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Characterization of porcine arterial endothelial cells cultured on amniotic membrane, a potential matrix for vascular tissue engineering

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Abstract

The existing of basement membrane improves the development of endothelium while constructing blood vessel equivalent. The amniotic membrane (AM) provides a natural basement membrane and has been used in ocular surface reconstruction. This study evaluated the molecular and cellular characteristics of porcine vascular endothelial cells (ECs) cultured on AM. ECs cultured on AM expressed the endothelial marker vWF and exhibited normal endothelial morphology. Here, we demonstrated that AM enhanced the expression of intercellular molecules, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and adhesion molecule VE-cadherin at the intercellular junctions. The expression level of integrin was markedly higher in ECs cultured on AM than on plastic dish. Furthermore, the AM down-regulated the expression of E-selectin and P-selectin in both LPS-activated and non-activated ECs. Consistently, adhesion of leukocytes to both activated and non-activated cells was decreased in ECs cultured on AM. Our results suggest that AM is an ideal matrix to develop a functional endothelium in blood vessel equivalent construction.

Keywords: Amniotic membrane; Cell adhesion molecules; Endothelial cell; Matrix

In vascular bypass graft, hemocompatibility is an important consideration, especially in grafts involving small-diameter (<6 mm) blood vessels because of their low flow velocities [1,2]. Although autologous vessels, including the saphenous vein and internal mammary artery, remain to be the standard for small-diameter grafts, the source of vascular grafts is limited in patients with vascular disease, amputation, and history of previous harvest. Furthermore, venous grafts develop atherosclerosis or mechanical failure more easily when implanted as arteries [3]. Tissue-engineered blood vessels provide a promising way to generate suitable vascular grafts. One of the critical components for a successful tissue-engineered blood vessel equivalents (BVEs) [4], is to have an intact, non-activated endothelium in the lumen surface [1], so that the BVE is hemocompatible. ECs have been seeded in various BVE constructs however, the endothelium formed exhibited low patency upon grafting due to a lack for well-developed basement membrane [1,2]. Other researchers have used fibroblast sheets with extracellular matrix (ECM) as a substitute for natural basement membrane [5–7], but the long fabrication period made it impractical. Amniotic membrane (AM) is a natural extracellular matrix, and has been used to culture limbal epithelial cell for transplantation [8,9]. Several studies showed that AM possesses anti-angiogenic and anti-inflammatory properties [10,11], and is able to preserve limbal epithelial stem cells in culture [12]. It is thus of interest to assess if AM is also a good substrate for EC growth. Hence in this study, we use AM as a

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substrate to assess EC growth, and several molecular and cellular features related to endothelial function.

To evaluate the integrity of endothelial monolayer cultured on AM, we analyzed the expressions of endothelial junctional proteins, including the platelet-endothelial cell adhesion molecule (PECAM) and vascular/endothelial-cadherin (VE-cadherin). PECAM and VE-cadherin are expressed in an endothelium-specific fashion, and are of great importance in preserving the integrity as well as diffusion barrier function of endothelial monolayer [6,13]. We also analyzed integrins for assessing the interaction between EC and AM. Integrins are involved in the signaling axis linking ECM and cytoskeleton, which is required for EC morphogenesis in the process of tube formation [14]. Endothelial behavior is influenced by the interaction of EC with its underlying basement membrane (BM), consisting predominantly of collagen IV (Col IV) and laminin [15]. In several studies, the adhesion of basal ECs to Col IV was shown to be mediated by two major integrins: α2β1 and α3β1 [16–18]. We therefore used integrin β1 in this study to assess EC-AM adhesiveness.

Furthermore, we examined the effects of AM on the expressions of endothelial cell adhesive molecules (CAMs). The CAMs play an important role in the binding and activation process of leukocytes, and are of importance in the inflammatory immune response [19]. The main endothelial CAMs involved in the leukocyte recruitment during inflammation are E- and P-selectin, and two members of the Ig-gene superfamily, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Bacterial lipopolysaccharide (LPS), an inflammatory mediator associated with Gram-negative infection and sepsis, induces the endothelial expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 [20–23]. Therefore, we also checked how AM would modulate the LPS-activated upregulation of endothelial CAMs.

