INTRODUCTION:
Avascular necrosis of the femoral head (FHN) is a common complication after high dose corticosteroid treatment. In the current study we investigated the possible role of vascular endothelial growth factor (VEGF) and its receptors VEGFR-2 in steroid induced femoral head necrosis and disordered bone remodeling by biochemical and immunohistochemical methods. Using cultured human osteoblasts as a model, our aim was to investigate altered VEGF levels due to GC treatment, and steroid induced femoral head necrosis.

MATERIALS AND METHODS:
Femoral heads (n=6) were obtained from patients undergoing total hip arthroplasty (age 32-54) for steroid related late stage (ARCO IV) femoral head necrosis, and six patients undergoing THA for end-stage primary osteoarthritis. Human osteoblasts were derived proliferating from Oligene (Berlin, Germany) and cultured under standard conditions. Cells were incubated for 24 hours or 24 and 72 hours with dexamethasone (100 µMol) and epidermal growth factor (EGF; TEBU, Offenbach, Germany), diluted to 10 ng/mL. Stimulation experiments were carried out in triplicates. Cell culture experiments were repeated for n=6 times.

For histology and immunohistochemistry, tissue samples were fixed in 3% paraformaldehyde, embedded in paraffin, sectioned, dewaxed, irradiated at 750 W in a microwave oven in 0.01 M sodium citrate buffer, pH 6.0 (twice for 5 min), sections blocked with 3% hydrogen peroxide (endogenous peroxidases) and subsequently with normal serum (1:5 in Tris-buffered saline) of the species in which the primary antibody was raised, immunostained with anti-VEGF or anti-VEGFR-2 followed by biotinylated secondary antibodies and a peroxidase-labelled streptavidin-biotin staining technique; nuclei were counterstained with hemalum. AZAN, Goldner and van Giesson staining were performed according to standard protocols.

For ELISA, culture supernatants and bone samples of necrotic femoral heads were analysed by a sandwich ELISA (R&D Systems, Minneapolis, MN, USA) that detects all VEGF splice forms. Human recombinant VEGF165 (PreproTech, Rocky Hill, NJ, USA) served as standard.

RESULTS:
Within all sections of the penumbra and the periphery of ON femoral heads, VEGF could be immunostained within the intra- and pericellular matrix of osteoblasts and osteocytes. VEGF immunostaining was negative after preincubation of the primary antibody with recombinant VEGF or by omitting the primary antibody. Osteoblasts of the periphery were less immunoreactive to VEGF antibody. Osteoblasts and endothelial cells of the periphery were also immunostained for VEGFR-2. VEGF expression appeared upregulated in the penumbra of steroid-related femoral head ON. In the penumbra, we detected immature vessels. In the periphery, we identified VEGFR-2-positive endothelial cells and osteoblasts. Using densitometry, immunohistochemistry revealed a fivefold upregulation of VEGF in osteoblasts and osteocytes from the penumbra of ON femoral heads compared to the necrotic area, the periphery, or OA nonnecrotic area (p < 0.012) or compared to nonnecrotic OA bone (p < 0.017). The strong increase of VEGF in the penumbra of ON femoral heads compared to the necrotic area (p < 0.015) or nonnecrotic area (p < 0.012) or compared to nonnecrotic OA bone (p < 0.017). The strong increase of VEGF in the penumbra of ON femoral heads compared to the necrotic area, the periphery, or OA control bone could be attributed to the neovascularization of this area. ELISA experiments revealed an increase (p < 0.001) of VEGF in the penumbra of ON of the femoral head (510.32 pg/1,000,000 cells) compared to the necrotic area. The VEGF amount per 1,000,000 cells in the necrotic area (107.18 pg) was similar (p < 0.537) from the VEGF amount per 1,000,000 cells in the periphery (106.47 pg). The VEGF amount in the nonnecrotic OA bone (173.72 pg/1,000,000 cells) was also similar to that in the periphery of ON area (p < 0.135) and the necrotic area (p < 0.132). VEGF mRNA and VEGFR-2 were expressed in human non-ON osteoblasts. RT-PCR experiments revealed osteoblasts expressing the splice variants VEGF121 and VEGF165. RT-PCR experiments further revealed the existence of VEGFR-2 (555 kb) but not VEGFR-1.

The 72-hour incubation revealed a nearly threefold increase (p < 0.013) in VEGF (from 164.45 ± 24 pg/mL to 476.5 ± 64.85 pg/mL [mean ± standard deviation]) in culture supernatant. We observed decreasing amounts of VEGF after 24-hour incubation with increasing doses of dexamethasone. VEGF expression levels were decreased (p < 0.001) in the collected cell supernatants (control: 164.06 pg/mL; 100 µM/L dexamethasone: 61.5 pg/mL). Stimulation with 1000 µM/L dexamethasone did not further decrease (p = 0.5) VEGF expression (50.9 pg/mL).

DISCUSSION:
The current study demonstrates an increase in vascular endothelial growth factor in the reactive interface (penumbra) of late-stage femoral head necrosis which may reflect a secondary phenomenon. The observed high amount of vascular endothelial growth factor in later-stage osteonecrosis might stimulate the ingrowth of reparative arterioles into the femoral head. Ohzono et al. examined human femoral heads at Ficat’s stages 2 to 4 histopathologically, and by microangiography (1). They described that reparative arterioles grew on the trunk of the intracapital nutrient arteries. They also described an avascular zone directly subchondrally, an underlying reparative vascular zone, and a normal vascular zone. In the reparative vascular zone, they described an advancing ingrowth of reparative vessels from stage 2 to 4. These were described as sparse in stage 2, a fine network in stage 3, and a dense network in stage 4. The new results of increased VEGF expression in the penumbra of ON femoral heads of the present study well correlate with the findings of Ohzono et al. Vascular endothelial growth factor is increased, and stimulates the ingrowth of reparative vessels into the necrotic femoral head.

On the other hand, the decrease in VEGF detected in the in-vitro part of the present study in osteoblasts incubated with GC may support the development of initial osteonecrosis. This may be an additional pathogenetic factor in the early stage of femoral head necrosis. GC impairs vessel ingrowth into the endarterial bed of the femoral head (18), and makes it more vulnerable to ischemia. Vessels have been shown to be essential to osteogenesis (3). In our study of GC induced femoral head necrosis and GC induced VEGF decrease in osteoblasts we indeed observed strong significant hints for participation of VEGF in femoral head necrosis.

By a range of different methodological approaches we have demonstrated that VEGF is expressed by osteoblasts and is affected by dexamethasone. These data strongly suggest that VEGF plays an important role in the initial stage of femoral head necrosis. Since VEGF is a potent angiogenic peptide, it is likely to be responsible for the neovascularisation observed in remodeled bone. Furthermore, we have demonstrated a strong GC-associated reduction of VEGF expression in human osteoblasts. The observed increase of VEGF in necrotic bone areas of femoral heads seems to stimulate the ingrowth of reparative arterioles into the necrotic femoral head.

REFERENCES:

ACKNOWLEDGEMENT:
The study was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft) grants no. 449/ DR 2-1, VA 2202-1 and PU214/4-2 and PU214/3-2 and by a grant from the Medical Faculty Kiel (FoFo) and by the Forschungsschwerpunkt Muskel- und Skelettsystem (Musculo-Skeletal Science Kiel, MSS-Kiel) of the University Hospital Schleswig-Holstein, Kiel.