Improvement of Function and Morphology of Tumor Necrosis Factor-\(\alpha\) Treated Endothelial Cells With 17-\(\alpha\) Estradiol

A Preliminary Study for a Feasible Simple Model for Atherosclerosis

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Background  Dysfunction of endothelial cells (EC) to produce endothelial nitric oxide synthase (eNOS) by tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) causes critical features of vascular inflammation associated with several disease states (eg, atherosclerosis including increased platelet aggregation and adhesion on EC, elevated adhesion molecules and enhanced inflammatory cells binding to EC). 17-\(\alpha\) estradiol (E2) can stimulate eNOS production and improve the critical features of atherosclerosis. Using TNF-\(\alpha\) and E2, we attempted to develop an in vitro vascular model for studying atherosclerosis.

Methods and Results  Human umbilical vein endothelial cells (HUVEC) grown in transwells were cocultured with smooth muscle cells in a 24-well plate to mimic the major components of the vascular wall. The model was incubated with TNF-\(\alpha\) (10 ng/ml) for 12 h, prior exposed to E2 (100 pg/ml) for 6–12 h, then investigated by transmission and scanning electron microscopes. The result indicated recovered morphology with good tight junction, and decreased platelet adhesion was noted in defective HUVEC after E2 treatment.

Conclusion  17-\(\alpha\) estradiol was represented as an antiatherosclerogenic agent to demonstrate feasibility of the model. Although our finding focused only on the endothelium, this would be the basis for our future studies to develop ex vivo continuous perfusion of human vessel segments for a further atherosclerosis study.  

Key Words: Atherosclerosis; Endothelial cell; Endothelial nitric oxide synthase; 17-\(\alpha\) Estradiol; Tumor necrosis factor-\(\alpha\); Ultrastructure

I
n normal vasculature endothelial cells (EC), a small amount of NO synthesized by endothelial nitric oxide synthase (eNOS) maintains normal vascular physiological function controls. 1,2 Endothelial cell dysfunction in eNOS expression by tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is involved in the pathogenesis of vascular diseases, for example atherosclerosis. 3 The related pathologic changes are increased expression of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1, and E-selectin that enhances inflammatory cell infiltration in the vascular wall, platelet aggregation and adhesion on EC surfaces, and smooth muscle cell proliferation and migration. 4 In contrast, 17-\(\alpha\) estradiol (E2) upregulates eNOS production in EC, protects against atherosclerosis and recovers EC after arterial injury in animal models. 5–7

At present, experimental models for pathological and pharmacological studies in atherosclerosis have been developed only in laboratory models, eg, primates, rats and mice. 5,6,8 The important defects in the animal models are their complicated metabolisms with systemic variation and the difficulty of controlling the concentrations of drugs acting directly on the target cells. To date, no in vitro vascular model has been demonstrated for pharmacological studies in atherosclerosis, even though there have been several studies with other vascular diseases. 9–11 In the present study, we first constructed the in vitro model vascular wall with endothelial cell dysfunction in eNOS production by TNF-\(\alpha\) to mimic the important basic early stage pathology of atherosclerosis, and determined the potential of the model by using E2 as an antiatherosclerogenic agent.

Methods

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVEC) were separated from fresh umbilical cord veins, as previously described. 12 The HUVEC were cultured in human endothelial-SFM basal growth medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 40 \(\mu\)g/ml gentamycin and glutamine. The HUVEC were incubated at 37°C in a 5% CO\(_2\) atmosphere. Positive staining for CD31 confirmed the endothelial cell identity.
lial cell origin. The HUVEC passages 2-5 were used in the current study.

**Smooth Muscle Cell Culture**

Human smooth muscle cells (SMC) were separated from fresh umbilical vein that was traumatized by repeatedly clamping it with a hemostat before collagenase digestion, as described previously.12 Spindle shaped SMC cells over-grew the HUVEC within 2 weeks. The SMC were cultured in M199 supplemented with 10% FBS, 1% pyruvate, 40μg/ml gentamycin at 37°C in a 5% CO2 atmosphere. The SMC were identified by positive immunostaining to α actin. Smooth muscle cells were continuously passaged and used in the study.