We demonstrate in this study that AM promotes the proliferation and junctional formation of co-cultured endothelium. While the endothelial expressions of PECAM, VE-cadherin and integrins are upregulated in the presence of AM, those of the pro-inflammatory CAMs are however downregulated. The downregulation of endothelial CAMs by AM is correlated with decreased adhesion of endothelium to leukocytes, even as challenged with LPS. These results strongly argue for a potential application of AM as an anti-inflammatory matrix, which supports endothelial growth and morphogenesis in the process of vascular tissue engineering.

Materials and methods

Preparation of the AM. The AM was obtained from full-term pregnant sows in Yorkshire, Landrace, and Duroc breeds. AM was rinsed with phosphate-buffered saline (PBS) containing 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma–Aldrich, USA), 0.25 μg/ml amphotericin B (Gibco, USA), 25 μg/ml gentamicin (Gibco, USA) and then preserved in PBS containing 50% glycerol at −80 °C. Before use, the AM was washed with PBS and incubated with 2.4 U/ml dispase II (Roche, Germany) at 37 °C for 30 min. The amniotic epithelial layer was scraped off and the AM was washed three times with PBS, spread out in a sterile dish, cut into 2 × 2 cm pieces and used in the following experiments.

Cell culture. Porcine aorta was rinsed with PBS containing 150 U/ml penicillin, 150 μg/ml streptomycin and cut into several pieces. The luminal side of the aorta was treated with 0.25% trypsin (HyClone, USA) and the endothelial cells (ECs) were scraped off. The cells were then incubated in Media 199 (Gibco, USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml amphotericin, 25 μg/ml gentamicin, and 2.5 μg/ml pituitary extract at 37 °C in a humidified atmosphere (95% O_2/5% CO_2) and used between passages 3 and 7. The media were changed every 2 days thereafter. The ECs were confirmed by von Willebrand factor expression.

Cell proliferation. ECs were seeded at 5×10^3 cells/cm^2 on AM and on plastic surface. At 0, 12, 24, 48, and 72 h after seeding, the cells were fixed and stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1:1000, v/v) (Molecular Probes, USA). Three areas were taken randomly from each sample and photographed by a fluorescent microscope. The cell numbers on three photographs for each sample were counted and summed. All experiments were carried out in triplicate. Student's *t*-test was used for statistical analysis.

Immunofluorescent staining. For visualizing the intercellular molecules localized at cell–cell junctions and integrins of ECs cultured on the AM, cells were seeded at 10^5 cells/cm^2 on AM. At 10 days after seeding, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with BSA in PBS. Actin was labeled with 0.2 M Alexa Fluor-phalloidin (Invitrogen, USA) at 37 °C for 30 min. vWF was visualized by antibody. PECAM-1 and VE-cadherin were visualized by staining with mouse anti-rat CD31 antibody (1:50, v/v) (BD bioscience, USA) and rat anti-porcine CD144 antibody (1:20, v/v) (Serotec, UK) at 37 °C for 30 min followed by fluorescein-conjugated goat anti-mouse IgG antibody (1:200, v/v) (Chemicon, USA) and Texas red-conjugated goat anti-rat IgG antibody (1:200, v/v) (Jackson ImmunoResearch, USA) at 37 °C for 30 min. Integrin β1 was stained with mouse anti-porcine integrin β1 antibody (1:50, v/v) (BD Biosciences, USA) followed by fluorescein-conjugated goat anti-mouse antibody (1:200, v/v) (Chemicon, USA). The nuclei were stained with either DAPI (1:1000, v/v) or propidium iodide (PI) (1:1000, v/v) (Molecular Probes, USA). Fluorescent images were taken by the LSM 510 confocal microscope (Zeiss, Germany) and the Eclipse E400 fluorescence microscope (Nikon, Japan).

