Human astrocytes/astrocyte conditioned medium and shear stress enhance the barrier properties of human brain microvascular endothelial cells

Venkatraman Siddharthan,
Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, Baltimore, MD 21287

Yuri V. Kim,
Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, Baltimore, MD 21287

Suyi Liu, and
World Precision Instruments Inc., 175 Sarasota Center Blvd, Sarasota FL 34240 U.S.A.

Kwang Sik Kim[#]
Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, Baltimore, MD 21287

Abstract

The blood-brain barrier (BBB) is a structural and functional barrier that regulates the passage of molecules into and out of the brain to maintain the neural microenvironment. We have previously developed the *in vitro* BBB model with human brain microvascular endothelial cells (HBMEC). However, *in vivo* HBMEC are shown to interact with astrocytes and also exposed to shear stress through blood flow. In an attempt to develop the BBB model to mimic the *in vivo* condition we constructed the flow-based *in vitro* BBB model using HBMEC and human fetal astrocytes (HFA). We also examined the effect of astrocyte conditioned medium (ACM) in lieu of HFA to study the role of secreted factor(s) on the BBB properties. The tightness of HBMEC monolayer was assessed by the permeability of dextran and propidium iodide as well as by measuring the transendothelial electrical resistance (TEER). We showed that the HBMEC permeability was reduced and TEER was increased by non-contact, co-cultivation with HFA and ACM. The exposure of HBMEC to shear stress also exhibited decreased permeability. Moreover, HFA/ACM and shear flow exhibited additive effect of decreasing the permeability of HBMEC monolayer. In addition, we showed that the HBMEC expression of ZO-1 (tight junction protein) was increased by co-cultivation with ACM and in response to shear stress. These findings suggest that the non-contact co-cultivation with HFA helps maintain the barrier properties of HBMEC by secreting factor(s) into the medium. Our *in vitro* flow model system with the cells of human origin should be useful for studying the interactions between endothelial cells, glial cells, and secreted factor(s) as well as the role of shear stress in the barrier property of HBMEC.

Correspondence: Prof. Kwang Sik Kim, Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, 600 North Wolfe Street, Park 256, Baltimore, MD 21287. Email: E-mail: kwangkim@jhmi.edu, Phone: 410-614-3917, Fax: 410-614-1491.

Keywords

Brain endothelial cells; astrocytes; astrocyte-conditioned medium; *In vitro* BBB model; permeability; tight junctions

1. Introduction

The blood-brain barrier is a unique capillary barrier that is formed by brain microvascular endothelial cells and separates the blood from the central nervous system. The barrier plays an important role in the homeostatic regulation of the brain microenvironment. In mammals and higher vertebrates, the complex cell-cell contacts between microvascular endothelial cells comprised of tight and adherens junction proteins prevent the paracellular migration of hydrophilic molecules from blood into the brain [10,59].

Tightness of the intercellular junctions can be monitored *in vitro* using several techniques which include the trans-membrane recording of electrical resistance and the diffusion of dyes or labeled macromolecules across an endothelial cell monolayer. The disruption of the blood-brain barrier integrity has been shown to occur in many neurological disorders such as Alzheimer's disease, stroke and HIV-1 encephalopathy [34,54,63] but the mechanisms involved in BBB disruption remain incompletely understood. A major limiting factor is the lack of reliable models of the human blood-brain barrier.

The structure and physiological properties of the BBB have been extensively studied in isolated microvessels and in primary cultures of brain microvascular endothelial cells derived from non-human origin such as rodents and cows [30,39,45,75]. We have previously developed the *in vitro* BBB model with HBMEC [25,53,72]. The HBMEC were found to be >99% pure endothelial cells, based on specific marker studies [72]. However, *in vivo* HBMEC are shown to interact with astrocytes and also exposed to shear stress through blood flow. Several lines of evidence suggest that non-human brain and non-brain endothelial tight junctions are enhanced when co-cultured with astrocytes or in the presence of ACM [1,5,56,61,62,69,81], and blood flow associated shear stress have been shown to modify the endothelial barrier [67,70,74].

The purpose of the paper was to examine the effects of co-cultivation of HBMEC with HFA/ ACM and to investigate the effect of shear stress on the barrier properties of HBMEC in the presence of HFA/ACM. We designed the in *vitro* flow chamber made of thick plastic chamber connected with peristaltic pump and reservoir. The whole setup was kept inside the CO_2 chamber. The advantages of our *in vitro* flow cell culture system include 1) comparison between the dynamic and stationary system is possible by using the snapwell inserts; 2) continuous medium exchange like *in vivo*; 3) continuous oxygenation of the cell culture medium within the chamber by keeping inside the CO_2 chamber; 4) opportunity to perform continuous or repeat exposure to a test compound; 5) continuous sampling of out-flowing medium for analysis of the metabolic products; 6) the whole system is sterilize-able and reusable; 7) the volumes on the two sides of the membrane can be easily controlled which allows for drug transport studies; 8) electrophysiology studies are feasible because a uniform current can be applied and 9) different combination of experiments can be performed using the multiple chambers attached together.

These advantages allowed us (a) to measure the permeability across the HBMEC monolayer exposed to shear stress by influx or traversal of fluorescent molecules, (b) to assess the role of ACM and shear stress on the barrier property of HBMEC (c) to fix the snap well membrane with paraformaldehyde for immunofluorescent studies of the spatial distribution and

expression of cellular proteins and tight junction proteins and (d) to set up the whole system with tissues derived from humans. In our model system, we measured the transendothelial permeability, TEER and expression of tight junction protein ZO-1, as the markers for the BBB characteristics. We showed that HBMEC co-cultivation with HFA/ACM and exposure to shear stress exhibited decreased permeability and increased resistance and also exhibited increased expression of ZO-1 compared with HBMEC without ACM and flow. Our findings suggest non-contact co-cultivation of HBMEC with HFA/ACM and shear stress enhances the barrier functions, by up-regulating the tight junctional protein ZO-1, and reducing the transendothelial permeability across the HBMEC.

2. Results

2.1 Co-cultivation with HFA decreased the permeability of HBMEC

It has been previously shown that co-cultivation of bovine, rat and porcine brain and non-brain endothelial cells with rat brain astrocytes decrease the permeability of endothelial cells [13, 37,52,70]. However, there are no reports for examining the effects of human astrocytes on the HBMEC barrier properties such as transendothelial permeability and TEER. In the present study, we examined the effect of HFA co-cultivation on the permeability of HBMEC monolayers. To investigate the effect of HFA, HBMEC were cultivated on transwell inserts and HFA on the lower chamber of the 24-well plates as described in the Materials and Methods section, without direct contact and the results were compared with HBMEC without HFA. As shown in Fig 2a penetration of low molecular weight propidium iodide across HBMEC monolayer was significantly reduced by HFA co-cultivation by 30% when compared to the HBMEC alone, suggesting HFA co-cultivation decreased the permeability of HBMEC. This suggests that the co-cultivation of HBMEC with human astrocytes can enhance the barrier properties such as decreased permeability.