**NO Conduction in HUVEC Culture**

To establish the condition of defective EC to produce eNOS, confluent monolayer of HUVEC on coverslips inserted in 24-well plates were cultured with M199+10% FBS+TNF-α at concentrations of 3, 5, 10, 15 and 20 ng/ml. The HUVEC were cultured with M199+10% FBS+E2 at the concentration of 50, 100, 150, 300 and 500 pg/ml to stimulate eNOS expression in HUVEC. The normal condition of HUVEC for eNOS synthesis was cultured in M199+10% FBS. At different times of the incubation intervals, the cover slips with HUVEC were harvested, fixed in methanol:acetone (1:1) and the amounts of eNOS expression were determined by immunoperoxidase technique.

**Determination of eNOS Expression by Immunoperoxidase**

Detection of eNOS production was confirmed by immunoperoxidase technique, as described previously.13 Briefly, endogenous peroxidase was blocked by incubating the HUVEC for eNOS expression was cultured in M199+10% FBS. At different times of the incubation intervals, the cover slips with HUVEC were harvested, fixed in methanol:acetone (1:1) and the amounts of eNOS expression were determined by immunoperoxidase technique.

**Preparation of Washed Platelets**

Platelets were obtained from a healthy donor who had never taken anti-platelet drugs, then washed using the method modified from the previous studies by Cooke and Nash.16 Briefly, anticoagulant was added to whole blood, 9 volumes of blood to 1 volume of acid-citrate-dextrose (90 mmol/L sodium citrate, 7 mmol/L citric acid, pH 4.6, 140 mmol/L dextrose and supplemented with 70 mmol/L theophylline). Platelet-rich plasma was obtained by centrifugation of whole blood at 1,000 rpm for 10 min. The platelet pellets were sedimented at 3,000 rpm for 8 min and resuspended in platelet washing buffer (403 mmol/L NaHPO4, 24.3 mmol/L Na2HPO4, 4.3 mmol/L K2HPO4, pH 6.5, 113 mmol/L NaCl, 5.5 mmol/L glucose, 0.5% BSA and 10 mmol/L theophylline, then the pellet was washed and resuspended in tyrode buffer (12 mmol/L NaHCO3, 10 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl and 5.5 mmol/L glucose).

**Determination of Platelet Adhesion on EC**

We plated 1×10^6 HUVEC in 3 mm dishes with EC growth medium for 3 days. After overnight quiescence in M199 supplemented with 1% FBS, HUVEC were co-cultured with 2×10^6/ml of platelets17 and 3–20 ng/ml of TNF-α or 50–500 pg/ml of E2 in M199 supplemented with 10% FBS. After incubation for 6, 12, 24, or 36 h, 3×10^6 HUVEC for each treatment and interval time were harvested by cell scrapers. The cells were washed in PBS twice by centrifugation at 10,000 G for 5 min each. The pellet was lysed in 200 μl of lysis buffer (PBS containing 1% sodium dodecyl sulfate (SDS), 1 mmol/L NaVO4, 100 μm aprotinin, 1 μg/ml leupeptin and 1 mmol/L phenyl methyl sulphonyl fluoride).14 The cell debris was separated by centrifugation at 10,000 G for 10 min. The total protein in the supernatant was determined by BCA protein assay (Pierce Chemical Co, Rockford IL, USA). A total of 25 μg of proteins were size-fractionated on 5% stacking/10% separating SDS/-polyacrylamide gel electrophoresis at 20 mA for 6 h.15 The resolved proteins were transferred to a 0.2 μm nitrocellulose membrane on a semi-dry electrophoresis transfer system (Atto, Japan). Blots were blocked for nonspecific proteins for 2 h in 3% dry skimmed milk solution and incubated with rabbit polyclonal antibodies to human eNOS, or human VCAM-1, mouse monoclonal antibodies to ICAM-1 (Santa Cruz Biotechnology) or E-selectin (Southern Biotechnology, Birmingham, AL, USA) at a dilution of 1:1,000. After washing and incubating with biotinylated goat antirabbit mouse IgG or mouse IgG at the dilution of 1:2,000 for 2 h, blots were added as strepavidin-alkalinephosphatase (Mabtech, Stockholm, Sweden) at the dilution of 1:2,000 for 1 h. Immunoreactive bands were visualized by adding BCIP/NBT substrate (KPL, Gaitherburg, Maryland, USA) for 5–10 min. The experiments were carried out at room temperature.