Western blotting. Cell lysates were collected from cells which had been cultured for 5–10 days with or without LPS on the AM and on plastic for 20 h. Proteins were separated by 12% SDS–PAGE and electrotransferred to a PVDF membrane (Millipore, USA) and blocked with skimmed milk. Blots were probed with mouse anti-rat CD31 antibody (1:1500 v/v), rat anti-porcine CD144 (1:500 v/v), mouse anti-human active form integrin β1 (1:5000, v/v) (Chemicon, USA), mouse anti-porcine integrin β1 (1:10,000, v/v), mouse anti-human E-selectin (1:1000, v/v) (Serotec, UK), mouse anti-human ICAM-1 (1:1000, v/v) (Serotec, UK), and mouse anti-β-actin (1:10,000 v/v, Sigma, USA) primary antibodies. Appropriate HRP-conjugated secondary IgG antibodies were visualized using enhanced chemiluminescence reagent (Perkin-Elmer Life Sciences, USA). The intensity of reaction bands was analyzed by an Image Gauge system (Fuji, Japan).

Quantitative RT-PCR analysis. ECs were seeded to reach immediate confluence on the AM. The ECs were cultured for 5 days, and then subject to treatment with or without LPS (10 μg/mL) for 20 h before harvesting for RNA analysis. The RNA transcripts of E-selectin, P-selectin, ICAM-1, and house keeping internal control RNA polymerase II were quantified by real-time PCR. The quantified values were generated from the average results from three independent experiments, and the value of each experiment was from the average of triplicate PCR runs. Total RNAs of the cultured ECs were extracted by TRIzol reagent (Invitrogen, USA), and cDNAs were prepared using SuperScript II Reverse Transcriptase (Invitrogen, USA). Primers for quantitative PCR were designed using the LightCycler probe design software 2.0 (Roche, Germany) and summarized in Table 1. Quantitative PCRs were performed by the LightCycler Fast-Start DNA Master SYBR Green I kit and on a LightCycler 1.5 PCR machine (Roche, Germany). The data are expressed as means ± SE.

Table 1
Sequences of RT-PCR primers used in this study

Name	Sequence	Nucleotide position
pE-selectin (f)	5′-GTGTTCAAGTTTGGAGGT-3′	1198–1215
pE-selectin (r)	5′-AGTCAGAAGGCATTTGGTA-3′	1549–1531
pP-selectin (f)	5′-GAGAGTGGTCAACTACG-3′	1706–1722
pP-selectin (r)	5′-TGGTCAAGTCTATCGGG-3′	2027–2011
pICAM-1 (f)	5′-TGCTCAGTGTCCTGTATG-3′	1207–1224
pICAM-1 (r)	5′-ATCTTCCGCTGGTAGTTA T-3′	1570–1552
pVCAM-1 (f)	5′-ATTTACCTTTACTCCTACCG-3′	637–656
pVCAM-1 (r)	5′-ATCTTCCATCCTCATAGCA-3′	946–928
pRPII (f)	5′-CCCTGACATCATCATCAAC-3′	507–525
pRPII (r)	5′-CCATTTCTCCAAAACGCA-3′	889–872

Note: Accession numbers of the genes are: pE-selectin: NM_214268; pP-selectin: NM_214078; pICAM-1: NM_213816; pVCAM-1: NM_213891; pRPII: AF265351.

Statistical analysis of the data was performed using analysis of variance, followed by Student's *t*-test.

Leukocyte adhesion to ECs cultured on AM. Leukocytes were isolated from fresh porcine blood with Histopaque 1119 (Sigma–Aldrich, USA) and spread on ECs in culture plates (2×10^5 cells/well in M199 plus 10% FBS) with or without LPS for 20 h. After incubation under static adhesion assay conditions at 37 °C for 15 min, non-adherent leukocytes were removed by washing with PBS. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with DAPI. For each experiment, the numbers of leukocytes from three fields per well were counted under a fluorescence microscope and summed. All experiments were performed in 6–8 times.

Electron microscopy analysis. ECs were seeded to reach confluence on the AM. After a growth period of 5 days, cells were fixed with 4% paraformaldehyde and 5% glutaraldehyde, followed by 1% osmium tetraoxide. The specimens were embedded in Epon. Thin sections (80 nm) were stained with uranyl acetate and lead citrate and viewed under a Hitachi H-600 transmission electron microscope (Hitachi, Japan).

