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TNF-α and IL-1β Increase Pericyte/Endothelial Cell Co-Culture Permeability

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Background. **Pericytes (PC) have a unique synergistic relationship with microvascular endothelial cells (MVEC) in the regulation of capillary permeability. This study investigates the effect of TNF-α, IL-1β, and IL-6 on the microvasculature by measuring changes in PC contractility, and also, albumin permeability across MVEC/PC co-cultures.**

Materials and methods. **Semi-permeable inserts were plated first with rat lung MVEC and then PCs (on the fourth day) at a ratio of 10:1 MVEC/PC. On day 5, 50 ng/ml of TNF-α, IL-1β, and IL-6 were added with or without a secretory phospholipase A_2-IIA ($sPLA_2$-IIA) inhibitor for 24 h. After treatments, albumin clearances were quantified. For measuring contractility, PCs were cultured on collagen matrices and exposed for 24 h to TNF-α, IL-1β, and IL-6 at 1 ng/ml, 10 ng/ml, and 50 ng/ml with/without inhibitors for $sPLA_2$-IIA, phospholipase A_2 (PLA_2), and cyclooxygenase-II (COX-II). After treatments, the surface area of the collagen disks was digitally quantified.**

Results. **TNF-α and IL-1β significantly increased albumin clearance in MVEC/PC co-cultures ($P < 0.05$) and induced dose-dependent relaxation of PCs ($P < 0.05$). PC relaxation was completely attenuated with the $sPLA_2$-IIA and pLA_2 inhibitors; the COX-II inhibitor provided partial blockade. IL-6 had no effect on PC contractility or permeability.**

Conclusion. **TNF-α and IL-1β directly increased microvascular permeability in co-cultures. They also induced relaxation of PCs through a $sPLA_2$-IIA dependent mechanism. Interestingly, IL-6 had no effect, although its presence in high levels has been demonstrated in inflamed lungs. These findings may help elucidate the significance of PC in regulating the capillary response to various pro-inflammatory cytokines.**

Key Words: **pulmonary microvasculature; permeability; sepsis; pericytes; endothelial cells; co-cultures; ARDS**

INTRODUCTION

Inflammatory mediators cause a wide array of dysfunction, particularly in the pulmonary system where changes in vascular dynamics and increases in microvascular permeability can lead to the formation of edema and subsequent respiratory failure.

Previous studies have suggested that the microvascular barrier is composed of endothelial cells surrounded by pericytes (PC) and that both cell types contribute to the formation of the basal lamina (basement membrane) [1–3].

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are well known mediators of shock [4–7]. TNF-α and IL-1β are present in high concentrations in bronchoalveolar lavage fluid of patients with sustained ARDS [6], and IL-6 has been shown to be up-regulated in the lungs of septic patients [7]. Studies have shown that TNF-α and IL-1β can directly alter vascular tone [8] and increase microvascular permeability in endothelial monolayers [9–11]. Traditionally, *in vitro* studies of permeability have focused around changes in the barrier function of endothelial cell monolayers. Our laboratory has successfully co-cultured pulmonary microvascular PC and endothelial cells on gelatin coated semipermeable inserts and shown that PC augment the barrier of endothelial cell monolayers [12].

This study investigates changes in permeability because of TNF-α, IL-1β, and IL-6 in endothelial cell/PC co-cultures compared to endothelial monolayers. Addi-

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tionally, the direct effects of these cytokines on PC contractile tones were measured along with the use of inhibitors for secretory phospholipase A_2-IIA (sPLA$_2$-IIA), phospholipase A_2 (PLA$_2$), and cyclooxygenase-II (COX-II) to help elucidate mechanistic pathways.

MATERIALS AND METHODS

PC Isolation and Culture

Microvascular PC from rat lungs were isolated by methods previously modified by this laboratory [13] in accordance with The National Institutes of Health guidelines and using procedures approved by the Animal Investigation Committee of Wayne State University. In summary, peripheral portions of rat lungs were minced and digested with collagenase, filtered through a 100 μM mesh to isolate microvessels, and then plated. Platelet deficient serum (PDS) along with the use of non-coated plates assured the preferential growth of PC. Morphologically, PC were identified by their large, irregular shape with dendritic processes and a lack of "hill and valley" morphology typical of smooth muscle cells at confluence [1]. PC identity was confirmed by the presence of smooth muscle actin observed by indirect immunofluorescent microscopy with monoclonal anti-alpha smooth muscle actin. For further characterization, the average resting membrane potential of PC was determined to be approximately -30 mv by patch-clamp [14, 15].

Endothelial Culture

Rat pulmonary microvascular endothelial cells were purchased (VEC Technologies, Rensselaer, NY) and grown to confluency in MCDB 131 media (VEC Technologies, Rensselaer, NY). Endothelial cell monolayers demonstrated typical cobblestone appearing morphology and were positively stained with acetylated low-density lipoprotein (Sigma-Aldrich, St. Louis, MO).

