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Environmental Arsenic Exposures Stimulate Hepatic Vascular Cell Remodeling *In Vivo* and *In Vitro*

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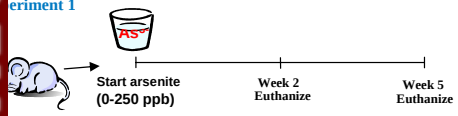
Overview

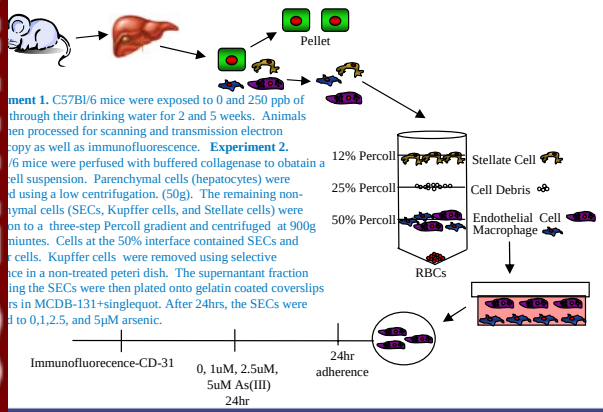
c is a naturally- occurring environmental toxicant that causes a wide range of organ specific diseases ncers in 50-100 million people worldwide. Epidemiological evidence demonstrates that drinking water inated with arsenic enhances vascular remodeling in humans and contributes to the pathogenesis of seases through poorly defined mechanisms. Since a significant amount of liver disease results from r changes and chronic environmental exposures to arsenic enhance angiogenesis and vascular eling, we examined the hypothesis that arsenic induces hepatic sinusoidal endothelial cell remodeling. tial findings demonstrate that sub-chronic exposure to arsenic enhances sinusoidal endothelial cell eling, leading to vascular capillarization, a process by which sinusoidal endothelial cells (SECs) lose ae, gain platlet endothelial cell adhesion molecule (PECAM) expression, and develop a basement ane. This phenotype can lead to a wide range of diseases such as diabetes, atherosclerosis, portal s and portal hypertension. Also, this study may be first in showing the mechanism by which arsenic re initiates the development of these diseases. This study is first to identify a mouse model in which amental levels (250 ppb) of arsenic contribute to liver pathology and sinusoidal endothelial cell ction. This work was supported by NIEHS grant E07373 (AB) and EPA STAR Fellowship FP-201 (AS).

Approach

eriment 1



periment 2



ment 1. C57Bl/6 mice were exposed to 0 and 250 ppb of through their drinking water for 2 and 5 weeks. Animals hen processed for scanning and transmission electron copy as well as immunofluorescence. **Experiment 2.** /6 mice were perfused with buffered collagenase to obatain a ell suspension. Parenchymal cells (hepatocytes) were d using a low centrifugation. (50g). The remaining non-hymal cells (SECs, Kupffer cells, and Stellate cells) were on to a three-step Percoll gradient and centrifuged at 900g inuntes. Cells at the 50% interface contained SECs and r cells. Kupffer cells were removed using selective nce in a non-treated peteri dish. The supernantant fraction ing the SECs were then plated onto gelatin coated coverslips rs in MCDB-131+singlequot. After 24hrs, the SECs were d to 0,1,2.5, and 5μM arsenic.

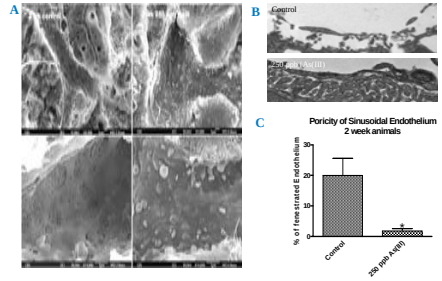


Figure 1. Arsenic-stimulated capillarization of the liver sinusoidal endothelium. **A.** Scanning electron microscopy (SEM) images of sinusoidal vessels were captured from ~200 micron sections from control and 250 ppb As(III) for 2 weeks. Defenstration(loss of open mesh seives) and formation of a basement membrane was observed in three mice from control and 250ppb of As(III). Magnification is given in each image. **B.** Transmission electron microscopy (TEM) images of sinusoidal vessels demonstrating 1). loss of fenestrae, gain of a basement membrane, and increased hepatocyte microvilli in As(III) exposed mice in comparison to control. **C.** Quantification of porocity of sinusoidal endothelium from three animals from control and 250 ppb As(III).

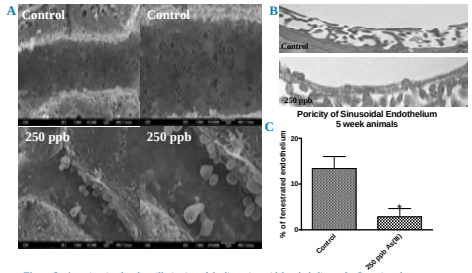


Figure 2. Arsenic-stimulated capillarization of the liver sinusoidal endothelium. **A.** Scanning electron microscopy (SEM) images of sinusoidal vessels were captured from ~200 micron sections from control and 250 ppb As(III) for 5 weeks. Defenstration(loss of open mesh seives) and formation of a basement membrane was observed in three mice from control and 250ppb of As(III). Magnification is given in each image. **B.** Transmission electron microscopy (TEM) images of sinusoidal vessels demonstrating 1). loss of fenestrae, gain of a basement membrane, and increased hepatocyte microvilli in As(III) exposed mice in comparison to control. **C.** Quantification of porocity of sinusoidal endothelium from three animals from control and 250 ppb As(III).