Results

The proliferation of ECs cultured on AM

As the ECs were seeded at subconfluent density, there was a significant increase ($p < 0.05$) of cell growth in the AM-based culture at 24, 48, and 72 h after seeding, as compared with plastic culture controls. This result indicated that AM improved the proliferation of ECs (Fig. 1A and B).

Enhancement of cell–cell junctional proteins and integrins in ECs cultured on AM

The intracytoplasmic vWF was present in all ECs resting on AM as detected by immunostaining, indicating conserved endothelial characteristics (Fig. 2A). Immunostaining of PECAM-1 and VE-cadherin showed the restrictive localization of these proteins at the intercellular junctions, demonstrating the integrity of a well-formed monolayer (Fig. 2B and C). Integrin β1 was found to be present at the junction of EC and AM, suggestive of well-formed focal contacts (Fig. 2D). Consistently, electron-dense hemidesmosome-like structures between EC and AM, which are representative of cell–matrix adhesion, were revealed by transmission electron microscopy (Fig. 2E).

The expression levels of VE-cadherin, PECAM-1, and integrin β1 were found to be significantly elevated in AM-based culture. As normalized with housekeeping β-actin gene, AM-based culture demonstrated 1.64- and 2.42-folds of increase in the expression of VE-cadherin and PECAM-1, respectively (Fig. 2F and G). The active integrin β1 demonstrated a higher elevation in AM-based culture (3.34-fold) than total integrin β1 (1.2-fold) (Fig. 2H and I). This suggests the involvement of active interaction between EC and AM, which might correlate

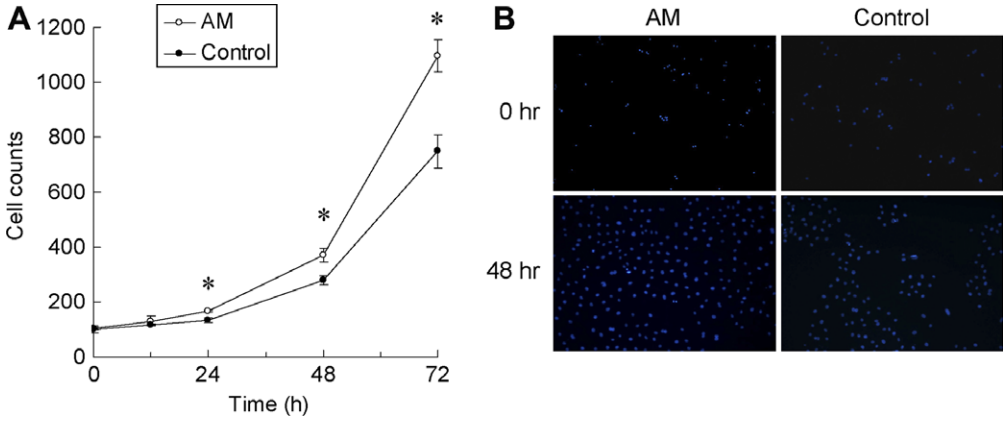

Fig. 1. The cell numbers increased gradually with time both in the AM and the plastic control groups. The AM group had higher cell numbers at 24, 48, and 72 h than that of control group (A,B). Means ± SE, $n = 3$, $^*p < 0.05$.

