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# Appearance of vascular endothelial growth factor (VEGF) in femoral head in the growing rat

**M. Ichigatani, T. Saga, K. Yamaki and M. Yoshizuka**

Department of Anatomy, Kurume University School of Medicine, Kurume, Japan

**Summary.** In this study, we examined the appearance of vascular endothelial growth factor (VEGF) in the femoral head of the growing rat using an immunocytochemical technique. Our results showed VEGF-immunopositive cells existed in the inner region and peripheral region of the femoral head at each developmental stage. In the 19-day-old fetus, immunopositive mesenchymal cells were demonstrated in the peripheral region of the femoral head. At 1 to 10 days after birth, VEGF immunoreactivities were observed in the osteoblasts, osteoclasts, periosteum, perichondrium and cartilage matrix of the femur. At 15 days after birth, VEGF immunoreactive chondrocytes appeared in the apex area of the femoral head. In this stage, the femoral head is still constituted by chondrocytes and no apparent vascular formation has been observed. Thereafter, the immunopositive chondrocytes in the femoral head increased in number. The penetration of capillaries was recognized within the ligament of the femoral head at 60 days after birth. The results indicate that some chondrocytes in the femoral head produce VEGF before the beginning of ossification, and that VEGF may play an important role in the penetration of blood vessels into the femoral head from the ligament of the femoral head.

**Key words:** VEGF, Femur, Developmental biology, Vascularization, Rat

## Introduction

Vascular endothelial growth factor (VEGF) is known to stimulate endothelial cell growth and angiogenesis (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989; Leung et al., 1989), and to act as a vascular permeability factor (VPF) to induce microvascular hyperpermeability (Senger et al., 1983; Connolly et al., 1989). Furthermore, Horner et al. (1999) reported the immunolocalization of VEGF in human neonatal growth plate cartilage. They demonstrated that hypertrophic chondrocytes produced VEGF and suggested that VEGF may play a key role in the regulation of vascular invasion of the growth plate. Recently, Gerber et al. (1999) reported the role of VEGF in endochondral bone formation of the mouse. They indicated that VEGF is an essential coordinator of chondrocyte death, chondroclast function, extracellular matrix remodeling, angiogenesis and bone formation in the growth plate. Furthermore, chondrocytes originate from pluripotent mesenchymal progenitors which also give rise to osteoblasts, adipocytes and other cell types (Rodan and Noda, 1991). The expression of VEGF is upregulated with increasing differentiation of osteoblasts, adipocytes and myoblasts, suggesting that the production of VEGF is a common event linked to the differentiation of mesenchyme-derived cells (Claffey et al., 1992; Harada et al., 1994).

It is also well known that VEGF is produced in various normal tissues (Fan and Iseki, 1998), embryos and tumors (Ferrara and Davis-Smith, 1997). However, little information is available about the occurrence of VEGF during bone formation in the femoral head of the growing rat.

In this study, therefore, we investigated the appearance of VEGF in the femoral head of the growing rat using an immunocytochemical technique to clarify the role of VEGF in the bone formation and angiogenesis.

## Materials and methods

This study was carried out after permission had been granted by the Committee on Animal Experimentation, Kurume University. Wistar strain rats (19-day fetus and 1-60 day-rats) were used in this study.

At least five animals at each stage were sacrificed by decapitation under deep anaesthesia at 10 o'clock am. The femurs of each stage were fixed in Bouin solution for 24 hr - 2 weeks. Tissue samples were dehydrated through a series of solutions with increasing concentrations of ethanol. Tissues were embedded in paraffin, and serial sagittal sections at 4 $\mu$m were mounted on glass slides. The preparations of the femur

*Offprint requests to:* T. Saga, Department of Anatomy, Kurume University, School of Medicine, Asahi-machi, Kurume 830-0011, Japan. Fax: 81-942-33-3233. e-mail: saga@med.kurume-u.ac.jp

were stained with Haematoxylin-Eosin and toluidine blue to permit observation of the general structure of the femur at each developmental stage.

*Immunocytochemistry*

Primary antibodies generated against human recombinant VEGF165 were used as immunocytochemical probes (obtained from NeoMarkers, USA, diluted 1:500). This polyclonal antibody recognizes multiple isoforms of VEGF containing 206-, 188-, 165- and 121-amino acid residues in human, mouse and rat.