**Determination of Adhesion Molecules and eNOS Expression by Western Blot**

We cultured 3×10^5 HUVEC in 25 cm² flasks in growth EC medium until a confluent monolayer was formed. After overnight quiescence in M199 supplemented with 1% FBS, HUVEC were treated with 3, 5, 10, 15 and 20 ng/ml of TNF-α or 50–500 pg/ml of E2 in M199 supplemented with 10% FBS. After incubation for 6, 12, 24, or 36 h, intact HUVEC were gently washed with PBS, fixed with absolute methanol for 5 min, washed with 0.1 mol/L phosphate buffered solution (PBS) for 15 min. After washing 3 times, the cells were incubated in 0.01% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, USA) in PBS for 15 min to block nonspecific proteins. The cells were then incubated with 0.1 μg of rabbit polyclonal antibody to human eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.01% BSA in PBS for 90 min. The cells were washed in 0.01% BSA in PBS and incubated with 30 μg of biotinylated F(ab)2 fraction of goat anti-rabbit IgG (Dako, Glostrup, Denmark) for 30 min, washed and incubated with strepavidin-biotin complex conjugated with peroxidase (Vector Laboratories, Burlingame, CA, USA) for 30 min. After washing, the peroxidase enzyme activity in the cells was detected by adding 3,3’-diaminobenzidine tetrahydrochloride and H2O2 (Vector Laboratories) for 10 min. The cells were counterstained with Meyer’s hematoxylin for 3 min, washed and mounted with Permount and examined under a light microscope. The staining was carried out at room temperature. The negative control for immunostaining was omission of primary antibody, and the positive control was 24 h lipopolysaccharide (1 μg/ml) stimulated HUVEC.
Fig 1. Expression of endothelial nitric oxide synthase (eNOS), detected by immunoperoxidase, in (a) normal or human umbilical vein endothelial cells (HUVEC) incubated with tumor necrosis factor-β (TNF-β) at the concentration of (b) 3 ng/ml; (c) 5 ng/ml; (d) 10 ng/ml; (e) 15 ng/ml and (f) 20 ng/ml for 6–36 h. White and black bars represent percentages of negative and positive eNOS staining HUVEC, respectively. The results express as mean ± SEM from 3 experiments.

Fig 2. Expression of endothelial nitric oxide synthase (eNOS), detected by immunoperoxidase, in (a) normal or human umbilical vein endothelial cells (HUVEC) incubated with 17-β estradiol (E2) at the concentration of (b) 50 pg/ml; (c) 100 pg/ml; (d) 150 pg/ml; (e) 300 pg/ml and (f) 500 pg/ml for 6–36 h. White and black bars represent percentages of negative and positive eNOS staining HUVEC, respectively. The results express as mean ± SEM from 3 experiments.
Estradiol Recovered Dysfunctional HUVEC

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No. 3413) coated the membranes with 4 μg of fibronectin in M199 and were air dried overnight in a laminar flow. After 1 h of equilibrating the precoated membranes with M199, 1.5×10⁵ HUVEC in 100 μl of growth EC medium were seeded in the transwells by insertion of a 24-well plate with 600 μl of growth EC medium/well. The HUVEC cultured for at least 72 h could form a confluent monolayer and a completely tight junction. Before the experiment the prepared HUVEC in the transwells were randomly checked for completely tight junction by determining the leakage of 125I labeled albumin through the monolayer on the membrane.¹⁸

