Published in final edited form as:

Atherosclerosis. 2010 October ; 212(2): 701–703. doi:10.1016/j.atherosclerosis.2010.06.030.

Baboon fetal arterial endothelial cells are more responsive to challenge by tumor necrosis factor α (TNF- α) than baboon fetal umbilical vein endothelial cells

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Keywords

endothelial cells; umbilical vein endothelial cells; arterial endothelial cells; tumor necrosis factor; atherosclerosis; fetus; nonhuman primate; CCL2

Dear Editor

Endothelial cells (ECs) serve many functions including responses to inflammatory mediators, e.g. TNF- α from macrophages and immune cells that is considered to play a role in the initiation of atherosclerosis (1). Human umbilical vein ECs (HUVECs) are readily available but not normal human fetal systemic arterial ECs (2–4). Comparisons of fetal and adult ECs do not address effects of altered fetal environments on EC function since fetal pO₂, glucose and blood pressure differ considerably from adults. The developing fetal vasculature is susceptible to damage predisposing to vascular disease including atherosclerosis. For example, maternal hypercholesterolemia in pregnant rabbits leads to development of fetal atherosclerotic lesions by term (5;6). To remove this barrier to progress we characterized and established fetal baboon UVEC (FBUVECs) and fetal aortic arterial EC (FBAECs) lines for study by ourselves and other investigators to investigate developmental programming of predisposition to atheroma

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and arterial disease by sub-optimal intrauterine conditions. We chose the terminal fetal aorta, a common site of atherosclerosis that can be biopsied in adult baboons, allowing follow up post-natally (7).

TNF- α induces robust HUVEC gene expression (8), via TNFR1 and TNFR2, cell surface receptors whose effects on EC include increased protein synthesis-independent cell permeability and motility changes, leading to localized inflammatory responses, vascular leakage and edema (9). Although TNFR1 expression is generally lower than TNFR2, signals elicited by TNF- α are considered largely dependent on interaction with TNFR1 (10). Chemokines (e.g. CCL2) (1) produced by activated EC attract white cells to adhere to EC's penetrating the vessel intima potentially producing arterial wall damage and predisposing to atherosclerosis (11). We hypothesized differences between FBUVECs and FBAECs in basal activation state and CCL2 production following TNF-stimulation.

We studied term pregnant baboons (*Papio anubis*) in AAALAC approved facilities. Animal care procedures were approved by the Southwest Foundation for Biomedical Research IACUC (12;13). Sterile FBUVEC were released from 5 cm umbilical vein mid-portion and FBAEC from 2.5 cm distal aorta of the same fetus and collected by centrifugation, resuspended in medium and seeded into 25 cm² culture flasks (5% CO₂ at 37°C) (7;14). Cells were grown to confluence, detached with 0.25% trypsin placed in 15ml conical tubes containing EC basal medium-2, centrifuged at 100 rpm, media removed and the pellet resuspended in 0.5 ml 0.1% FBS in PBS and purified using magnetic CD31 antibody coated Dynabeads. The mixture was incubated for 20 min (2–8°C) under gentle agitation, volume doubled with buffer and placed in a magnet for 2 min. The supernatant was discarded and bead bound cells washed 3 times with buffer and seeded into culture flasks. Next day, cells were washed to remove unbound beads, seeded into culture flasks, grown to 70–90% confluence, detached using 0.25% trypsin, beads removed with a magnet and supernatant cells divided. Half of the cells were removed and seeded into 6-well chambers to grow to confluence (about 2 days) for the TNF- α challenge (10 ng/mL) and the rest frozen (–80°C) for later evaluation of maintenance of phenotype following freezing. Supernatant CCL2 production was assessed by ELISA. A separate set of cells was assigned for flow cytometry. Two hours after starting the TNF- α challenge, Brefeldin-A was added (10 μ g/ml) Brefeldin was not added to wells used to determine CCL2 secretion. After 4 hours medium was removed, cells washed with PBS, detached using EDTA solution and resuspended in 1.0 ml DMSO, FBS+ medium and frozen (–80°C) for later flow cytometry analysis. The cells which had been frozen to evaluate maintenance of phenotype were reconstituted, similarly grown to confluence and tested for their TNF- α response.