Immunocytochemical staining was performed with a Histofine kit (Nichirei, Japan). In brief, sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in phosphate-buffered saline (10 mM sodium phosphate, 0.15M sodium chloride, pH 7.5; PBS). All procedures were performed at room temperature, and incubations were performed in closed humid chambers. First, the tissue sections were incubated for 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activities, and were then washed in PBS. To reduce nonspecific staining caused by the biotinylated anti-rabbit IgG, sections were treated with normal goat serum for 30 min, and then washed in PBS. Primary antisera were applied to the sections for 2 to 24 h, and the biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin were applied for 1 h each. The final reactive products were visualized with 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxide (Hsu et al., 1981). The sections were then counter-stained with haematoxylin, washed in running water, dehydrated through an increased ethanol series, and mounted in Diatex (Ab Wilh Becker, Sweden). To confirm the specificity of the immunocytochemical staining, the primary antiserum was replaced by PBS.

## Results

In the 19-day-old fetus, VEGF-immunopositive cells were observed in the immature chondrocytes at the peripheral region of the femoral head. Moreover, mesenchymal cells around the femoral head immunoreacted with the VEGF165 antibody (Fig. 1). To confirm the specificity of this immunoreaction, the primary antiserum was replaced by PBS, as the results the immunopositive reactions were not demonstrated.

At one day after birth, immunoreactivities against this VEGF antibody were demonstrated in the periosteum and perichondrium of the femur. Moreover, osteoblasts and osteoclasts in the proximal end of diaphysis also immunoreacted with this VEGF-antibody (Figs. 2a,b). Immature chondrocytes, which were observed in the 19-day-old fetus, were not found in the femoral head. In this stage, the chondrocytes in this region exhibited no immunoreaction with the VEGF antibody.

At four days after birth, weak but apparent VEGF immunoreactivities appeared in the chondrocytes in the proliferating and hypertrophic zones and in the cartilage matrix of the femoral head (Fig. 2c,d). The immunopositive periosteum, perichondrium, osteoblasts and osteoclasts were observed like as one day after birth. Some microvessels were observed in the metaphysis and periosteum.

At seven days after birth, more intense immuno-

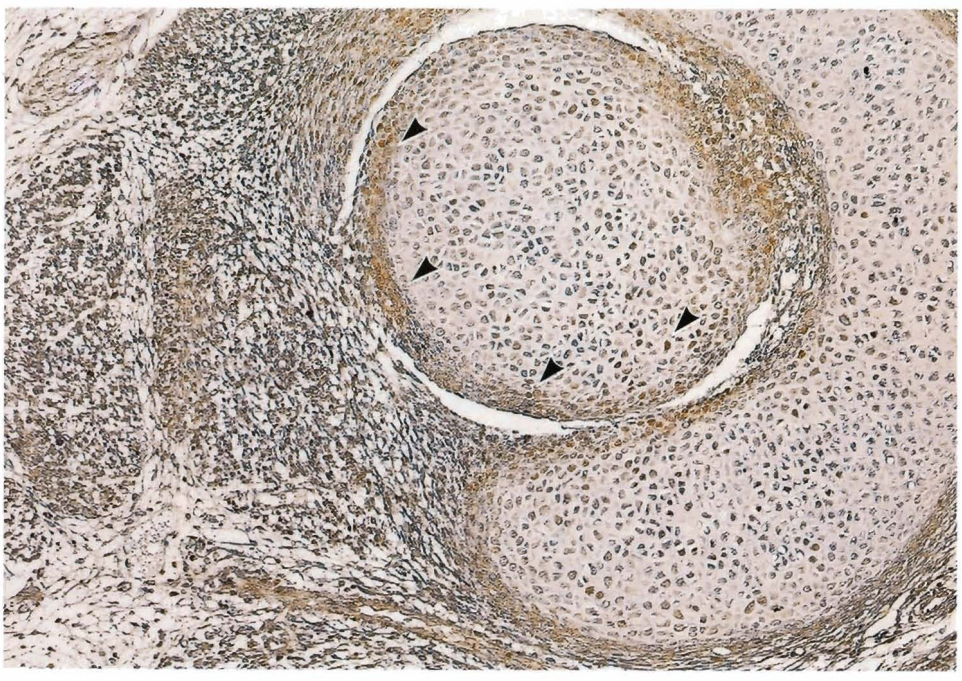

**Fig. 1.** Femoral head of the 19-day-old fetus. VEGF immunopositive immature chondrocytes at the peripheral region of the femoral head (arrow heads) and mesenchymal cells around the femoral head are shown. x 90