Ultrastructural Study of the HUVEC in the In Vitro Vascular Model

The transwells with completely grown HUVEC were then transferred for insertion into 24-well plates with SMC monolayers in the bottom wells. The HUVEC were cocultured with SMC for a further 24 h in growth medium in the presence of TNF-α at an optimal dose to decrease eNOS production for 12 h, then in a medium with E₂ at an optimal dose to increase eNOS production to normal levels. After 6 and 12 h of E₂ incubation, the transwells with treated HUVEC were then harvested, washed in 0.1 mol/L PB with sucrose pH 7.2, fixed with 2.5% glutaraldehyde in PB for 1–2 h, and postfixed in 1% osmium tetroxide in PB for 2 h. For scanning electron microscopic examination, the cells on the membranes of the transwells were gradually dehydrated in ethanol, and dried by using the critical point dryer, coated with gold particles and examined by a HITASHI S-2360N scanning electron microscope. For transmission electron microscopic examination, postfixed HUVEC in transwells were gradually dehydrated in acetone and embedded in epon-aradite. Semithin sections stained with toluidine blue were examined under a light microscope and a suitable area was selected for the preparation of 70 nm ultrathin sections by a diamond knife. These sections were then double-stained with uranyl acetate and lead citrate, and examined in a Joel 1200EX transmission electron microscope.

For ultrastructural studying in platelet adhesion to EC, 2×10⁵ of platelets in 100 μl of medium were added into each transwell as mentioned above. After the same incubation time the transwells with treated HUVEC were harvested and processed for scanning electron microscope.

Statistical Analysis

The results were expressed as mean±SEM. The differences between the groups were evaluated with the use of ANOVA. Differences were considered significant at a level of p<0.05.

Results

eNOS Expression by Immunoperoxidase

Endothelial nitric oxide synthase expression in HUVEC on cover slips was determined by immunoperoxidase. The percentages of EC that produced eNOS were compared among the cells incubated with varying concentrations of TNF-α and E₂ for 6–36 h. Expression of eNOS protein in HUVEC incubated with TNF-α (3–20 ng/ml) and E₂ (50–500 pg/ml) are shown in Figs 1, 2. TNF-α in particular, at a concentration of 5–20 ng/ml could significantly decrease eNOS production within 24–36 h after incubation, compared with HUVEC in normal conditions (p=0.00) (Fig 1). High concentrations of E₂ (300–500 pg/ml) could stimulate approximately 80% of HUVEC to express eNOS and the numbers of eNOS positive cells were significantly higher in only 6 h of incubation compared with those in normal conditions (p<0.05), and within 24–36 h of incubation, with all concentrations of E₂ (50–500 pg/ml) (p=0.00) (Fig 2).

Western Blot Analysis of eNOS and Adhesion Molecules Expressed by HUVEC

Protein expression was determined at 24 h incubation (Fig 3). Compared with the normal HUVEC, eNOS protein abundance was decreased in the cells incubated with TNF-α (3–20 ng/ml), whereas it was markedly elevated in the cells stimulated with E₂ (50–500 pg/ml). This was consis-
tent with the localization of eNOS expression in HUVEC under the same conditions by immunoperoxidase (Fig 3). ICAM-1, VCAM-1 and E-selectin protein abundance was markedly present in HUVEC incubated with TNF-α (3–20 ng/ml). By contrast, ICAM-1 and VCAM-1 expression was decreased in the cells cultured with E2 (50–500 pg/ml), whereas E-selectin protein abundance could not be observed in both normal cells stimulated with E2 (Fig 3).

Platelet-Endothelial Cell Interaction
To evaluate platelet-endothelial cell interaction after decrease and increase in eNOS expression, the number of platelets adhering to the HUVEC surface was calculated at 6, 12, 24, and 36 h of incubation with TNF-α (3–20 ng/ml) (Fig 4a) or E2 (50–500 pg/ml) (Fig 4b). At 6 h incubation with TNF-α at a concentration of 20 ng/ml, the number of platelets adhering to the HUVEC was significantly the highest compared with those incubated with TNF-α 3–15 ng/ml or normal HUVEC (p<0.01). The significant peak of platelet adhesion was observed in HUVEC incubated with TNF-α at a dose of 3–15 ng/ml for 24 h (p=0.00). Platelet adhesion on the EC surface was significantly decreased within 24 h in TNF-α incubated HUVEC. Meanwhile, E2 (50–500 pg/ml) could nonsignificantly decrease platelet adhesion on HUVEC within 24 h of incubation. Only HUVEC incubated with 100 pg/ml of E2 for 36 h showed a significant but slightly decreased platelet adhesion (p=0.008).