2.2 ACM decreased the permeability of HBMEC

Our co-cultivation system did not allow direct contact between HFA and HBMEC, suggesting that the above-noted effect of astrocyte co-cultivation may be mediated by secreted factor(s). Our next experiment was to use ACM derived from HFA instead of glial cells and to examine its effect on HBMEC permeability. HBMEC in the presence of ACM also showed decreased propidium iodide permeability across the HBMEC monolayer by 70% (Fig.2d), when compared to HBMEC alone. These findings support the concept that the secreted factor(s) into the medium by astrocytes helps maintain the barrier properties of HBMEC by decreasing the transendothelial permeability of small and large molecules.

2.3 Co-cultivation with HFA/ACM increased the resistance of HBMEC

We next examined the role of HFA or ACM in another barrier parameter, TEER. To examine the role of HFA and ACM on TEER, SMC and SMC-conditioned medium were used as a control in the TEER measurement to replace the HFA and ACM, respectively. Fig 3a using Endohm showed that both HFA and ACM exhibited the increased resistance, but the resistance decreased with SMC. Transendothelial resistance was also measured by ECIS. Figure 3b revealed that after an overnight incubation in low serum containing medium the resistance of HBMEC was gradually decreased but restored with the addition of ACM, not with SMC CM. We, however, cannot exclude the possibility that the failure to increase the resistance by rat SMC or SMC-conditioned medium might be related to xenogeneic issues. Taken together, these findings indicate that HFA and ACM were able to maintain the barrier properties of HBMEC via secreted factor(s) into the medium.

The above results with HFA/ACM were obtained from HBMEC derived from adults. We also examined whether HFA/ACM exhibit similar properties on the HBMEC derived from children.

HFA/ACM also caused a decreased permeability in these HBMEC (data not shown). In another experiment we replaced the ACM with human astrocytoma derived conditioned medium. The astrocytoma-conditioned medium had similar effects in increasing and maintaining HBMEC monolayer resistance similar to ACM (data not shown). These findings illustrate that co-cultivation of adult/children HBMEC with HFA/ACM or astrocytoma-derived conditioned medium helps maintain the barrier properties of HBMEC.

2.4 Shear stress decreased permeability of HBMEC monolayer

The static co-cultivation using transwell inserts or flat bottom wells or Petri dishes may not mimic the *in vivo* situation for endothelial cells, because endothelial cells are likely to be exposed to shear stress through blood flow *in vivo*. We developed the *in vitro* flow system to mimic the *in vivo* situation of BBB (Fig.1) where endothelial cells are exposed to shear stress on apical side and basolateral side is exposed to glial cells or astrocytes.

To examine the effect of flow on HBMEC permeability, our initial experiments were performed on the 12-mm snap well insert. The results obtained from the cells grown under the static condition were set at 100%. The permeability was significantly reduced by confluent HBMEC monolayer exposed to fluid shear stress of 1-2 dyne/cm^2 when compared to the static condition. (Fig.4a). These results suggest that shear stress contributes to decreasing permeability of HBMEC monolayers.

2.5 Shear stress and HFA decreased permeability on HBMEC monolayer

Using the hollow-fiber *in vitro* BBB model, it has been shown that co-cultivation of rat, bovine and human brain endothelial cells with astrocytes from rat brain and C6 glioma increased the resistance after the introduction of flow with the shear stress of 1 dyne/cm^2 [70]. Our earlier experiments using static co-cultivation showed that permeability of HBMEC was decreased with HFA (Fig.2a). Our next experiments were designed to culture HFA on the lower chamber of the flow system in the presence of shear stress. HBMEC exposed to shear stress was considered as a control and the obtained value was arbitrarily set at 100%. Co-cultivation with HFA significantly decreased the permeability by 40% when compared to HBMEC exposed to flow alone (Fig.4a). These findings indicate that HFA decreased the permeability of HBMEC monolayer with and without shear stress.

2.6 Shear stress and ACM decreased permeability of HBMEC monolayer

As shown earlier with our static co-cultivation experiments, ACM decreased the permeability of HBMEC by 70% (Fig.2b). We next examined the effect of ACM along with shear stress on the permeability of HBMEC. HBMEC that are exposed to flow in the presence of ACM decreased the permeability by 50% when compared to HBMEC exposed to flow alone (Fig. 4b). Taken together, these findings indicate that HFA/ACM and shear stress are additive in exhibiting decreased permeability of HBMEC monolayer.

2.7 Up-regulation of tight junction protein, ZO-1 in the HBMEC co-cultured with HFA/ACM and exposed to shear stress

Previous studies suggest that permeability of endothelial and epithelial cell is regulated by components of tight and adherens junction proteins, which include occludin [32] VE-cadherin [2,17,38], claudin [26], ZO-1 [29,62,71] ZO-2 [31], cingulin [16], 7H6 antigen [65,82] and symplekin [40]. We examined whether the permeability changes occurred in response to astrocyte/ACM and shear stress are correlated with the changes in expression of tight and adherens junction proteins (e.g., ZO-1 and β-catenin, respectively). Immunofluorescent analysis of HBMEC exhibited ZO-1 and β-catenin expression around intercellular junctions (Fig.5a, b). Cell lysates from the HBMEC co-cultivated with ACM or exposed to shear stress

cells were examined for the expression of ZO-1 and β-catenin by Western blot analysis. As shown in Fig 5c & d, ZO-1 expression was increased by 100% in HBMEC exposed to ACM when compared to the control (HBMEC without ACM). Similarly, cell lysates from shear exposed HBMEC also showed the up-regulation of ZO-1 by more than 50% (Fig.5e & f) when compared to the static control. These findings are similar to those of bovine retinal vascular endothelial cells, which showed increased barrier properties and ZO-1 expression in the presence of conditioned media from rat astrocytes [29]. In contrary, the adherens junction protein β-catenin of HBMEC did not show any significant changes in response to ACM and shear stress (Fig 5c & g, and e & h, respectively). These findings suggest that the observed decreased permeability changes with the ACM and shear exposed HBMEC may be related to increased expression of ZO-1.

3. Discussion

The BBB is composed of a specialized microvascular endothelium and glial cell elements, including astrocytes and microglia that are in physical proximity to the endothelium and a basement membrane. All these elements contribute to maintaining the neural microenvironment. The BBB cells containing tight junctions serve to restrict and control the movement of substances between the systemic circulation and brain extracellular fluid and are characterized by a high TEER and low permeability [8,11,14,18,49,59]. BBB disruption leads to extravasation of plasma proteins into the brain through transcellular and paracellular routes [34,54,63]. Our understanding of the mechanisms that lead to the disruption of the BBB is limited. This is due, in part, to the lack of reliable in vitro models of BBB.