positive reactions were observed in chondrocytes, osteoblasts and osteoclasts. Especially, chondrocytes in the proliferating and hypertrophic zones exhibited more intense immunoreactions. Cartilage matrix around the immunopositive chondrocytes also demonstrated VEGF immunoreactivities (Figs. 3a-c).

At 10 days after birth, more intense immunoreactivities were detected in the cartilage matrix at the peripheral region of the femoral head (Fig. 3d). Intense immunoreactivities appeared at this time in the chondrocytes and cartilage matrix around these chondrocytes at the apex area of the femoral head (Fig. 3e).

At 15 days after birth, immunoreactive VEGF cells appeared in the apex area of the femoral head. Moreover, the perichondrial cells at the apex of the femoral head exhibited VEGF immunoreaction (Fig. 4a). In this stage, the femoral head still consisted of chondrocytes, and no apparent vascular formation was observed in the femoral head.

At 20 days after birth, VEGF-immunopositive chondrocytes in the apex area of the femoral head increased in number. Moreover, these immunopositive chondrocytes developed and further increased in number at 30 days after birth (Fig. 4b).

At 60 days after birth, a few capillaries were observed in the ligament of the femoral head. These capillaries penetrated into the apex area of the femoral head where VEGF-immunopositive chondrocytes had previously been identified (Fig. 4c).

## Discussion

This is the first report demonstrating the appearance of VEGF in the femoral head in the growing rat. It has been reported that VEGF stimulates the differentiation of mesenchyme-derived cells (Claffey et al., 1992; Harada et al., 1994). In this study, many VEGF-immunopositive mesenchymal cells were found around the femoral head in the 19-day-old fetus. These mesenchymal cells demonstrated no immunoreaction with the PBS which replaced by primary antiserum. Therefore, we consider that these VEGF immunoreactions are specific. Moreover, the results indicate that VEGF produced by