Evaluation of Optimal Conditions of TNF-α and E2 to Use in the Vascular Model
Based on the results of eNOS expression in HUVEC incubated with TNF-α and E2 by immunostaining, TNF-α at a
concentration of 10 ng/ml for 12 h was selected to cause mild dysfunction of HUVEC to decrease eNOS producing cells approximately 10% compared with the control (Fig 1), although the morphology observed under a light microscope was still normal. To recover the defective HUVEC, we chose E2 at the concentration of 100 pg/ml, which could stimulate HUVEC to produce eNOS at a similar ratio for the positive/negative eNOS-producing cells to those in normal HUVEC (Fig 2). To investigate the early change in the HUVEC in the model, HUVEC in the transwells were harvested twice at 6 and 12 h E2 incubation.


In the present study, we evaluated the normal morphology of the confluent monolayer from the continuous tight junctional integrity of the HUVEC attached to the membrane of the transwells. During the 24 h of the experiment, the control HUVEC could form and maintain a confluent monolayer condition (Fig 5a) determined by a complete intercellular border (Fig 5b) and firm attachment to the membrane (Fig 5c). After incubation with TNF-α (10 ng/ml) for 12 h (Fig 6a), some HUVEC showed disruption of intact intercellular border and detached from the membrane (Fig 6b), while the cells incubated with E2 (100 pg/ml) for 12 h appeared to retain their normal morphology at the intercellular area (Fig 7a,b). In contrast, 12-h preincubated HUVEC with TNF-α (10 ng/ml) could recover their morphology and arrangement after incubation with E2 for 6 h (Fig 8a), and could again form a tight junction and attach to the membrane within 12 h (Fig 8b,c).

In the presence of the cultured platelets we found moderate numbers of platelets adhering to the surface of normal HUVEC (Fig 5d), while numerous activated platelets with long pseudopodia were observed on 12 h TNF-α incubated HUVEC (Fig 6c). In contrast, very few platelets adhered to the surface of HUVEC incubated with TNF-α prior to E2.
for 12 h (Fig 8d). The numbers of platelet adhesion to HUVEC related to an increase of platelet endothelial cell adhesion molecules on the surface of HUVEC.

Discussion

The present study aimed to develop an in vitro model of vascular wall with main and basic pathologic atherosclerosis change. Comparing the permeability of confluent HUVEC cultured in various types of transwells coated with gelatin, collagen, or fibronectin, we found HUVEC seeded on fibronectin (4–10 μg) precoated polycarbonate porous membrane for 3 days could form a continuous tight junctional integrity, which was determined by no leakage of 125I-labeled albumin through the monolayer of the HUVEC on the membrane (unpubl data). Coculturing of HUVEC in transwells inserted in a 24-well plate with confluent monolayers of SMC is a basic model of vascular wall, which has been demonstrated in other previous studies.9,10,11,21 In the present study, SMC proliferation and migration of SMC to the endothelial cell layer1 could not be demonstrated in our short time of investigation. The previous study in saphenous vein culture demonstrated TNF-α regulated SMC proliferation and migration within 7 days.22 During development of an atherosclerotic plaque, arterial SMC change to secretory phenotype and migrate into plaque. Secretory SMC generate several agents eg, interleukin (IL)-8, monocyte chemoattractant protein-1, IL-1 and TNF-α that could induce adhesion molecule expression on EC and promote leukocyte infiltration of the arterial wall.31 Therefore, our SMC coculture was necessary to allow the assessment of cell-cell interaction and soluble mediator activity, although the system lacked of natural contact between HUVEC and SMC.

TNF-α is able to downregulate eNOS expression by enhancing the degradation of its mRNA23 and decrease activity of eNOS gene promoter24,25 We, therefore, used the recombinant human TNF-α to cause dysfunction of HUVEC in our model. The optimal concentration of TNF-α used in our model was determined from the ranges of those modified from previous studies9,26 that were safe from its cytotoxicity.27 The consequent changes in HUVEC were consistent with other studies and could represent important changes of NO involved EC dysfunction in atherosclerosis, including decrease in eNOS expression, increase in adhesion molecules, particularly ICAM-1, VCAM-1, and E-selectin and platelet adhesion to the EC surface.1 Our Western blot analysis showed an overall semiquantitative result, but not the comparison between amount of protein expression and doses of TNF-α or E2. The abundance in adhesion molecules in HUVEC was increased after 24-h incubation with TNF-α (3–20 ng/ml), while the expression of eNOS decreased. This finding was in part to confirm the validity of our model.