There is considerable body of evidence, *in vitro* and *in vivo* to indicate that astrocyte interaction with the cerebral endothelium helps determine the BBB function, morphology (i.e. tightness), and protein expression [3,6,12,58,81]. However, it remains unclear whether or not promotion of the endothelial barrier function by astrocytes requires a direct contact between astrocytes and endothelial cells. For example, contact co-cultivation of bovine adrenal capillary, aortic endothelial cells, rat brain microvascular endothelial cells with neonatal rat brain astrocytes increased the transendothelial electrical resistance and the expression of tight junctional protein such as occludin at endothelial cell junction [28]. A recent study showed that contact co-cultivation of astrocytes help bovine endothelial cells maintain the BBB properties by up-regulating the P-glycoprotein (P-gp) on the endothelial cells [27]. Since astrocytes do not express P-gp, this finding suggests that contact communication between the endothelial cells and astrocytes is necessary for the up-regulation of P-gp.

In contrast, non-contact co-cultivation of astrocytes has been shown to enhance the transendothelial resistance and decrease the transendothelial permeability in *in vitro* model of blood-brain barrier. [1,35,39,44,61,62]. Astrocytes do not make direct contact with endothelial cells *in vivo*, but rather, are separated from endothelial cells by an extracellular matrix. The ~20 nm gap between adjacent astrocytes and endothelial cell is readily diffusible by various proteins, such as horseradish peroxidase [9]. Previously, it was shown that cultivation of human and bovine brain endothelial cells in the presence of ACM further reduced diffusion of FITC-BSA and sucrose permeability [56,62]. Moreover, the soluble factors present in the ACM exhibited the upregulation of HT7, UEA lectin binding sites and angiotensin receptors on the bovine and human adult brain endothelial cells that help to define the phenotype of brain endothelial cells [56]. These findings suggest that the influence of astrocytes may be mediated by a secreted factor(s) [62]. The conditioned medium derived from astrocytes has been shown to be capable of maintaining the BBB characteristics, suggesting that an astrocyte derived soluble factor is responsible for the endothelial cells to develop a BBB property [50,62]. We also showed that non-contact co-cultivation with HFA as well as ACM decreased the

permeability (Fig.2) and increased TEER of HBMEC (Fig.3a.b), suggesting that soluble factor (s) present in the ACM is likely to contribute to the enhanced barrier properties of HBMEC.

Several reports on aortic endothelial cells suggest that the application of shear stress induces morphological and functional changes. The modulation is mediated in part by the regulation of genes encoding for many proteins including vasoactive substances, growth factors and proto-oncogenes [15,33,47,60], affecting cell structure and function in endothelium [21,48]. Recent studies have shown that shear stress affected the brain endothelial cell surface [20,43,70]. In our flow model system, the comparison between static *vs* flow systems using the same size snapwell exhibited significantly greater reduction in the permeability of dextran molecules with the flow system (Fig.4a), suggesting that laminar shear stress enhances the barrier properties of HBMEC monolayer. In contrast, shear stress was shown to increase the dextran permeability and decrease the TEER in bovine aortic endothelial cells [55].

Since we observed the decreased permeability of HBMEC with shear stress when compared to the static cultivation, we next examined the effects of astrocyte/ACM on the barrier properties of HBMEC monolayer using our flow system (Fig.1). Co-cultivations of rat astrocytes and rat glioma cells with rat brain endothelial cells and bovine aortic endothelial cells have been shown to increase the resistance in the presence of shear stress when compared to the endothelial cells alone grown on the hollow fiber apparatus [43,70]. In our shear stress model, non-contact co-cultivation of HBMEC with HFA/ACM also exhibited a decrease in permeability, supporting the roles of HFA/ACM and shear stress in maintaining/exhibiting the barrier properties of HBMEC. Earlier it has been reported that non-contact co-cultivation of bovine and human brain endothelial cells with rat brain astrocytes and conditioned medium enhances the barrier properties [56,62]. Similarly, co-cultivation of bovine, human and rat brain endothelial cells with rat brain astrocytes and C6 astroglioma in the hollow fiber apparatus also increased the resistance, suggesting that both shear stress and glial cells contribute to the maintenance of barrier properties of endothelial cells [70].

Junction complex in the brain microvascular endothelial cells comprises tight and adherens junctions [22,64]. Tight junctions in general, fulfill the two functions. First, they contribute to the maintenance of cell polarity [77,79], and second they seal the gap between adjacent cells and prevent uncontrolled paracellular exchange of small and macromolecules [66,77]. However, the sealing properties of tight junctions vary between endothelial cells of different locations. The mechanisms regulating cell junction organization in endothelial cells are incompletely understood. Several possibilities, such as changes in the expression level, phosphorylation status and distribution pattern of junctional proteins, have been proposed [41,42,51]. The tight junction consists of three integral membrane proteins, namely claudin, occludin and junction adhesion molecules, and a number of cytoplasmic accessory proteins including ZO-1, ZO-2 and ZO-3, and cingulin [4]. ZO and occludin molecules are shown to be primary regulatory proteins of tight junction that modulate BBB permeability [4]. Increased numbers of tight junctions in the presence of astrocytes or astrocyte CM have been demonstrated by the presence of inward ZO-1 expression [29,44,62]. We found that ZO-1 expression of HBMEC was increased in the presence of ACM (Fig. 5 c) and in response to shear stress (Fig. 5e). Moreover, penetration of low molecular weight molecule across the monolayer of HBMEC was decreased, suggesting that increased expression of ZO-1 can be correlated with enhanced barrier properties. Similarly, non contact co-cultivation of retinal endothelial cells with rat brain astrocytes significantly increased the ZO-1 content and also increased the resistance [29], suggesting that up-regulation of ZO-1 can be correlated with enhanced barrier properties. In contrast, we showed that β-catenin, which is part of adherens junction complex did not exhibit any significant changes in the presence of ACM and in response to shear stress. These findings suggest the differential responses of tight and adherens junction proteins in our HBMEC in response to ACM and shear stress.

The chemical nature of the glial cell-produced signal(s) in induction and maintenance of the barrier properties of HBMEC is unclear. Several candidate molecules have been identified, such as TGF β [7,76], GDNF [36,78] and bFGF [69], which can up-regulate the barrier properties by increasing the resistance and decreasing the paracellular permeability in brain endothelial, cells. Several lines of evidence suggest that the enhancement of endothelial barrier properties by astrocytes/ACM and shear stress may be related to TGF-β1 [19,28,76,80]. For example, astrocyte regulation of endothelial cell function has been shown to be mediated in part by astrocyte-derived TGF-β1 and TGF-β1 was shown to be present in ACM collected from rat astrocytes [23,28,76]. In addition, TGF-β1 expression is shown to be increased when human umbilical vein and bovine aortic arterial endothelial cells are exposed to shear stress [19,80]. In our preliminary experiments, treatment of HBMEC with TGF- β1 at a physiological relevant concentration (1 ng/ml) exhibited an increased resistance of the HBMEC monolayer (data not shown). Studies are in progress to determine whether the barrier properties of HBMEC observed with HFA/ACM and shear stress are related to TGF β-1.