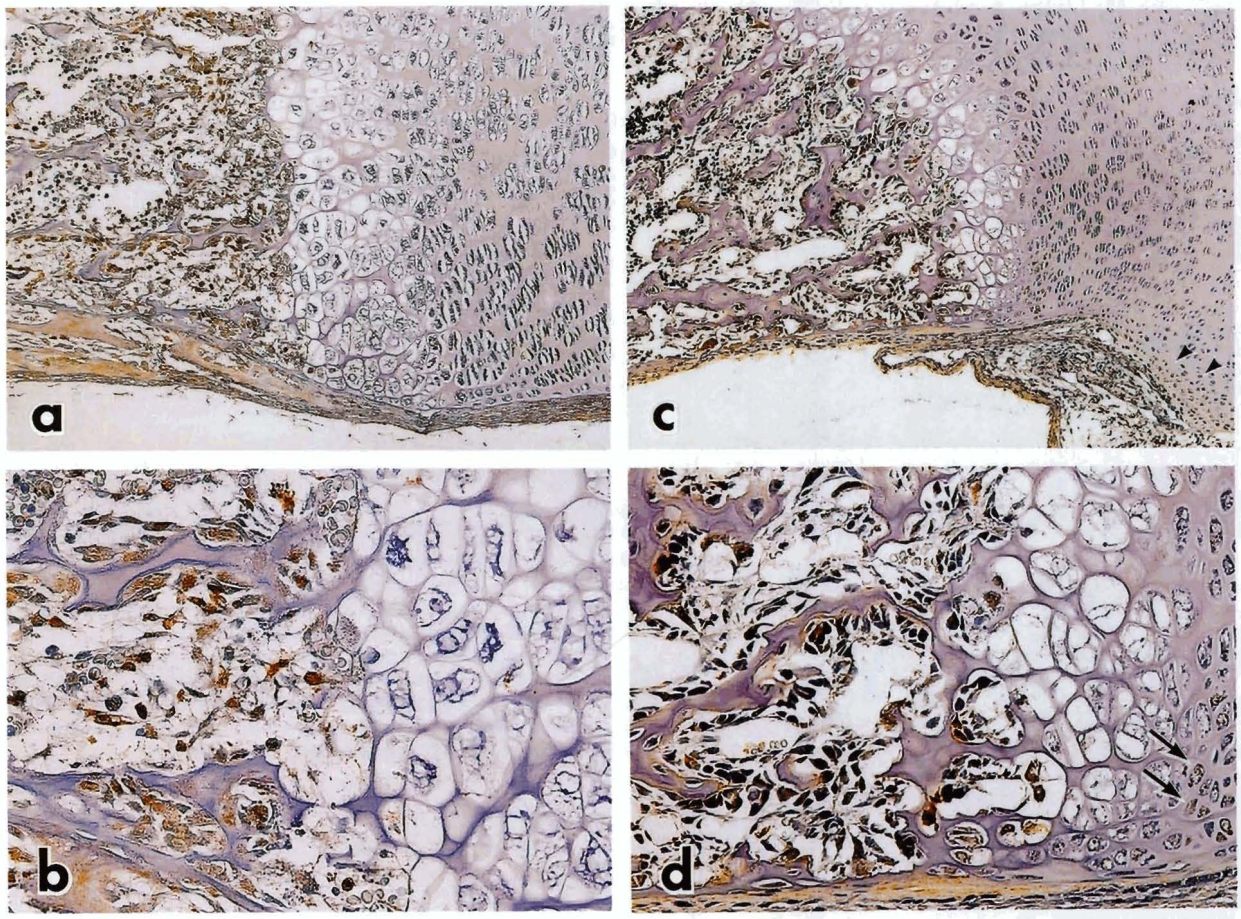


**Fig. 2. a and b.** Boundary area of the femoral head and metaphysis immunostained with VEGF antibody. Immunopositive osteoblasts, osteoclasts, periosteum and perichondrium are present in the one-day-old rat. a, x 70; b, x 200. **c and d.** Immunopositive cartilage matrix (arrow heads) and chondrocytes (arrows) appeared in the 4-day-old rat. c, x 60; d, x170

mesenchymal cells around the femoral head might induce angiogeneis toward the femur during early bone formation.

After birth, VEGF was demonstrated in the early stage in the osteoblasts, osteoclasts, periosteum and perichondrium around the femoral head. VEGF, the endothelial cell-specific mitogen, is an essential mediator of angiogenesis (Ferrara and Davis-Smith, 1997), and angiogenesis is essential for bone growth and healing (Streeten and Brandi, 1990; Mori et al., 1998; Ryan et al., 1999), and the recruitment of osteoblasts coincides with vascular invasion (Holder, 1978; Carrington and Reddi, 1991). Therefore, VEGF produced in osteoblasts and osteoclasts might play a key role in the formation of microvessels at the metaphysis. Moreover, the existence of VEGF in the periosteum and

