**EXPRESSION OF GAS6 AND AXL IN DEVELOPING EMBRYO LIMBS**

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**Introduction:** We recently reported that growth arrest specific 6 (Gas6) is downregulated during the initiation of chondrogenesis and negatively regulates the process of differentiation from mesenchymal cells [1]. Gas6 is a member of the γ-carboxylated glutamic acid (Gla) protein family which includes coagulation factors, osteocalcin and matrix Gla protein (MGP). It is known that Gas6 is a ligand for Axl which is a receptor tyrosine kinase expressed in several types of cells, and Gas6-Axl pathway plays an important role in cell proliferation and anti-apoptosis. Co-expression of Gas6 and Axl has been detected in vascular tissues, synovial tissues and cartilage, whereas that during limb development in vivo has remained unknown. In this study, to obtain additional evidence by Gas6-Axl pathway in chondrogenesis, we attempted to detect the expression of Gas6 and Axl in developing embryo limbs using immunohistochemistry.

**Materials and Methods: Immunohistochemistry**

Immunohistochemical analysis was performed in paraffinized sections obtained from C57BL/6 mice (E11.5, E13.5 and E18.5 embryos). The sections were treated with 2.5% hyaluronidase (Sigma) for 30 min and treated with 0.3% H2O2 in PBS for 30 min. They were incubated with polyclonal rabbit antibodies against rat Gas6 and Axl at a dilution of 1:100 for overnight at 4°C. As negative controls, we used non-immune rabbit Immunoglobulin fraction (DakoCytmation, Kyoto, Japan). The sections were rinsed in wash buffer and incubated with horseradish peroxidase (HRP)-conjugated rabbit IgG (DakoCytmation) for 30 min at room temperature. Visualization of immunoreactivity was performed by diaminobenzidine (DAB) staining with hematoxirene counterstaining.

**Results:** In order to reveal the expression pattern and course of Gas6 and Axl in developing mouse limbs in vivo, we performed immunohistochemistry using longitudinal sections of E11.5 to E18.5 embryo limbs. At E11.5, only low level of Gas6 expression was observed in the condensed mesenchymal tissues with in styalopod and autopod elements (Fig. B). At E13.5, differentiated chondrocytes with intense Safranin-O staining were apparent in the proximal part of the limb (Fig. D). Gas6 expression was detected within this chondrogenic tissue, as well as in non-chondrogenic mesenchymal tissue in the limb (Fig. E). Immunostaining for Gas6, however, was absent in the pre-chondrogenic cells surrounding the differentiated chondrocytes (Fig. E, E1). Axl expression was most marked in the differentiated chondrocytes within the limbs (Fig. F). At E18.5, within the growth plate of long bones, Gas6 expression was abundant in proliferative chondrocytes and cells in the mineralized zone, but was weak in the resting and hypertrophic chondrocytes (Fig. H). Axl expression was present from resting to hypertrophic chondrocytes, and was marked in the mineralized zone and primary spongiosa (Fig. I).

**Discussion:** We showed in this study the expression pattern of Gas6 and Axl in developing embryo limbs in vivo. Gas6 was strongly expressed in the proliferative chondrocytes followed by moderate decrease in the expression during chondrocyte hypertrophy. This terminal differentiation of chondrocytes is regulated by a number of secreted peptides including BMPs, FGFs, Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP). Suppression of Gas6 may support their effects by mediating intracellular signaling pathways. It has reported that MGP, a vitamin K-dependent protein, inhibits the mineralization of hypertrophic chondrocytes [2]. On the other hand, Vitamin K antagonist, warfarin, has been shown to stimulate matrix mineralization of chick hypertrophic chondrocytes. MGP is expressed in the proliferative and mineralized chondrocytes, but not in the intervening chondrocytes. Thus, the expression pattern of Gas6 is similar to that of MGP. It may be speculated that Gas6 inhibits matrix mineralization through the present of Gla residues.

**References:**