In summary, co-cultivation of HBMEC with HFA/ACM and HBMEC exposed to shear stress decreased the permeability and increased the resistance, and co-culitvation with HFA/ACM and shear stress were additive in decreasing the permeability of HBMEC. Western blot analysis of the HBMEC exposed to ACM and shear stress showed the increased expression of ZO-1, whereas β-catenin did not show any significant changes. We speculate that the observed changes of the HBMEC permeability in response to co-culture with HFA/ACM and shear stress may be mediated through tight junction protein(s) and studies are in progress to elucidate the mechanisms.

4. Experimental Procedures

4.1. Materials

Alexa fluor® 488 goat anti-mouse IgG, Alexa fluor® 568 goat anti-rabbit IgG, were purchased from Molecular Probes Inc. (Eugene OR). Monoclonal anti-ZO-1 antibody and polyclonal anti-β-catenin antibody were obtained from Zymed laboratories (San Francisco, CA). Rabbit-anti-actin antibody, protease inhibitors, Tween 20, Propidium iodide and Bovine Serum Albumin (BSA) were obtained from Sigma (St. Louis, MO). Horseradish peroxidase secondary antibody was purchased from Cell Signalling Technology (Beverly, MA). Medium 199, Modified Eagle's Medium (MEM), penicillin and streptomycin, trypsin, Hanks Balanced Salt Solution (HBSS) and SDSPAGE gels were purchased from Invitrogen Corporation (Carlsbad, CA). Heat inactivated fetal bovine serum (FBS) was purchased from Omega scientific Inc. (Tarzena, CA), and Nu Serum was from BD Biosciences (Bedford, MA). L-glutamine was purchased from Irvine Scientific (Santa Ana, CA). 6.5 mm transwell inserts and 12 mm snapwell inserts (0.4 μm pore size) were obtained from Corning Incorporated (Corning, NY). Pronectin-F was purchased from Deepwater Chemicals (Woodward, OK). M-PER extraction buffer was purchased from Pierce (Rockford, IL). Protein assay reagent and peristaltic pump fittings kit were purchased from Bio-Rad (Hercules, CA). PVDF membranes and sterile disposable vacuum filtration system with 0.22 _m Millipore Express Plus Membrane were obtained from Millipore (Bedford, MA). ECL Plus detection system and Hyperfilm™ were obtained from Amersham (Piscataway, NJ). Endohm™ (TEER measurement chambers), EVOM™ (Epithelial Volt ohmmeter), silicone tubing and Peri-Star peristaltic pump were obtained from World Precision Instruments (Sarasota, FL). Nalgene reusable in-line filter holder was purchased from Fisher-Scientific (Newark, DE). ECIS culture-ware™ (8W10E) was purchased from Applied Biophysics Inc. (Troy, NY).

4.2 Cell lines and culture conditions

Primary HBMEC from adult human brain were isolated and characterized as previously described [72]. Briefly, the HBMEC were cultured in medium 199 and supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS), Nu Serum (10%), 100 U/ml penicillin and 100 μg/ml streptomycin. Confluent HBMEC were lifted with 0.05% trypsin-0.5 mM EDTA solution, and resuspended in fresh medium 199. HFA were cultured in MEM medium containing 2 mM L-glutamine and 10% FBS as described previously (HFA medium) [25]. Rat smooth muscle cell (SMC), human astrocytoma (SW1783), and L15 medium were obtained from ATCC (Manassas, VA). SMC were cultured in the HFA culture medium and astrocytoma cultured in the L15 medium.

4.3 Preparation of conditioned medium

To obtain the conditioned medium, HFA were grown on the 75-cm culture flask until confluency. Thereafter, every 24 hours the old medium was collected as ACM and replaced with fresh HFA medium. ACM was sterile filtered and stored at -70 C until use. Rat SMC were grown in the same HFA medium and the conditioned medium from smooth muscle (SMC CM) was also collected like ACM. In the same way, conditioned medium from astroglioma cells using L15 medium was also collected.

4.4 Static co-cultivation of HBMEC and HFA/ACM

Frozen stocks (passage number 13-15) of HBMEC were thawed and cultured on the 75cm culture flasks. For the experiments, medium 199 supplemented with 10% FBS, 10% Nu serum and L-glutamine (2 mM) were used. 2×10 cells were seeded onto the 6.5mm transwell inserts (0.4 μm pore size) coated with Pronectin-F (10 μg/ml). Frozen stocks of HFA, passage number 6-10, were thawed and cultured in MEM supplemented with 10% (vol/vol) heat-inactivated FBS, L-glutamine (2 mM), 100 U/ml penicillin and 100 μg/ml of streptomycin as described previously [25]. Confluent HFA was detached using the 0.05% trypsin-0.5 mM EDTA solution and resuspended with HFA medium. 2×10 HFA were seeded onto the Pronectin-F coated 24 well plate. Once the HBMEC became confluent, the transwells containing HBMEC were transferred to 24 well plate containing confluent astrocytes. Fresh 250 μl of medium 199 was added to the upper chamber of the transwell inserts and 1 ml of HFA medium was added into the lower chamber of the 24 well plate. In separate experiments, astrocytes were replaced with ACM, for which1:1 ratio of ACM and HFA medium was used. HBMEC and HFA or ACM were allowed to grow for 5 more days. Fresh medium was provided every other day for both upper and lower chambers. On day 5, the permeability assays were performed as described below.

4.5 Preparation of the flow system

In vitro flow chambers were fabricated in collaboration with World Precision Instruments (Sarasota FL). The dynamic *in vitro* flow system consists of a transparent chamber, made of thick plastic having hollow space, in which a snapwell insert with 12 mm diameter can be housed, that separates the upper from lower chamber. The attachment of the upper and lower chamber was sealed by Teflon O-ring and was tightened with the stainless steel screws. As shown in the figure 1, the upper chamber has the inlet and outlet ports through which multiple individual chambers are connected to the speed-controllable peristaltic pump with the gas-permeable, platinum-cured silicone tubes that run through the medium 199 reservoir and back to the chambers. The medium passing through the upper chamber applies the shear stress on the confluent HBMEC growing on the snapwell membrane. The in-line filter holder with 0.22-μm filters was placed between the chamber and the medium reservoir to remove the dead cells floating from the system. The whole setup was kept inside the temperature-controlled, water-

jacketed CO_2 incubator. The flow chamber, gas permeable platinum-cured silicone tubes, in-line filters and accessories used for the flow system were sterilized by ethylene oxide.