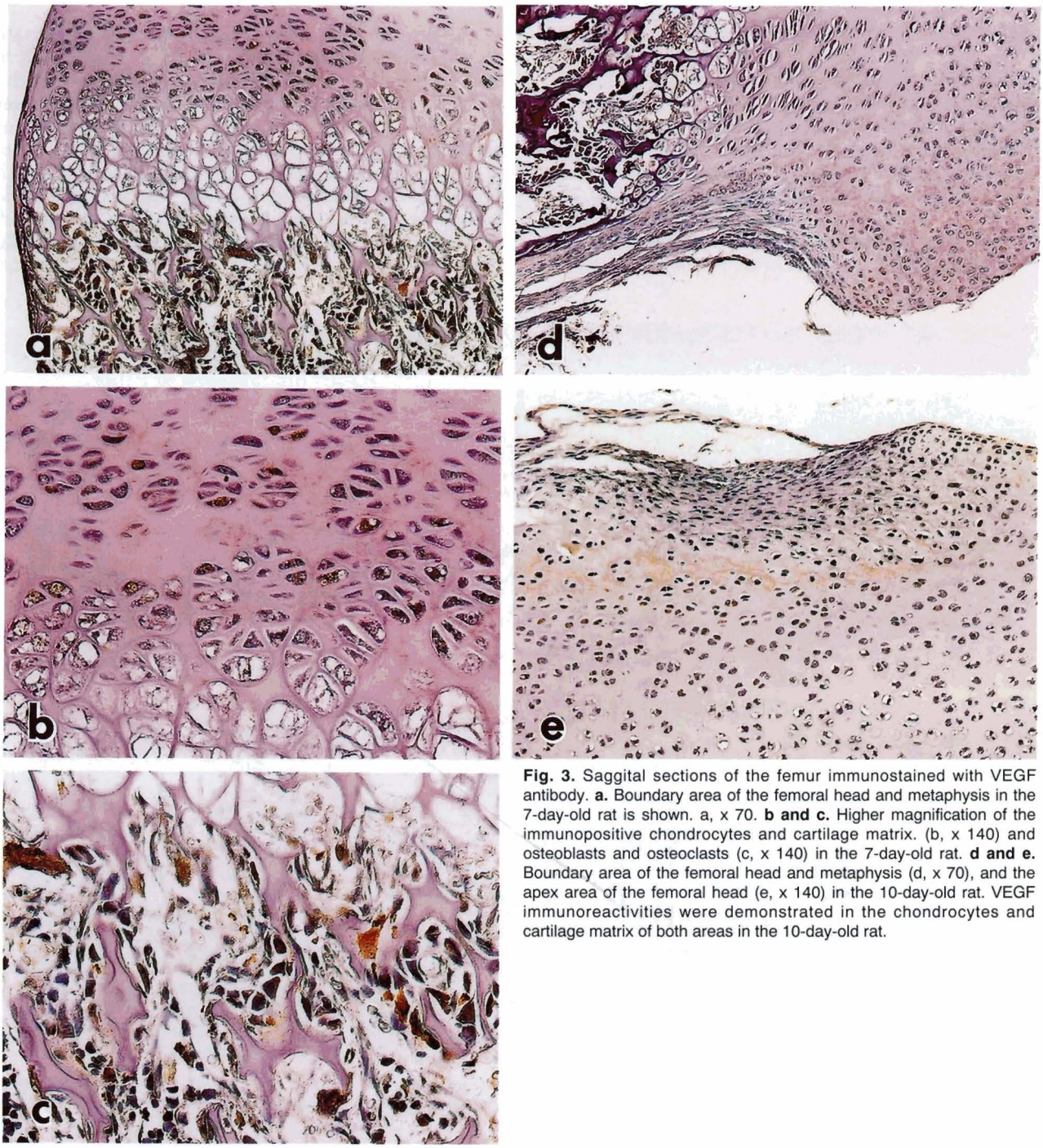


**Fig. 3.** Saggital sections of the femur immunostained with VEGF antibody. **a.** Boundary area of the femoral head and metaphysis in the 7-day-old rat is shown. a, x 70. **b and c.** Higher magnification of the immunopositive chondrocytes and cartilage matrix. (b, x 140) and osteoblasts and osteoclasts (c, x 140) in the 7-day-old rat. **d and e.** Boundary area of the femoral head and metaphysis (d, x 70), and the apex area of the femoral head (e, x 140) in the 10-day-old rat. VEGF immunoreactivities were demonstrated in the chondrocytes and cartilage matrix of both areas in the 10-day-old rat.

