Regulation of Vascular Endothelial Growth Factor (VEGF) Expression by Hypoxia-inducible Factor-1 in the Femoral Head Cartilage Following Ischemia Osteonecrosis

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ABSTRACT INTRODUCTION:
Legg-Calve-Perthes disease (LCPD) is a common juvenile form of ischemic osteonecrosis of the femoral head that affects children between the ages of 2 to 14 years and has the attack rate of 1 in 740 boys and 1 in 3700 girls. LCPD is due to blood supply disruption to the femoral head. Ischemic osteonecrosis of the femoral head remains one of the most challenging conditions to treat due to the lack of understanding of the biology of the disease and our inability to modulate the repair process. Pig model of juvenile ischemic osteonecrosis of the femoral head has been shown to have radiographic and histopathologic changes resembling Legg-Calve-Perthes disease. In the model, the induction of total femoral head ischemia produced extensive cell death in the hypertrophy zone of the epiphyseal cartilage and produced growth arrest of the secondary center of ossification, the bony epiphysis.

Angiogenesis is an essential component for the healing of damaged head. Hypoxia-inducible factor-1α (HIF-1α) is a master regulator of cellular response to hypoxia. Our preliminary histological studies showed increased vessel formation in cartilage in the ischemic group compared to the control group in a pig model of femoral head osteonecrosis. The mechanism underlying this angiogenesis response to ischemic in the cartilage and its significance to the repair process is an interesting area of research that may provide new insights into how we can stimulate revascularization and repair following femoral head ischemia.

METHODS:
Animal and cartilage isolation. This study was approved by the local institutional animal care and use committee. Yorkshire immature pigs aged 5 to 6 weeks old (6 to 8 kg) were used. Ischemic osteonecrosis of the femoral head was surgically induced by applying a ligature tightly around the femoral neck and transecting the ligamentum teres, as previously described. Only the right side was operated and the unoperated left femoral heads were used as controls.

Histology and Immunohistochemistry. Femoral heads were cut into four-millimeter thick sections in a coronal plane, fixed, decalcified, and embedded. Five-micron sections were cut and mounted. Sections were stained with hematoxylin-eosin. For immunohistochemistry, Deparaffinized sections were digested and washed. Endogenous peroxidase activity was deactivated. A rabbit polyclonal antibody against HIF1α or VEGF was used as a primary antibody. Poly-HRP anti-Rabbit IgG was used. All sections were examined under a Nikon Eclipse E800M Microscope. Cells were counted at 40x magnification in the mid-thickness of the cartilage. A paired t-test was performed comparing the normal and the contralateral ischemic sides.

RNA isolation and Microarray. Femoral heads were retrieved and cartilage samples were removed from articular cartilage of the femoral head. Total RNA was extracted using Trizol reagent. Affymetrix GeneChip Porcine Genome Array containing 23,937 probe sets to interrogate 23,256 transcripts in pigs (20,201 genes) was used to compare gene expression profiles between ischemic group and control group.

Quantitative Real-time Reverse Transcription-PCR. RNA was subjected to quantitative RT-PCR using the TaqMan One-Step RT-PCR Master Mix reagent. Relative transcript levels were measured by real-time PCR on 96-well plates using an ABI PRISM 7000 sequence detection system. House-keeping gene expression heat-shock protein 90 was tested for the stability of the expression on the ischemic side compared to the normal side and was used to normalize the expression levels of various genes studied using primers from Applied Biosystems.

Hypoxia experiment. Primary pig chondrocytes and Rat chondrosarcoma (RCS) cell line were cultured in DMEM, and maintained in normoxic (20%O2) or hypoxia (1%O2) condition with 5%CO2 and the balanced N2 using a humidified hypoxia workstation. All endpoints measured in hypoxia cells were compared with those in cells kept under normoxic condition.

Statistical Analysis. All experiments were carried out with a minimum of n=3. We reported data as the mean with standard error. For histology studies, two histology sections from the central region of femoral head were used, and 3 pigs each group were analyzed. Comparisons were made between groups of equal size by Student’s t test with p<0.05 considered statistically significant.

RESULTS SECTION:
Our histological studies showed increased vessel formation in cartilage in the ischemic group compared to the control group in a pig model of femoral head osteonecrosis. To explore the mechanism underlying this angiogenesis response, porcine microarray was performed to compare gene profiles of cartilage from normal and ischemic femoral heads. In the ischemic side, the expression of VEGF, an important mediator of angiogenesis, was upregulated along with HIF-1α. Microarray results were confirmed by quantitative RT-PCR. Immunohistochemistry assay demonstrated that both HIF-1α and VEGF were upregulated in chondrocytes in ischemic femoral heads.

To examine the coordinate expression of VEGF and HIF-1α in vitro under hypoxia, pig primary chondrocytes and RCS were cultured in hypoxia station with 1% or 20% of O2. Both HIF-1α and VEGF were upregulated in two different chondrocytes. Interestingly, addition of deferoxamine (DFO), an HIF-1α activator, further increased VEGF expression significantly, suggesting HIF1α is involved in mediating VEGF expression in response to hypoxia. To investigate if HIF-1α is required for VEGF upregulation by hypoxia, we used siRNA directed against HIF-1α to knockdown HIF-1α expression. Expression of VEGF was reduced by 44% after HIF-1α siRNA transfection. Furthermore, transient transfection assays showed that HIF-1α activated VEGF promoter reporter expression in a dose-dependent manner.

DISCUSSION:
Taken together, our data indicated that upregulation of VEGF during hypoxia in chondrocyte is mediated partially through HIF-1α. VEGF expression was reduced by 44% after HIF-1α knockdown by siRNA transfection, suggesting that HIF-1α is responsible for VEGF upregulation and that other factors besides HIF-1α may also be involved in VEGF regulation during hypoxia. HIF-1α upregulation of VEGF activity may be one of the mechanisms for angiogenesis response following femoral head ischemia.

SIGNIFICANCE:
This study addresses the mechanism underlying this angiogenesis response to ischemic in the cartilage. The results may provide clues to help to develop therapeutic approach to promote femoral head repair by stimulating revascularization.

ACKNOWLEDGMENTS:
Work in Bone Research Laboratory is supported by Research Grant from Arthritis Foundation (To Chi Zhang) and RAP01 grant from Texas Scottish Rite Hospital for Children (To Chi Zhang).