Before applying the shear stress, 5×10 HBMEC were seeded on the Pronectin-F coated 12-mm snapwell inserts and allowed to attach and grow in a static condition. At the same time, HFA were seeded on the 8-mm glass cover slips that fit onto the bottom of the flow chamber. After 24-36 hours, the endothelial cells were transferred to the flow chamber and subjected to fluid shear stress 0.5 dyne/cm^2. Once the HBMEC reached confluence (based on the microscopic observation), the 8-mm cover slips with HFA were transferred to the lower chamber of the flow system. In other experiments, ACM was added instead of HFA. The HBMEC and HFA or HBMEC and ACM were allowed to grow for 5 days and subjected to physiologically relevant shear stress 1-2 dyne/cm^2. The medium 199 was replaced with the fresh medium every 24 hours. The fluid shear stress strength was calculated according to the formulae: $\tau = 6\mu Q/a^2 b$, where μ is the viscosity of the perfusate, Q is flow volume (ml/s), **a** and **b** is cross sectional dimension of the flow path in centimeters [68].

4.6 Permeability assay

In the case of *in vitro* flow chamber system 12-mm snapwell insert-containing HBMEC was removed from the flow chamber and transferred to the 6-well plate filled with 1.5 ml of HBSS without phenol red. The HBMEC were washed with medium 199. The final volume of the medium inside the snapwell was 300 μl. 150 μl of the medium (total volume 300 μl) was removed and replaced with 150 μl of HBSS containing 0.5 mg/ml of fluorescent-conjugated dextran (70,000 Da) and 0.5 mg/ml propidium iodide (668 Da). The plates were kept in temperature controlled CO_2 chamber at 37°C. After 60 minutes of incubation, 50-μl aliquots from the bottom of the 12 mm snapwell insert were collected and transferred to the 96-well plate. Fluorescent intensity of the assays were measured using the plate reader, Fluoroskan Ascent FL, having two different filters for fluorescent dextran and propidium iodide (Thermo Electron Corporation, Franklin, MA). The graphs were made using Microsoft Excel. For permeability assays using 6.5-mm transwell inserts, 0.5-mg/ml fluorescent-conjugated dextran and propidium iodide in a total volume of 100 μl was added. The absolute fluorescence intensity was used after background subtraction. HBMEC grown alone on the transwell or snapwell insert were used as a control.

4.7 Measurement of transendothelial electrical resistance (TEER)

To measure TEER across the 6.5 mm transwell, we used Endohm-EVOM measuring system (World Precision Instruments). Resistance was measured using an assembly containing current passing and voltage measuring electrodes. The resistance of blank inserts was subtracted and the results were calculated per cm of the membrane. The resistance readings were taken every 24 hours.

In addition, a new special ECIS™ (Electric Cell-substrate Impedance Sensing) Model 1600R Morphological Biosensor (Applied Biophysics) was used to measure the real-time TEER during exposure to ACM and SMC-CM. HBMEC were directly seeded on a collagen-coated eight-well gold electrode array in medium 199 supplemented with 10% FBS and Nu serum. Each well had one active electrode (250-μm diameter) and a large counter electrode. Both electrodes were connected to a phase-sensitive lock-in amplifier. The electrodes were fed with a constant current of 1μA supplied by a 1-V, 4,000-Hz AC signal. Each well contained 400 μl of the medium. Initial resistance of electrodes in medium 199 was 1400-1500Ω. After reaching maximal, steady-state readings of TEER that meant maximal confluence, cells were additionally incubated overnight in low-serum (0.5% FBS) medium 199, and then ACM and SMC-CM-induced changes in resistance of endothelial monolayers were monitored. The conditioned medium was mixed in an equal volume (1:1) with the fresh medium.

4.8 Immunofluorescent staining of cell junctional proteins

Primary HBMEC grown on cover slips were fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were washed three times with PBS for 5 min each. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 3 minutes and again washed three times with PBS. Then, the cells were blocked for 1 hr in a PBS solution containing 5% BSA (Sigma). This was followed by overnight incubation at 4°C with 1:200 dilution of the primary ZO-1 and β-catenin antibody. The cells were washed three times in PBS, 5 min each, and incubated for 2 h at room temperature with secondary antibodies (Alexa fluor® 488-conjugated anti-goat and Alexa fluor® 568 conjugated anti-rabbit antibodies) diluted 1:500 in PBS/BSA. Cover slips with the cells were again washed three times in PBS before being mounted onto the stage of a fluorescent microscope. Normal rabbit IgG, mouse IgG, and goat IgG were used as negative controls, which did not show any fluorescence increase above background.

4.9 Gel electrophoresis and Immunoblotting

Total cell lysates were obtained from HBMEC grown on the transwell or snapwell inserts under the flow and static conditions as described previously. Briefly, HBMEC rinsed with ice-cold PBS and lysed with MPER extraction buffer plus cocktail of protease inhibitors (20 mM Tris pH, 8.0, 150 mM NaCl, 1 mM DTT, 1% sodium deoxycholate, 0.5% SDS, 1% nonidet P40, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride and 10 mM sodium fluoride). Protein concentrations were determined with a protein assay reagent (Bio-Rad). For each condition, 10 μg protein was separated on 10% SDS-PAGE gel in an Xcell Surelock™ Electrophoresis Cell (Invitrogen) and transferred to PVDF membranes. The membranes were blocked for 1 hr at room temperature with 5% non-fat dry milk, and 0.05% Tween 20 in PBS, and subsequently incubated with the primary antibody against monoclonal ZO-1, polyclonal β-catenin and rabbit-anti-actin in 5% BSA and 0.05% Tween 20 for overnight at 4°C with shaking. The membranes were washed three times with 0.1% Tween 20 in PBS for 10 min each. The membranes were incubated with anti-mouse and anti-rabbit horseradish peroxidase secondary antibody (1:2000) for 1 hr at room temperature, and then washed as above. Antibody-antigen complexes were visualized using the ECL Plus detection system and detected on Hyperfilm™. The bands were normalized against their respective controls using Software Quantity One, version 4.2.2 (Bio-Rad, Hercules, CA).