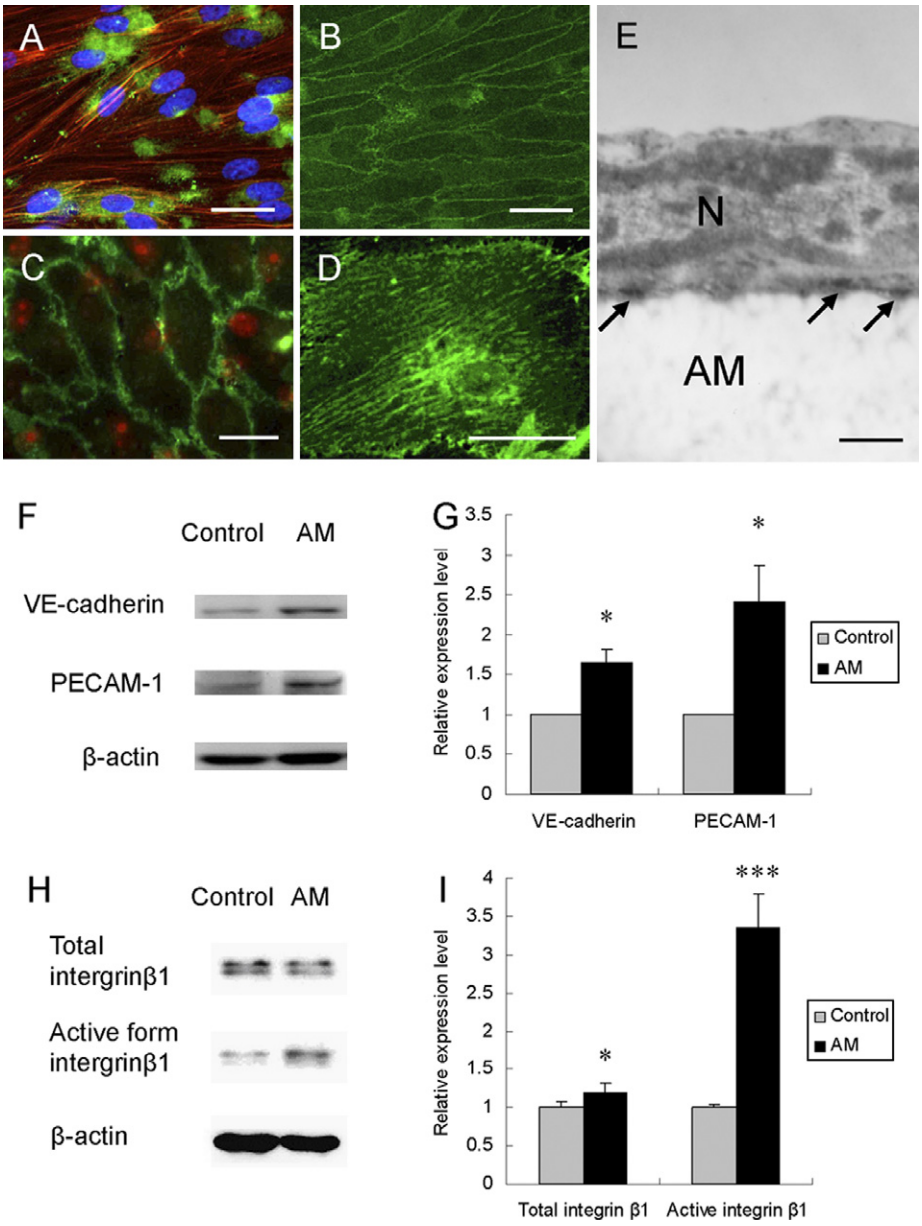

Fig. 2. ECs cultured on AM were labeled for vWF (green), f-actin (red), and nuclei (blue) (A). The intact cell–cell junctions were observed by the immunostaining of VE-cadherin (green) (B) and PECAM-1 (green) (C). Integrin β1 (green) was observed at the EC–AM junctions by confocal microscope (D). TEM image (E) showed a hemidesmosome-like structure (arrow) attched to the AM on the basal side of EC. (A–D, bar = 10 μm; E, bar = 0.5 μm.) The expression levels of VE-cadherin and PECAM-1 increased in the ECs cultured on AM (F,G). The level of total and active integrin β1 was higher in the ECs cultured on AM compared with those cultured on the plastic control (H,I). Means ± SE, $n = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

with the enhanced proliferation and junctional formation in AM-based EC culture.

Downregulation of adhesive molecule expressions in ECs cultured on AM with or without LPS activation

To assess the effect of AM on the expression of endothelial CAM molecules, we analyzed the RNA levels of E-selectin (Fig. 3A), P-selectin (Fig. 3B), ICAM-1 (Fig. 3C), and VCAM-1 (Fig. 3D) in ECs cultured on either plastic surface or AM. We also examined whether AM would modulate the expression of activated endothelial CAMs resulting from LPS challenge. The gene expressions of E-selectin, P-selectin, and ICAM-1 were significantly downregulated in ECs cultured on AM, while less significant change in VCAM-1 expression was detected. The relative RNA expression levels, as normalized by RNA polymerase II, revealed an average reduction by 56% for E-selectin, 31% for P-selectin, and 16% for ICAM-1, in ECs seeded on AM.

