted by coronary artery endothelial cells increased gradually as passage proceeded up to the fourth, whereas secretion by aortic endothelial cells varied greatly (Fig. 3a). The amount of endothelin-1 secretion by coronary artery endothelial cells tended to be greater than the amount secreted by aortic endothelial cells throughout the passages (Fig. 3b).

**Prostacyclin and endothelin-1 secretion by the cells at the second passage:** There were no differences between the amounts of prostacyclin secreted by coronary artery endothelial cells and by aortic endothelial cells. The amount of endothelin-1 secretion by coronary artery endothelial cells, on the other hand, was significantly greater than by aortic endothelial cells (Table 2).

**Identification of cells synthesizing prostacyclin and endothelin-1**

Immunofluorescence staining revealed that both prostacyclin and endothelin-1 were positive in the cytoplasm of the same coronary artery endothelial cell (Fig. 4), indicating that individual cells are able to synthesize these two substances simultaneously. The same was true of aortic endothelial cells (data not shown).

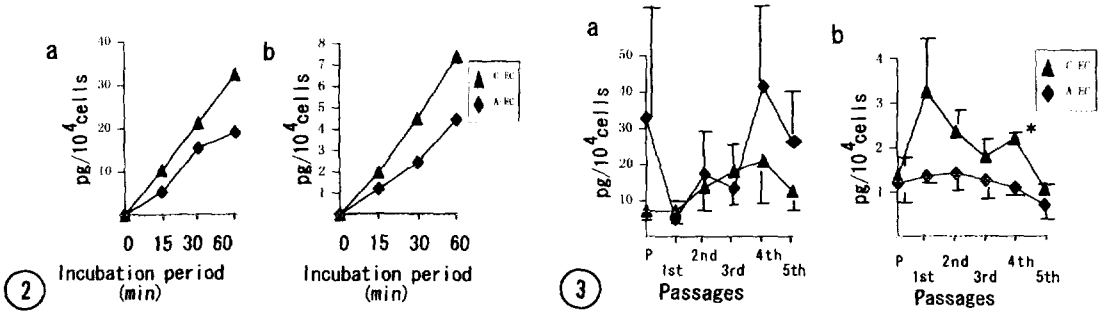


**Fig. 2.** Prostacyclin and endothelin-1 secretion in relation to time elapsed. Coronary artery endothelial cells (C-EC) and aortic endothelial cells (A-EC) were incubated in a 12-well culture plate with 2ml/ well culture medium, and a portion of the incubation medium was collected to measure of prostacyclin (a) and endothelin-1 (b). Data are shown as means of triplicate determinations.

**Fig. 3.** Prostacyclin (a) and endothelin-1 (b) secretion by both coronary artery endothelial cells (C-EC) and aortic endothelial cells (A-EC) in serial passages. P, primary culture. Data shown are means of three animal experiments performed in triplicate for each animal, with standard deviations indicated by vertical bars. *P<0.01.

**TABLE 2.** Secretion of prostacyclin and endothelin-1 by both coronary artery and aortic endothelial cells at the second passage

| Substances | Number of animals | C-EC | A-EC |
|---|---|---|---|
| Prostacyclin, pg/$10^4$ cells | 7 | 13.0 ± 6.3 | 14.7± 10.8 |
| Endothelin-1, pg/$10^4$ cells | 7 | 2.38 ± 1.15* | 1.59± 0.48 |

C-EC, coronary artery endothelial cells; A-EC, aortic endothelial cells; Data shown are means of seven animal experiments performed in triplicate for each animal, with standard deviations. * $P<0.05$, significant difference from aortic endothelial cells.

## Discussion

A number of previous studies have characterized the nature of endothelial cells from both aorta and human umbilical vein (3), however, there have been only a few reports specifically assessing the nature of coronary artery endothelial cells (6,8,9). In the present study, we established a culture of porcine coronary artery endothelial cells, and assessed some of the characteristics of the cells. We demonstrated that porcine coronary artery endothelial cells in

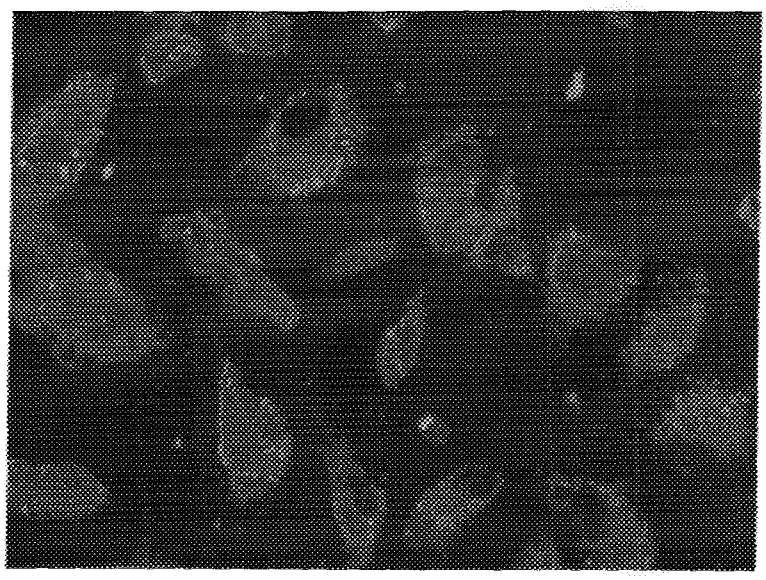

Fig. 4. Immunofluorescence staining of prostacyclin and endothelin-1 on cultured porcine coronary artery endothelial cells. Cells at the fifth passage plated on a Lab-tek chamber slide were fixed with 95% methanol for one hour and exposed to antibodies to 6 keto PGF1$\alpha$ and endothelin-1 followed by the second antibodies. The presence of both prostacyclin (red) and endothelin-1 (green) is demonstrated under a laser scanning fluorescence microscope.

culture differ from aortic endothelial cells in both morphology and the secretion of endothelin-1.

Coronary artery endothelial cells in primary culture tended to be larger than aortic endothelial cells, and this became more obvious as the passage proceeded. There was no significant difference in growth activity between these two different types of endothelial cells. To our knowledge, there have been no studies reporting morphological differences in endothelial cells derived from different arteries in the same species.

Endothelial cells have been known to produce a variety of biologically active substances, and we selectively measured two of the major substances, prostacyclin, a potent vasodilator (16), and endothelin-1, the most potent constrictor reported thus far (17). Under basal conditions, endothelial cells from the coronary artery and aorta secreted significant amounts of prostacyclin in both primary culture and succeeding passages. Secretion of prostacyclin by aortic endothelial cells varied greatly from cell to cell as well as in relation to the passage. The most significant observation in this study was that secretion of endothelin-1 by coronary artery endothelial cells was larger in amount than secretion by aortic endothelial cells. Although endothelial cells are known to secrete a variety of biologically active substances, whether an individual cell can synthesize both of these substances, or separate cells have different functions and synthesize different substances had not been examined in endothelial cells. We have clearly shown that individual endothelial cells synthesize both substances simultaneously.

In conclusion, our observation demonstrating that the characteristics of coronary artery endothelial cells and aortic endothelial cells differ, is of particular importance in the study of coronary artery physiology and pathology, and in particular further strengthens the significance of coronary artery endothelial cell culture.

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