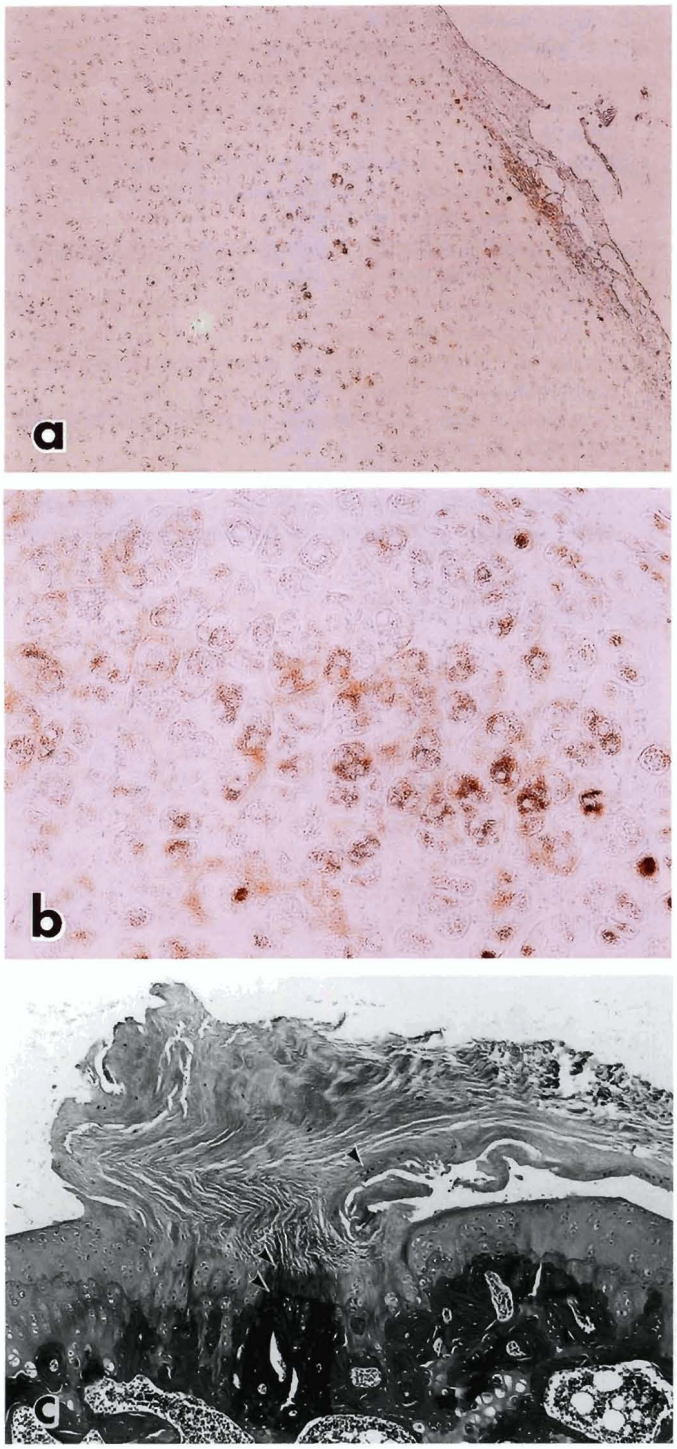


**Fig. 4. a.** Apex area of the femoral head immunostained with VEGF antibody in the 15-day-old rat. Immunopositive perichondrial cells and chondrocytes are shown. **b.** Apex area of the femoral head immunostained with VEGF antibody in the 30-day-old rat. Immunopositive chondrocytes developed and increased in number. **c.** Apex area of the femoral head stained with H&E in the 60-day-old rat. A few capillaries are present (arrow heads). a, x 50; b, x 250; c, x 60

the perichondrium suggests that VEGF has an intimate relation with the angiogenesis in the periosteum and perichondrium. At a more advanced stage, VEGF appeared in the cartilage matrix in the peripheral region of the femoral head and in the chondrocytes at the proliferative and hypertrophic zones. It seems that VEGF demonstrated in the proliferative and hypertrophic zones may play a role in the regulation of vascular formation in the elongating femur. On the other hand, VEGF in the cartilage matrix might be secreted by chondrocytes, but VEGF immunoreactivity was not always demonstrated in chondrocytes in the peripheral region. It is considered that the physiological situation of the chondrocyte at this region is different from the proliferative and hypertrophic zones, further investigation is needed in this problem.

In the young rat, VEGF was demonstrated in the chondrocytes at the apex area of the femoral head. The apex area of the femoral head is the presumptive secondary ossification area. On the surface of this region, a few capillaries penetrated within the ligament of the femoral head at 60 days after birth. Recently, Morini et al. (1999) reported that capillary invasion into the femoral head first occurred in 9-week-old rats. These results indicate that VEGF appearing in the chondrocytes at the apex area of the femoral head may play an important role in the microvascular invasion from the ligament of the femoral head. In fact, the blood vessels from the ligament of the femoral head is not abundant, the femoral head is mainly supported by blood vessels coming from the metaphysis. We consider that the vessels coming from the metaphysis are induced by the VEGF secreted from the chondrocytes in the proliferative and hypertrophic zones.

In conclusion, the early expression of VEGF at various sites during femoral head development indicates that VEGF plays a role in the regulation of angiogenesis at each site of endochondral bone formation.

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