When LPS was added to the endothelial culture, a significant increase in E-selectin (Fig. 3A), P-selectin (Fig. 3B), ICAM-1 (Fig. 3C), and VCAM-1 (Fig. 3D) mRNA was observed after 20 h of incubation. The expression of P-selectin was increased by 2.2-fold upon LPS

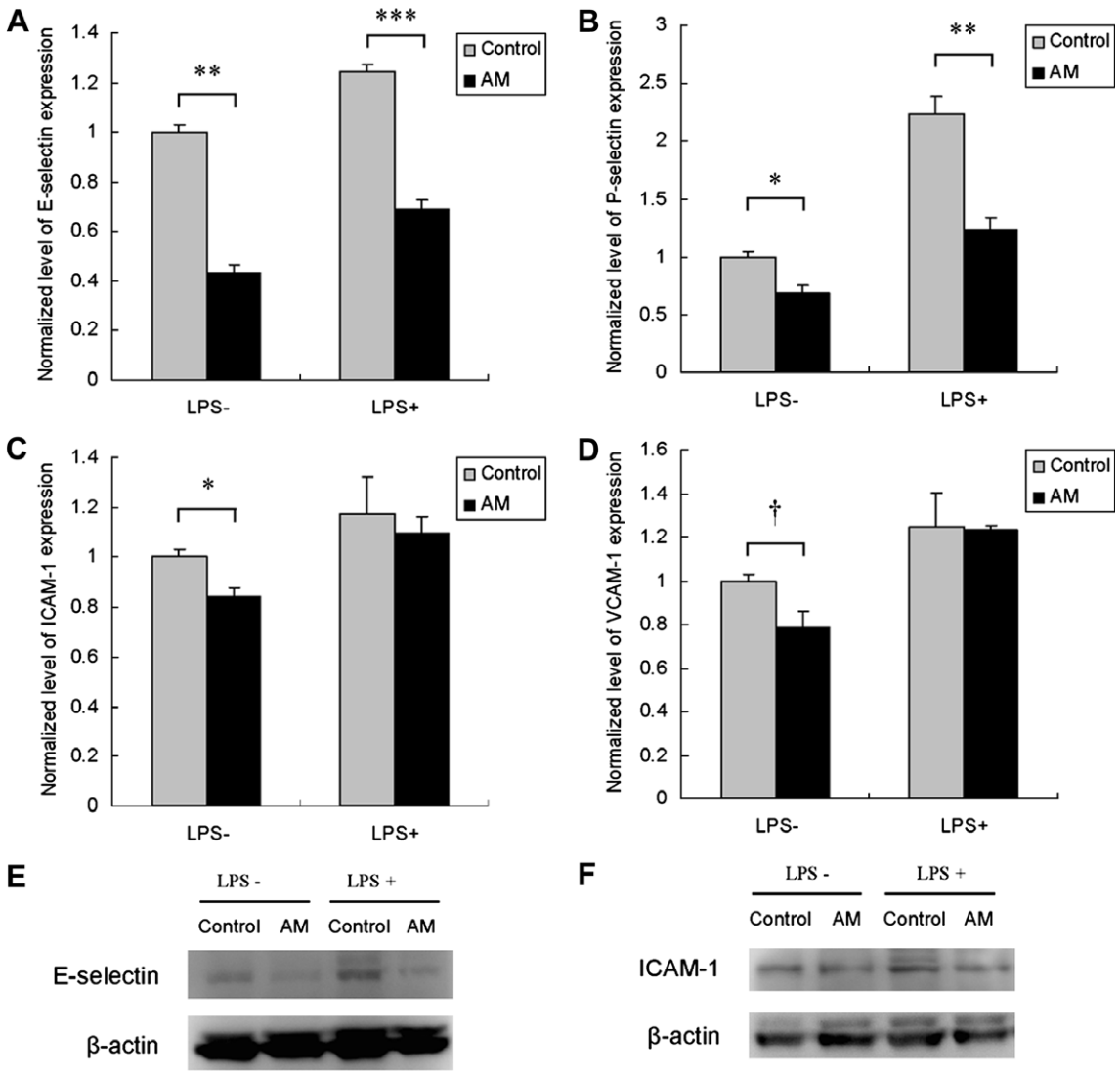

Fig. 3. The ECs cultured on either plastic or AM were treated with or without LPS, before being harvested for quantitative RT-PCR analysis for the expressions of E-selectin (A), P-selectin (B), ICAM-1 (C), and VCAM-1 (D). All mRNA levels were normalized against RNA polymerase II. The RNA levels of E-selectin (A) and P-selectin (B), in both non-activated and activated cells, were significantly decreased in ECs grown on AM as compared with plastic controls. The expression levels of ICAM-1 (C) and VCAM-1 (D) decreased in the ECs cultured on AM without LPS activation. (Means ± SE, $n = 3$, $^{\dagger}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.) The level of E-selectin was lower in the ECs cultured on AM than that in the plastic control with and without LPS treatment (E). The level of ICAM-1 in ECs cultured on AM was also decreased as compared with plastic control (F).

challenge compared to that of the untreated. In contrast, E-selectin, ICAM-1, and VCAM-1 were increased by 25%, 17%, and 25%, respectively, after LPS treatment. The LPS-activated E- and P-selectin expressions were suppressed in AM-based culture, in a fashion that was slightly higher than that of the ECs without LPS treatment. AM downregulated the LPS-activated E- and P-selectin expressions by 55% of that of the plastic-based culture. However, the LPS-activated ICAM-1 and VCAM-1 expressions were not significantly attenuated by AM. Taken together, our results demonstrated that endothelial expression of CAMs, notably E- and P-selectin, were downregulated in AM-based culture with and without LPS treatment.

The protein expression levels of E-selectin and ICAM-1 were analyzed by Western blot. In AM-based culture, E-selectin and ICAM-1 were less expressed than that of plastic-based culture (Fig. 3E and F). LPS treatment enhanced the expression of E-selectin and ICAM-1 compared to that of the untreated cultures. The enhancement by LPS was more prominent in plastic-based culture than in AM-based culture as compared to their respective controls (Fig. 3E and F). Both RNA and protein analyses showed that endothelial CAM expressions, notably after LPS stimulation, were downregulated in AM-based EC culture, implying the anti-inflammatory properties of AM as a matrix for endothelial monolayer.

Reduction of leukocytes adherence in AM-based EC culture

To further examine the anti-inflammatory properties of AM, we assayed the adherence of leukocyte to endothelial monolayer in both plastic control and AM-based cultures,