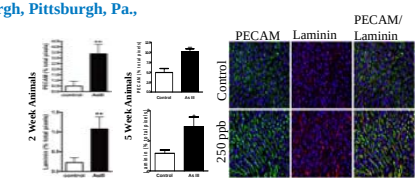


Figure 3 AsIII induced expression of sinusoidal PECAM and laminin protein. **A.** Sections of livers from control mice or mice exposed to 250 ppb AsIII for 1 week were immunostained for PECAM-1 (green channel) or laminin-1 (red channel). Confocal images were captured at 40x magnification. B. Quantitative morphometric analysis of confocal immunofluorescence images was used to measure PECAM-1 and laminin-1 protein expression in control mice and mice exposed to AsIII for 2 wk. Data are expressed as the mean ± s.d percentage of positive staining pixels per field (n=5 mice per group, ** = p<0.01 by t-test).

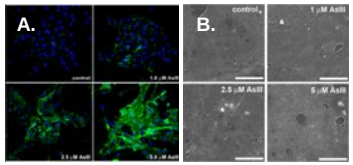


Fig.4 AsIII causes dose-dependent capillarization and defenestration of cultured SEC. After perfusion and collagenase digestion of mouse livers, non-parenchymal and parenchymal cells were separated by differential centrifugation. Stellate and Kupffer cells were removed from non-parenchymal cells by rapid plating on plastic. Nonadherent SECs were then plated on gelatin-coated coverslips and equilibrated for 24 h. The indicated concentrations of AsIII were added and the cells were incubated for an additional 24 h. Different coverslips were processed for **(A)** confocal immunofluorescence imaging for PECAM-1 expression or **(B)** SEM to determine the degree of fenestration in SEC sieve plates. The coverslips in A were co-stained with the nuclear dye, Draq 5, and the images in A were captured at a magnification of 40x. The white bar in B measures 10 microns.

Impacts and Public Health Relavence

-Hepatic sinusoidal endothelial cell phenotype becomes capillarized after environmentally relevant exposure to arsenic.
-This is the first low dose mouse study demonstrating that environmental levels of arsenic induce endothelial cell dysfunction within an endogenous vascular bed.
- CD-31 may be used as a biomarker for arsenic vascular toxicity within the liver.
-SECs are major regulators of liver function and SEC dysfunction and defenestration have major implications for both liver and systemic vascular diseases including diabetes and atherogenesis.

Future Studies

-Studies are being performed to demonstrate that reactive oxygen species stimulate hepatic capillarization.
-Demonstration that human SECs respond in the same manner as mouse SECs will also be determined.

Methods

Scanning and Transmission Electron Microscopy: To demonstrate whether AsIII stimulates SEC defenestration and capillarization *in vivo* and *ex vitro*, SEM and TEM images of the liver sinusoids ultrastructure were compared between control C57BL/6 mice and mice exposed for 2 or 5 wk to 250 ppb of AsIII in their drinking water. At the end of the respective exposure period, three mice in each group were euthanized by injecting sodium pentobarbital and opening the thoracic cavity. The livers were perfusion fixed by flushing with 10 ml of PBS and then perfused with 10 ml 2.5% gluteraldehyde in PBS. Livers were then removed and immersed in 2.5% gluteraldehyde overnight at 4oC. Samples for TEM were processed as described previously (1). Ultrathin, 70 nm sections were imaged on a JEOL 1210 TEM at 80 kV in the University of Pittsburgh Center for Biological Imaging. Samples for SEM were prepared as described (2) and images were captured in the CBI with a JSM-6330F scanning electron microscope (JEOL, Peabody, MA).

Ex Vivo Culture of Sinusoidal Endothelial Cells : To investigate direct AsIII effects on SEC defenestration and capillarization, primary mouse SEC were isolated using standard methods for separating liver cells as described in approach 2. The isolated cells were plated on gelatin-coated glass cover slips and cultured in MCDB 131 plus Cambrex EGM-2-MV SingleQuots (proprietary mixture of hEGF, hydrocortisone, GA-1000, 5% FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid) for 24 h before use in experiments. After equilibration, the cells were exposed to 1-5 mM of AsIII in complete medium for an additional 24 h. The cells were then fixed for immunofluorescence or SEM.

Immunofluorescence detection of proteins: Cryostat sections (10 micron) of excised liver were placed on charged glass slides and fixed for 5 minutes in cold methanol. After washing three times with 1X Phosphate Buffer Saline (PBS), primary antibodies CD31(PECAM) (BD Pharmingen) was diluted in PBS and incubated for 1 hour at room temperature. Slides were then washed three times with PBS and secondary antibodies Alexa Fluor ™Goat anti-rat 488 (H+L) (Invitrogen) diluted 1:500 were added for 1 hour at RT. Tissues were rinsed three times in PBS and nuclei were stained with Draq 5 nuclear stain for 1 hr. After three rinses with PBS, coverslips were mounted with Flouromount G (Southern Biotech). Confocal images were taken using an Olympus Flowview 500 confocal microscope (University of Pittsburgh Center for Biological Imaging).

References

1.) Nadler,E.P., Dickinson,E.C., Beer-Stolz,D., Alber,S.M., Watkins,S.C., Pratt,D.W., and Ford,H.R. 2001. Scavenging nitric oxide reduces hepatocellular injury after endotoxin challenge. *Am J Physiol Gastrointest.Liver Physiol* 281:G173-G181.

2.) Wack,K.E., Ross,M.A., Zegarra,V., Sysko,L.R., Watkins,S.C., and Stolz,D.B. 2001. Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver. *Hepatology* 33:363-378.

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