CARTILAGE-PERICHONDRIUM INTERACTIONS REGULATE ANGIOGENESIS DURING LONG BONE DEVELOPMENT

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Introduction. Angiogenesis mediates a pivotal event during endochondral ossification, when blood vessels grow into hypertrophic cartilage and initiate the degradation of its matrix and the migration of bone cells. In this study we examined the molecular and cellular mechanisms regulating angiogenesis during endochondral ossification, with a particular focus on the tissue interactions between perichondrium and cartilage that affect this process. Because of the inherent difficulty in studying the role of one tissue (perichondrium) in the presence of another (cartilage), we developed an ex-vivo approach in order to separate the roles of these two tissues during angiogenic invasion of embryonic cartilage.

Methods. Cartilage elements (humeri) were dissected from embryonic day 14.0 mouse limbs, then directly transplanted underneath the renal capsule of adult wild-type mice. The development and vascularization of the skeletal explants were initially assessed after 24 and 48h in the renal capsule. Subsequently, the explants were examined after 3 days (d), and up to 16 weeks, in the renal capsule of host mice.

To address the contribution of the perichondrium to vascularization of the cartilage anlage, the perichondrium was removed by enzymatic digestion followed by dissection. Cartilage elements, with or without their perichondrium, were cultured in vitro for 24 or 48h, or were directly transplanted in the renal capsule for 4d to 10d. For lineage analysis experiments, cartilage elements were isolated from embryos carrying the lacZ transgene (Rosa 26) and transplanted in wild-type host mice, or vice versa in order to follow cells derived from the graft and host separately. To evaluate the role of vascular endothelial growth factor, VEGF, cartilage elements were treated with a soluble form of the VEGF receptor, mFlt-IgG. At various time-points samples were removed from the renal capsule or culture dish and fixed in 4% paraformaldehyde and RT for 1h. Tissues were dehydrated in a graded ethanol series and embedded in paraffin. Five microm-thick sections were cut and collected on slides for histology, for immunohistochemistry using PECAM antibody and TRAP staining, and for in situ hybridization analyses using 35S-labelled riboprobes, including collagen type II, collagen type X osteocalcin, osteopontin, cbfa-1, mmp13, Ihh and vegf.

Results. To address the role of perichondrium-cartilage interactions in vascularization and ossification, we first compared the progression of chondrocyte differentiation and osteogenesis in skeletal elements implanted into the renal capsule with skeletal elements taken from growing embryos. In the renal capsule, ex vivo e14.0 cartilage elements became vascularized between 48 and 72h whereas this same process occurred after 24h in vivo. Following vascular invasion, ossification was initiated and a marrow cavity was established by d4 ex vivo, whereas the same processes occurred after an additional 24h in vivo.

We next tested the consequence of perichondrial removal. The domain of hypertrophic chondrocytes was enlarged in e14.0 humeri after 24h in culture, indicating that the rate of chondrocyte differentiation was delayed (Fig. 1E, F). Subsequent vascular invasion and bone formation were also postponed, or non-existent and very little endochondral bone was detectable at d10 (Fig. 1G, H). In addition, there was no evidence of a marrow cavity.

We hypothesized that the periosteum was the primary source of osteoblasts. To test this, we used e14.0 embryonic tissues taken from Rosa26 mice, which express the lacZ transgene under control of a ubiquitous promoter. If the perichondrium was derived from Rosa26 mice then osteoblasts were lacZ-positive. If host mice were carrying the lacZ gene, then few if any osteoblasts were lacZ-positive. No LacZ-positive osteoblasts were found in control samples, when wild-type cartilage elements were transplanted in wild-type host mice.

In another series of experiments, we evaluated the role of VEGF in regulating perichondrium-cartilage interactions that mediate vascular invasion. Ex vivo treatment of cartilage elements with mFlt-IgG delayed vascular invasion and ossification, as well as chondrocyte terminal differentiation.

Discussion. We have developed an ex-vivo approach that allows the recapitulation of all stages of normal long bone development, including terminal differentiation of hypertrophic chondrocytes, vascular invasion, the establishment of a marrow cavity, and ossification. Ex vivo culturing of embryonic skeletal elements in the renal capsule allowed these stages of endochondral ossification to be expanded in time compared to in vivo development. Therefore, we used this system to resolve the processes of vascular invasion and bone formation that occur in a relatively small developmental window in vivo. Our results show that the periosteum is required for proper chondrocyte differentiation, as well as for vascular invasion and ossification. Removing the perichondrium postpones chondrocyte differentiation, leading to an expansion of the zone of hypertrophic cartilage. These data suggest that the perichondrium is a source of signals that stimulate terminal differentiation of chondrocytes. Perichondrial removal also halted vascular invasion of the hypertrophic cartilage, raising the possibility that this tissue is also a source of angiogenic factors or a source of signals inducing the expression of angiogenic factors in hypertrophic cartilage. We also noted that perichondrial removal inhibited bone formation, implicating the perichondrium as a source of osteoblasts. Indeed, our subsequent lineage analyses showed that the perichondrium is a major source of precursors for bone cells. To explore the molecular basis of these cartilage-perichondrium interactions, we tested a candidate molecule, VEGF, which regulates angiogenesis and endochondral bone formation. VEGF is expressed in hypertrophic chondrocytes and can act via its receptors, Flt-1, Flk-1 and neuropilin expressed in the perichondrium. We found that the consequences of VEGF-inhibition mimic the consequences of perichondrial removal, suggesting that VEGF-signaling participates in the molecular interactions between the perichondrium and hypertrophic cartilage.

References.

Figure 1: Development of a e14.0 mouse humerus after 24h in culture (A, B, E, F) or 10 days after renal capsule transplantation (C, D, G, H), in the presence (A-D) or in the absence (E-H) of the perichondrium. (A, C, E, G) morphology, (B, F) in situ hybridization signals using a collagen type X (red) or a mmp13 (yellow) probe, (D, H) trichrome staining (arrows in D indicate periosteal bone and stars in H, absence of periosteal bone). Scale bars= (A, E) 500um, (B, F) 250um, (C, G) 1mm, (D, H) 200um.

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