Permeability Assay

Initially, endothelial cells were grown on semipermeable inserts (Becton, Dickinson and Company, Bedford, MA) at a concentration of 3×10^5 cell/ml and allowed incubate for 3 days until the monolayer was confluent. Pericytes were then added at a ratio of 1:10 and grown in co-culture for an additional 2 days. TNF-α, IL-1β, and IL-6 (Sigma-Aldrich) at 50 ng/ml were added to co-cultures for 24 h. The effect of a sPLA$_2$-IIA inhibitor (10 μM), c(2NapA)LS(2NapA)R TFA (Calbiochem, San Diego, CA) on these treatment groups was also tested. Bovine serum albumin at 5000 μg/ml was added to the abluminal compartment of co-cultures and a Bradford Protein Microassay was performed to determine the clearance of albumin across the barrier. Controls included co-cultures with no treatment, blank semipermeable inserts, and endothelial monolayers with treatment groups identical to co-culture couterparts. Albumin clearance was calculated as follows:

$$\text{Clearance} = \frac{\left[\text{albumin conc.} \times \text{volume}(\text{well})/\text{albumin conc.}(\text{insert})\right]}{\Delta\text{time}}$$ [12]

Contractility Assay

Collagen gel matrices were prepared as previously described [13]. Collagen type 1 (Calbiochem) was dissolved in 1% acetic acid overnight and stored at 4°C as stock (3 mg/ml). Stock collagen was added to plating medium [Dubelco's Modified Eagle Media (DMEM) with 10% fetal bovine serum (FBS) and 1% ABX, Sigma-Aldrich] at a ratio of 1:3 and plated onto 24-well plates (0.5 ml/well). The plates were then incubated at 37°C for 30 min to allow the collagen to solidify and gel. Cultured PC was plated on top of the collagen gels at a concentration of 7.5×10^4 cells/well in plating media. The collagen gels with PC were then incubated for 48 h and allowed to proliferate at 37°C and 5% CO_2. After 48 h, the original plating media was replaced with assay media containing DMEM and 15 μM bovine serum albumin (BSA) with the various experimental mediators.

Pericytes were incubated for 24 h in assay media with TNF-α, IL-1β, and IL-6 at concentrations of 1, 10, and 50 ng/ml. The effects of inhibitors for phospholipase A_2 (pLA$_2$; aristolochic acid, Calbiochem; 100 μmol/L), secretory phospholipase A2-IIA [sPLA$_2$-IIA; c(2NapA)LS(2NapA)R TFA, Calbiochem; 10 μmol/L], and cyclooxygenase-II [Methyl [5-methylsulfonyl-1-(4-chlorobenzyl)-1H-2-indolyl]carboxylate; Calbiochem; 1 μmol/L] were tested separately in the presence of 50 ng/ml of TNF-α, IL-1β, or IL-6. The inhibitors were added simultaneously with the various pro-inflammatory cytokines and were incubated together for 24 h in assay media.

After the incubation period, collagen gels were physically released from the sides of wells. A Kodak digital camera was then used, and the photographs were analyzed using ID Image analysis software (Kodak, Rochester, NY) to determine the surface area of the gels in pixels. Contractility was quantified by determining the magnitude of decreased or increased surface area in comparison to control collagen gels plated with PC receiving no treatment. The results of the various treatments were expressed as a percentage change in contraction in comparison to control gels.

Cell Viability

PC viability was determined after the 24 h assay periods were complete. The gels were digested in 10 mg/ml collagenase type 1 (Gibco, Carlsbad, CA) for 30 min and the remaining cells were washed with phosphate buffered saline (PBS; Sigma-Aldrich). The total number of PCs and the number of trypan blue-positive PCs were counted using a hemocytometer to determine percentage of viable cells.

Statistical Analysis

Results are expressed as a mean $\pm$ standard error of mean (SEM). Statistical analysis was performed using a one-way ANOVA comparing treatment groups to control. A value of $P < 0.05$ was defined as a significant difference.

RESULTS

Effects of TNF-α, IL-1β, and IL-6 on MVEC/PC Co-Culture Permeability

The albumin clearance measured through semipermeable inserts coated only with collagen and no cells (blank wells) was 0.213 ± 0.002. In contrast, the clearance for MVEC/PC controls with no cytokine treatment was 0.124 ± 0.004. These two clearances served as the upper and lower reference points for which to compare all subsequent treatment groups. The results represent an average of five separate assays and are represented in Fig. 1.

Exposure to TNF-α and IL-1β at 50 ng/ml for 24 h caused a significant ($P < 0.05$) increase in albumin clearance across co-culture barriers when compared to control wells with no treatment. TNF-α increased permeability by 54% (0.191 ± 0.002) and IL-1β by 52% (0.189 ± 0.002) compared to control (Fig. 1). After TNF-α and IL-1β treatment for 24 h, a barrier effect