Previous studies demonstrated that TNF-α, the important proinflammatory cytokine, alone could not stimulate inducible nitric oxide synthase (iNOS) expression in rat and HUVEC cultures28,29 although had little activity in murine30 and rat SMC.11 In addition, iNOS expression could not be detected in normal confluent monolayer and subcultures of HUVEC.22 Therefore, in the present study we did not determine the level of iNOS in the TNF-α stimulated HUVEC because the NO production was not much interfered by iNOS expression.

It is known that 17-estradiol can stimulate eNOS mRNA and protein expression3 and inhibit cytokine-mediated EC adhesion molecule transcription activation.33 To prove whether our EC dysfunction model can recover when the HUVEC receive an antiatheroslerogenic agent, E2 was replaced in the culture preincubated with TNF-α. We modified the optimal concentration and time of incubation according to a previous study7 which similarly reported 100 pg/ml of E2 induced mRNA eNOS expression in 6 h. Our data concerning the role of E2 in increasing eNOS expression and inhibiting adhesion molecules was in agreement with the other studies.5 The platelet-EC interaction revealed that the effect of E2 on inhibiting platelet adhesion was not significant (Fig 4b). It is possible that other factors may be combined with E2 to completely inhibit platelet activation.33 However, the ultrastructural morphology and approximate amount of platelets in our model indicated its activation (Fig 6c) and was consistent with those in other studies in platelet activation.17,34 In addition, few platelets adhering to EC in the recovered model after being exposed to E2 for 12 h (Fig 8d) supported the inhibition effect of E2 on platelet and EC interaction.5 In normal NO derived from eNOS, inhibits P-selectin expressed on platelet surface and platelet aggregation via activation of soluble guanylate cyclase, which increase cGMP35 TNF-α causes decreased cGMP and increased P-selectin expression to enhance platelet aggregation.36 In contrast E2 inhibits platelet aggregation by promoting Ca2+ extrusion or re-uptake activity that is dependent on the production of cGMP by increasing NO synthesis both in EC and platelets. The recent studies showed that platelet adhesion on endothelium played a critical role in the initiation of atherosclerosis38 and platelet activation was observed in different phases of atherosclerosis39.

Previous studies determined alteration of tight junction and permeability of endothelial cells by detecting the distribution of junctional protein expression eg, zonula occcludens and occludin at the intercellular area.40–42 In the present study, although we did not confirm the alteration of tight junctional proteins by immunocytochemistry or Western blot analysis; the ultrastructural change by scanning electron microscopy and transmission electron microscopy observed in our model could indicate obvious evidences of complete or impaired tight junction at intercellular borders. In our system we attempted to observe the changes in a short time, not longer than 24 h, because of the limitation of the HUVEC cultures in the transwells. However, our study time is suitable for investigating the early ultrastructural changes by electron microscopy. This is in agreement with the previous studies indicating that an ultrastructural study is the rapid determination of any change and superior to examination of only the permeability of the EC monolayer, eg, by detection of transendothelial electrical resistance or leakage of dye through the monolayer, without investigation of morphological changes.33,44 However, the disadvantage of the model is the required culturing expertise and the high cost of the transwells and the electron microscopic process. In addition, our study was conducted under static condition, which is not normal conditions for EC in vessels. We attempted to focus only on the dysfunction of EC by decrease in eNOS expression without the factor of shear stress. Our simple atherosclerotic model induced by TNF-α was successfully recovered by E2 that represented as an example of NO-related antiatheroslerogenic agents, although the inhibition effect on platelet adhesion was not complete. We could only demonstrate the early change only in EC, but not include SMC proliferation and migra-

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tion. However, this finding will be the basis for our further studies to develop ex vivo continuous perfusion of human vessel segments for studying atherosclerosis. This ex vivo model has more advantages because the changes of the whole vascular wall under flow conditions and longer time of study can be observed in serial section by histopathology, immunohistochemistry and ultrastructural pathology. In conclusion, (i) the tight junction of the HUVEC in the model was the most important criterion for evaluating the early pathologic changes; and (ii) our basic atherosclerosis model showed feasibility to modify ex vivo vein culture for pharmacological studies in atherosclerosis.

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