Acknowledgments

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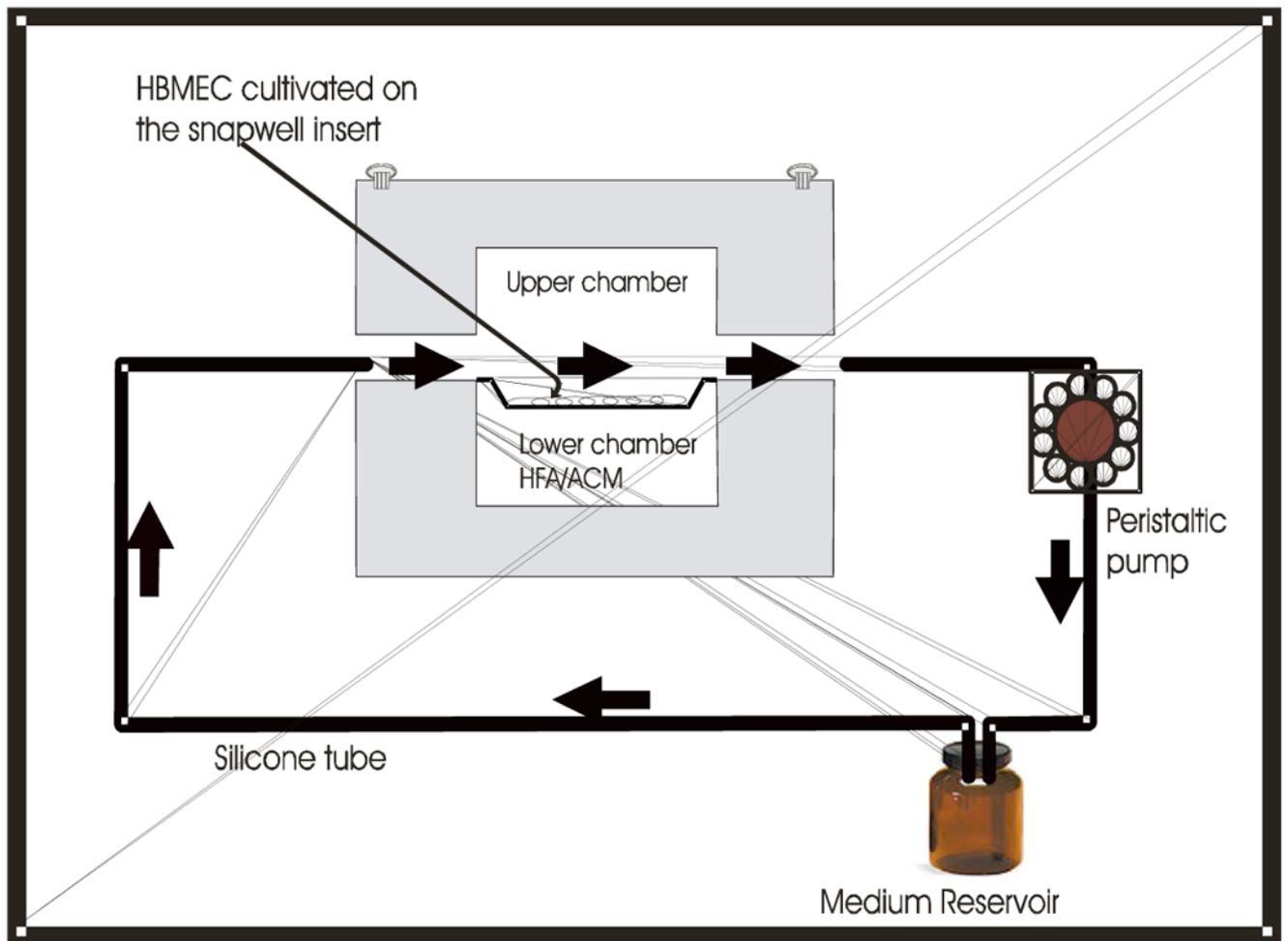
Fig.1.
Diagrammatic representation of the *in vitro* blood brain barrier model. The dynamic *in vitro* flow system consists of a transparent plastic chamber, with upper and lower chamber, having hollow space in between where a snapwell insert with 12 mm diameter is housed. The upper and lower chambers are tightly sealed with Teflon O-ring and stainless steel screws. Flow chamber is connected to the speed-controllable peristaltic pump with the gas-permeable, platinum-cured silicon tubes. The whole set up is kept inside the temperature controlled CO_2 incubator. The arrow indicates the direction of the flow.
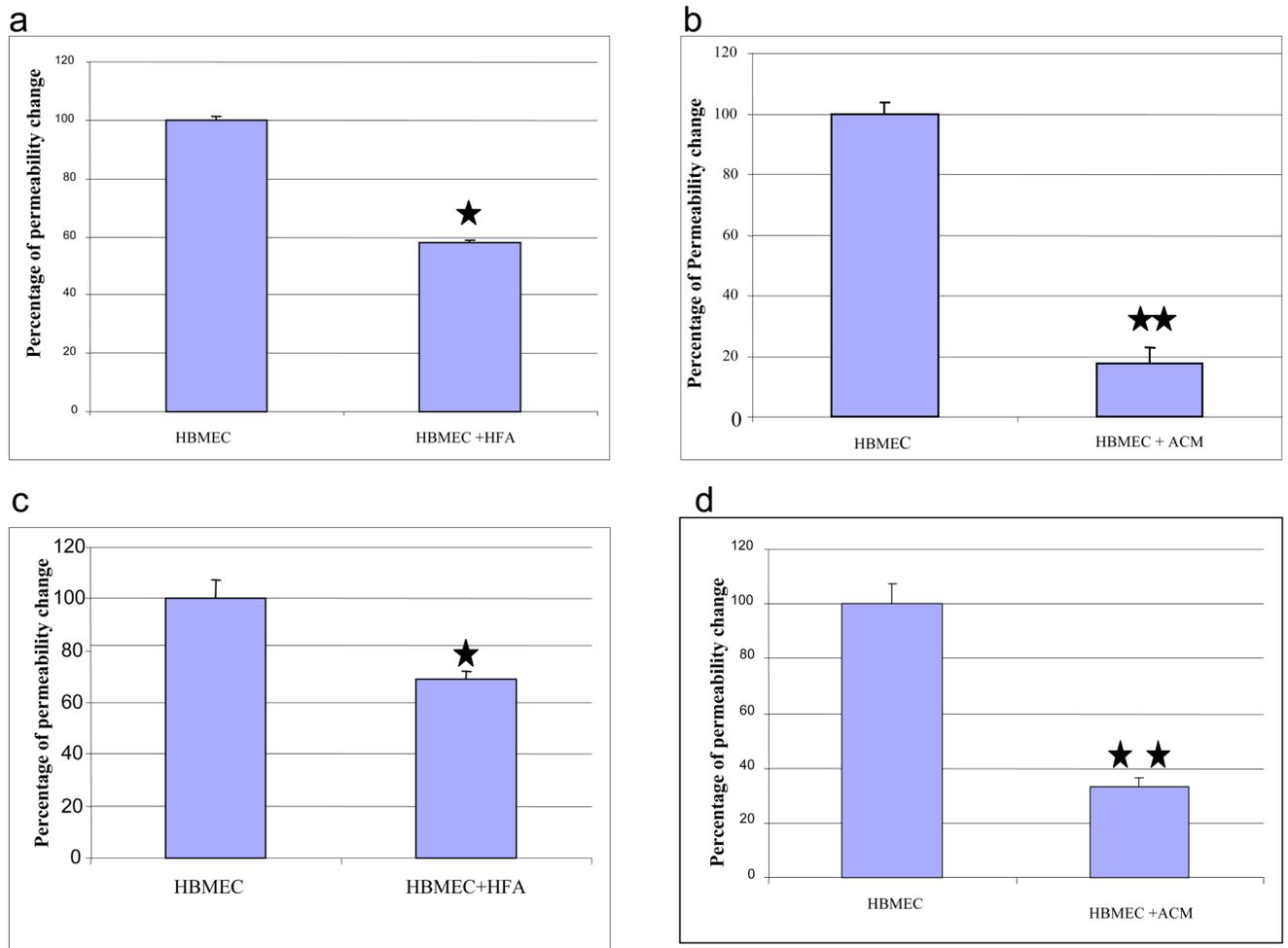
Fig.2.
Static co-cultivation of HBMEC with HFA or ACM significantly reduces the permeability of fluorescein-conjugated dextran (a, b) and propidium iodide (c, d) across the endothelial cell monolayer. HBMEC grown on the transwell inserts were co-cultivated with HFA (a, c) and ACM (b, d). The