Frozen cells were thawed slowly at room temperature (RT), 8.0 mL EC medium added and centrifuged (4°C, 1000 rpm for 10 min), medium aspirated and discarded. The pellet was resuspended in 0.3 mL of EC medium. 500,000 cells/tube were incubated with medium, ECs were mixed with 3 μ L of anti-CD31 FITC or 3 μ L of Isotype antibodies IgG1-FITC, for 15 min (RT in the dark). Subsequently, 500 μ L of 1:10 diluted distilled water FACS lysing solution was added and incubated (10 min RT), followed by centrifugation (900xg for 5 min at RT). Medium was decanted and the pellet re-suspended in 500 μ L of FACS permeabilizing solution diluted 1:10 with distilled water. After 10 min incubation at RT, cells were centrifuged medium removed and cells resuspended in 200 μ L PBS. Cells were stained with the following antibodies: anti-CCL2-PE or Isotype control antibodies IgG1-PE. Finally, the cell suspension was incubated (30 min at RT) in the dark, centrifuged, medium removed, the pellet resuspended in 100 μ L of PBS, and analyzed by flow cytometry. Data were analyzed using Summit software. Adequate adjustment of voltages for channels FSC, SSC, and each fluorescence signal was made based on isotype stained control cells. TNFR1 and TNFR2 protein expression were analyzed on cell lysates by Western analysis (15) using rabbit polyclonal antibodies to TNFR1,

TNFR2 and beta-actin-HRP, and polyclonal anti-rabbit-IgG-HRP. Comparisons were by two-way repeated measures ANOVA with Bonferroni correction, α level 0.05

The percentage cells expressing CD31 (Fig 1A) and MFI (Fig 1B) were similar in FBUVECs and FBAECs in media and after TNF- α . Percentage CCL2 producing CD31+ cells was higher in FBAECs than FBUVECs following TNF- α (Fig 1C). However, intracellular CCL2 MFI was higher in FBUVECs than FBAECs (Fig 1D). FBAEC CCL2 production (Fig 1E) was higher than FBUVEC agreeing with flow cytometry data. Importantly for potential further studies, cell phenotype was retained following freezing. CCL2 production by TNF- α in fresh or frozen cells was similar and higher in FBAEC (Fig 1F). TNFR2 (Fig 1H) but not TNFR1 (Fig 1G) protein was higher in FBAEC than FBUVEC and levels were unchanged with TNF-stimulation.

In summary, these data are the first direct comparison of umbilical venous and systemic arterial basal and TNF-induced CCL2 secretion by fetal primate ECs. We assured ourselves of an enriched EC population by initial CD31+ purification and characterization of phenotype by flow cytometry. Our results show no TNF- α induced shift in percentage CD31+ cells or production indicating that the TNF- α dose tested did not trigger EC proliferation or apoptosis. The higher FBAEC response to TNF- α is associated with increased TNFR2 but not TNFR1 protein compared with FBUVEC suggesting that TNFR2 is responsible for the greater FBAEC response. Our demonstration that FBAEC exhibit increased responsiveness to TNF- α compared with FBUVEC may point the way towards one mechanism that potentially puts the developing arterial system at greater atherosclerotic risk when developmental conditions are adverse (5; 6). However, further studies with blocking antibodies for the receptors are needed. Importantly, phenotype is maintained following freezing thus making this model system suitable for more in depth studies involving batch processing and we would be pleased to make these cells available to other investigators in this area.

Acknowledgments

This work was supported by NIH grant HD 21350-17 and in part by the Perinatology Research Branch, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS. The authors thank the staff of the CPNR and Southwest National Primate Research Center for their assistance.

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Figure 1.

Responses in fetal baboon arterial (FBAEC – open) or umbilical vein, (FBUVEC – filled) endothelial cells cultured 4h in medium alone (Medium) or with 10 ng/mL TNF- α (TNF- α). A) and B) Flow cytometry data; C) Percentage CCL2 expressing cells and D) CCL2 MFI; E) CCL2 production *in vitro*; F) Comparison of results obtained in fresh and frozen cells to demonstrate lack of effect of freezing. Western analysis of lysates expressed as intensity value (OD) of each protein normalized to total Beta-actin protein; G) TNFR1 (50 kDa protein); H) TNFR2 (~75 kDa protein); All N's=9; data presented as mean $\pm$ SEM; P< 0.05: * compared to medium same vessel; ^a compared to FBAEC same treatment.