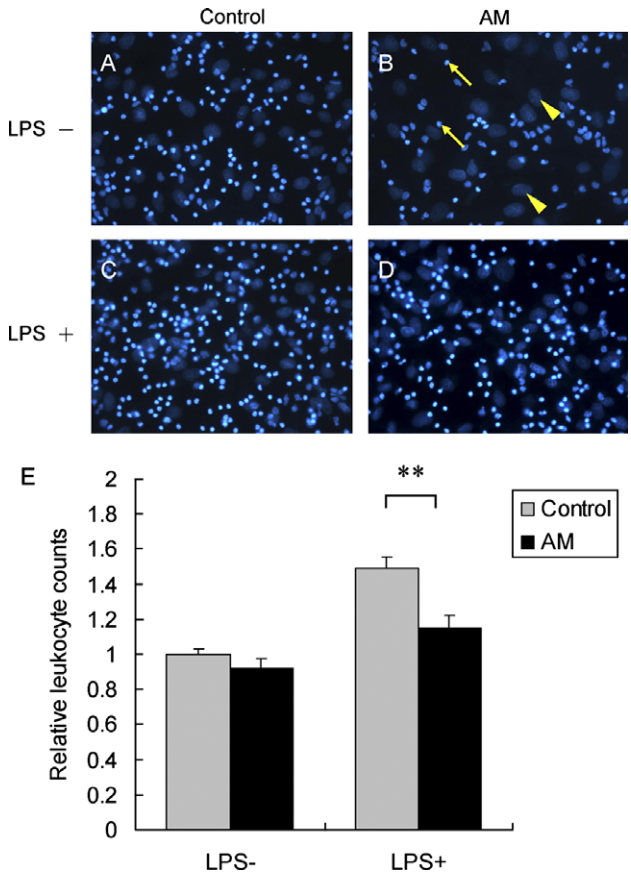

Fig. 4. Leukocyte suspensions (2×10^5/ml) were added to the ECs monolayer cultured on plastic control (A,C) and to that on AM (B,D) which was treated with or without LPS. Nuclei of leukocytes (arrows) were smaller and more condensed as compared with that of ECs (arrow heads). After LPS treatment, there was a significant reduction in leukocytes adhering to ECs cultured on AM (B,D) compared with those in the plastic control (A,C). The relative leukocyte counts were shown in (E) as means ± SE, $n = 6$–8, $^{**}p < 0.01$.

with or without LPS stimulation. In the absence of LPS, the leukocyte adherence to ECs appeared to be mildly attenuated in AM-based culture (Fig. 4A, B, and E). However, the enhanced leukocyte adherence to ECs resulting from LPS challenge was significantly reduced in AM-based culture (Fig. 4C–E). The results were consistent with the lowered CAM expressions in ECs cultured on AM, and suggest the potential anti-inflammatory properties of AM.

Discussion

In most BVE constructs reported thus far, ECs were seeded as the last step of the construction, and therefore, the endothelial functions can only be tested by functional assays. The endothelium formed in the BVE construct exhibited low patency upon grafting due to a lack for well-developed basement membrane. Grafting studies of these BVE constructs have been limited by thrombosis (due mainly to an endothelial defect), chronic inflammation and poor mechanic properties [4,5,24]. In this study, we demonstrated that the expressions of junctional proteins were increased, while those of the adhesive inflammatory molecules were decreased in porcine arterial ECs grown on AM.

ECs are regulated by their interaction with ECM, particularly the BM. It has been reported that the pre-existing BM provided by AM enhances cell growth and morphogenesis in corneal [8] and skin [25] epithelium. Our results showed that AM can enhance cell growth and improve the cell adhesion to the matrix by increasing the expression of integrin. The increased level of integrin expression and the formation of hemidesmosome-like structures indicated that ECs formed firm basal adhesions. These results suggest that AM enhances endothelial morphogenesis with well-defined basal-apical polarity.

Changes in endothelial permeability and leukocyte adhesion is thought to be involved in the initial step of atherosclerosis [26], which is also one of the major causes of BVE failure. In this study, immunofluorescence microscopy showed that ECs form an intact monolayer and express lateral junctional proteins, PECAM-1 and VE-cadherin. The complete lateral cell–cell junction indicated that a stable endothelium had been formed on AM. It has been demonstrated that the expression of adhesive molecules is correlated with the interaction between ECs and ECM. The expression of adhesive molecules was reported to be lower in ECs cultured on natural BM components, collagen IV and laminin, than in ECs cultured on collagen I [27]. The stromal matrix of AM was also noted to suppress the inflammatory response [11]. In our study, the expressions of leukocyte adhesive molecules, E-selectin, P-selectin, and ICAM-1, were lower at the mRNA level as well as at the protein level in ECs cultured on AM than in ECs grown on the plastic surface. After LPS activation, the levels of E-selectin and P-selectin were notably increased, however, the cells cultured on AM were less affected by LPS than cells cultured on plastic. Functional assay of leukocyte adhesion also demonstrated that fewer leukocytes adhered to ECs which had been cultured on AM. These results strongly suggested that AM-based endothelium possesses good anti-inflammatory and anti-thrombogenic properties.

A stable, quiescent and non-activated endothelium is the most critical characteristic of BVEs, especially in small-diameter vascular grafts. In this study, we provide evidence that AM is an ideal matrix to support in vitro vascular endothelium formation with structures and functions more resemble to that of the intima in vivo. Our result suggests that AM could be used as a matrix to develop a functional endothelium in BVE construction.

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