fluorescein dextran and propidium iodide permeability was determined as described in Materials and Methods section. An average of 3 experiments are shown in each panel and the results are presented as mean + SEM. *P<0.05, **P<0.01.
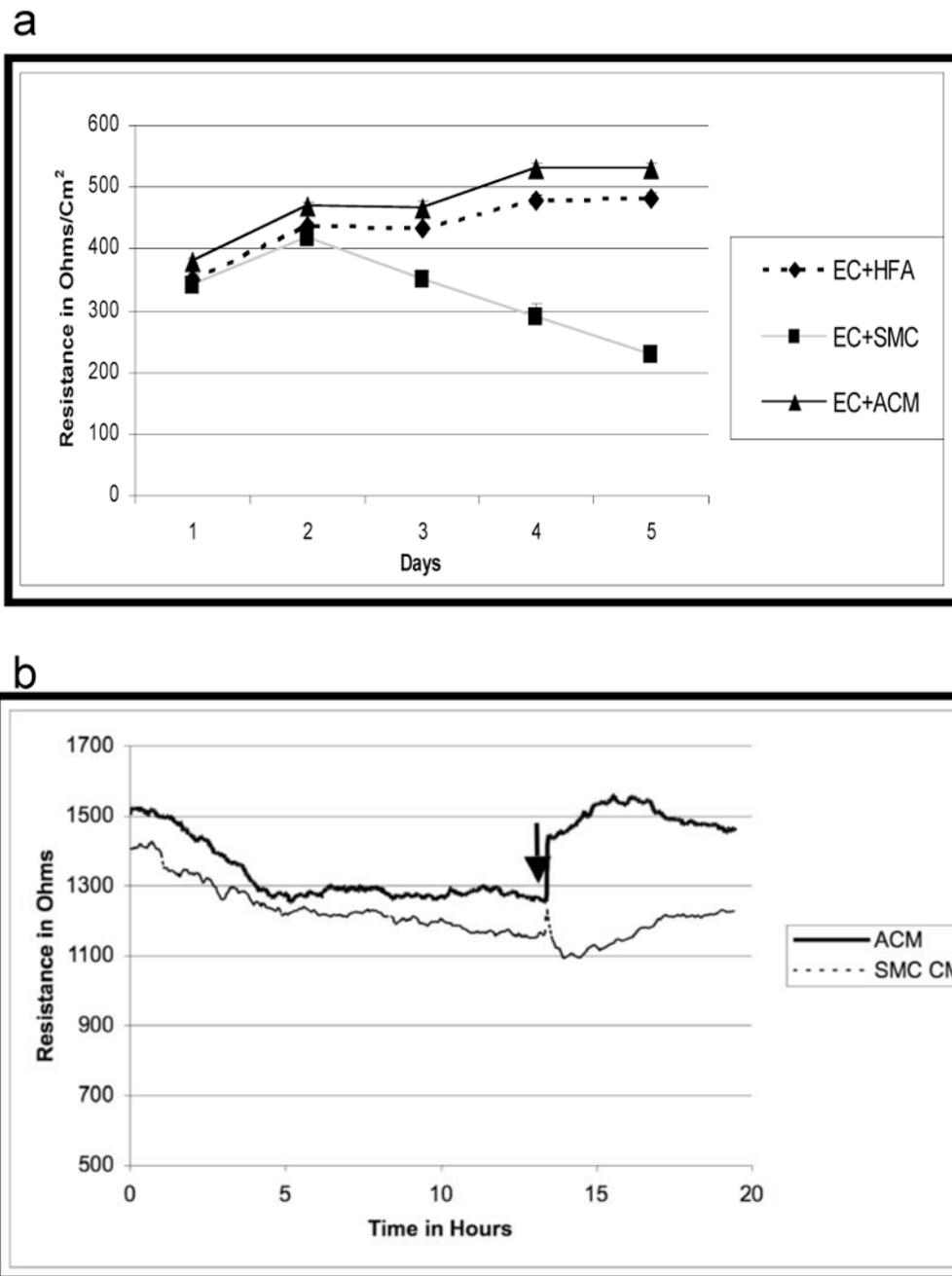
Fig.3.
Static co-cultivation of HBMEC with HFA and ACM increases the TEER. (a) Endohm TEER measurements across HBMEC monolayer grown on the transwell insert membrane in the presence of HFA, ACM or SMC. (b) HBMEC grown on collagen-coated gold electrodes of the ECIS culture-ware were maintained on low serum containing media for overnight prior to the addition of ACM or SMC-CM. Arrow represents the addition of conditioned medium. Representative traces of 3 independent experiments are shown.
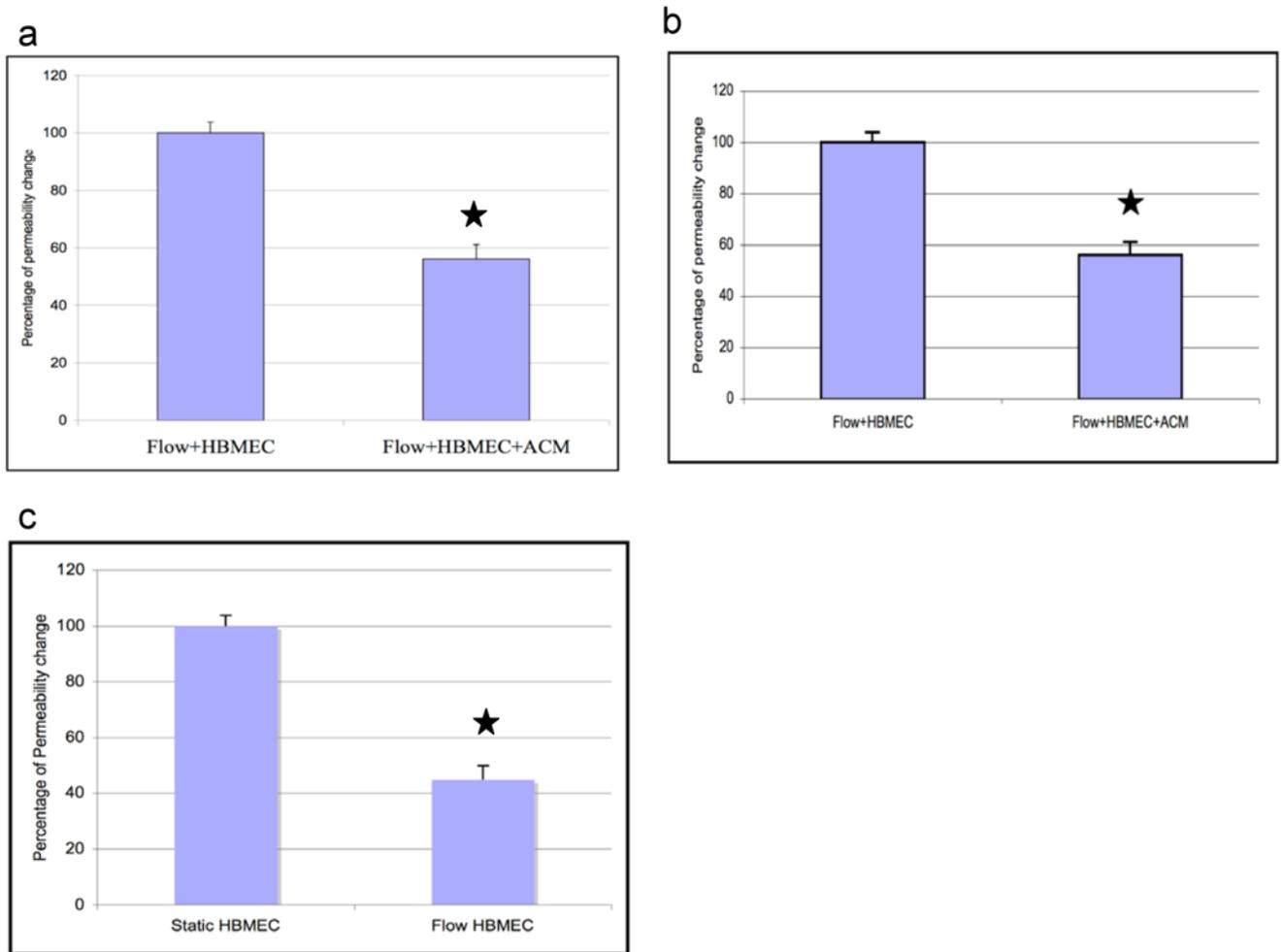
Fig.4.
Application of shear stress enhances the barrier properties of HBMEC monolayer. Fluorescein dextran permeability was measured across the HBMEC monolayer grown on snapwell inserts for 5 days under shear stress with HFA or ACM. Flow-exposed HBMEC monolayer (a) significantly decreased the dextran permeability when compared to the static cultured HBMEC. Static values were arbitrarily set at 100%. Flow-exposed HBMEC monolayer co-cultivated with HFA (b) and exposed to ACM on the lower chamber (c) significantly decreased the dextran permeability compared to HBMEC exposed to shear stress. The results are normalized against the permeability of the HBMEC exposed to shear. An average of 3 experiments are shown in each panel and the results are presented as mean + SEM. *$P<0.05$.
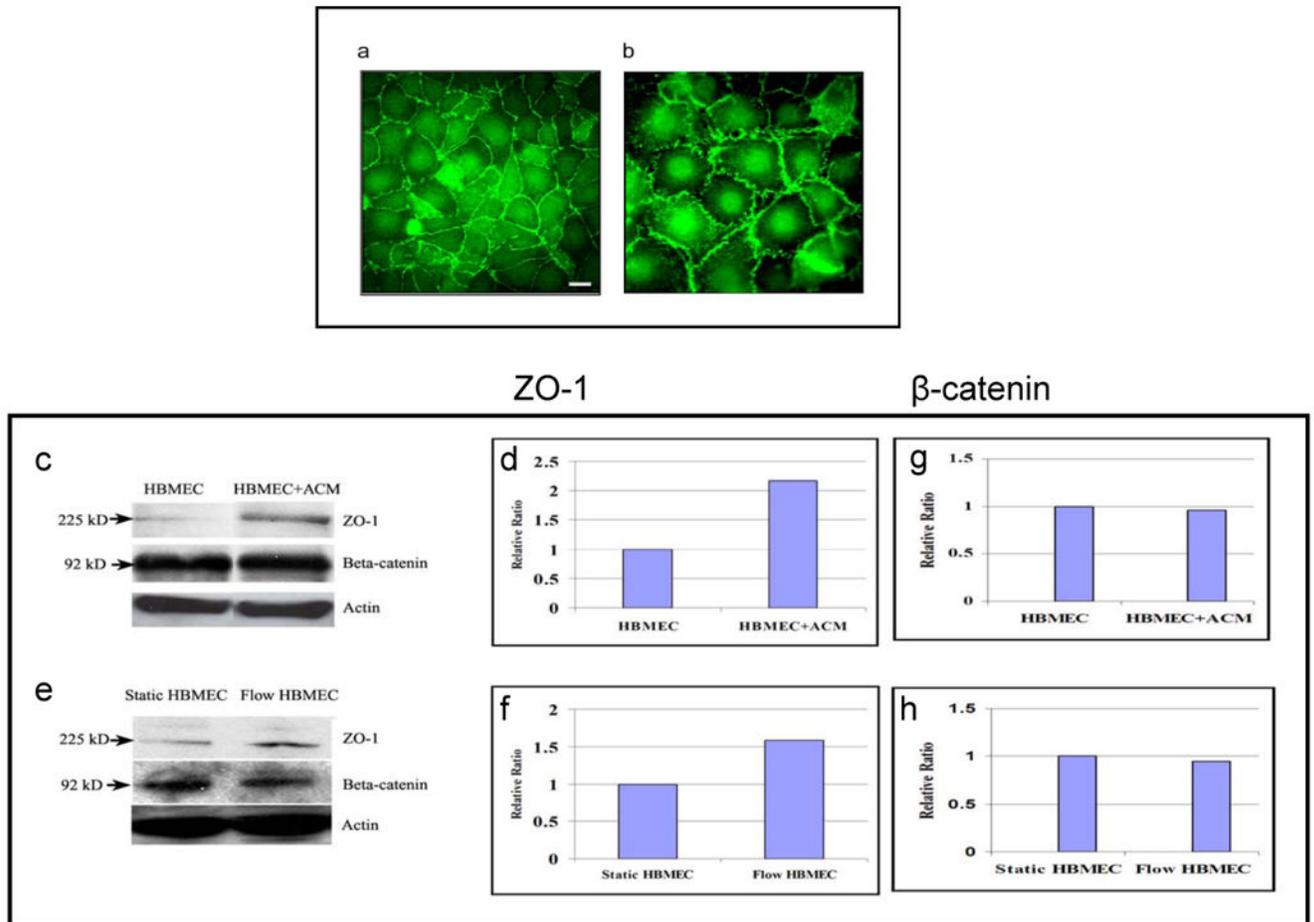
Fig.5.
ACM and shear stress increases the expression of ZO-1 in HBMEC. (a) Immunofluorescence staining of tight junction protein (ZO-1) and (b) adherens junction protein (β-catenin). Western blot analysis of tight junction protein, ZO-1 and β-catenin from cell lysates of (c) HBMEC co-cultivated with ACM (e) HBMEC exposed to shear stress. (d, g) and (f, h) show relative expression ratio of the bands presented on (c) and (e) obtained using gel densitometer. Scale bar = 40 μm.