学 位 論 文 の 要 旨

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学 位 論 文 名　*In Vivo* Analysis of Arg-Gly-Asp Sequence/Integrin α5β1-Mediated Signal Involvement in Embryonic Enchondral Ossification by *Exo Utero* Development System

発 表 雑 誌 名　Journal of Bone and Mineral Research
（巻，初頁～終頁，年）　in press

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論 文 内 容 の 要 旨

INTRODUCTION

Enchondral ossification is a fundamental mechanism for longitudinal bone growth during vertebrate development. In this process, chondrocytes near the articular surface (periarticular chondrocytes; Pe) proliferate, differentiate into flat column-forming proliferating chondrocytes (columnar chondrocytes; Co), stop dividing, and finally differentiate into hypertrophic chondrocytes (HT). Co have a characteristic shape and secrete matrix rich in type 2 collagen (Coll 2), whereas enchondral ossification requires the hypertrophic differentiation of chondrocytes, which is characterized by the secretion of type X collagen (Coll X).

In mature cartilage, the integrin family of cell surface receptors appears to play a major role in mediating cell–extracellular matrix (ECM) interactions that are important to cartilage homeostasis and repair, including cell attachment, growth, and differentiation. The Arg-Gly-Asp (RGD) amino acid sequence has been found to consist of a binding site with integrins in a wide variety of ECM components. In the embryonic mouse limb culture system, functional blockade with RGD peptides or with an antibody that interferes with integrin α5β1–ligand interactions inhibited pre-hypertrophic chondrocyte (PHT) differentiation. These *in vitro* reports suggest that the integrin α5β1-mediated ECM signal through the RGD sequence is involved in the regulatory mechanisms of chondrocyte proliferation, differentiation, and apoptosis in enchondral ossification. However, the precise function of this signal *in vivo* remains unclear.

The purpose of this study is therefore to elucidate *in vivo* the roles of the integrin α5β1-mediated signal through the RGD sequence in the cell-ECM interaction during embryonic enchondral ossification.

MATERIALS AND METHODS

Jcl:ICR female mice aged 8–20 weeks (CLEA Japan, Tokyo, Japan) were used. We injected Arg-Gly-Asp-Ser (RGDS) peptides and anti-integrin α5β1 antibody (α5β1 ab) in the upper limbs of mouse embryos at embryonic day (E) 15.5 (RGDS-injected limbs; n = 30, α5β1 ab-injected limbs; n = 27) by an *exo utero* development system, and compared the effects on chondrocyte proliferation, differentiation, and apoptosis in enchondral ossification with those found in the control limbs (n = 30 for Arg-Gly-Glu-Ser peptide-, or vehicle-injected, and no surgery, n = 27 for mouse IgG-injected) at E16.5. Methods used are histology, immunohistochemistry, immunoblotting, TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay, and real-time reverse transcription-polymerase chain reaction.

RESULTS AND DISCUSSION

In the present study, whereas body weight and crown rump length (CRL) were not affected in either group after *exo utero* development, the ratio of brachium length to CRL was significantly decreased in the RGDS-injected limbs, suggesting the region-specific influence of RGDS injection. Humerus length was shorter in the RGDS-injected limbs than in the control limbs, suggesting that the ratio of brachium length to CRL was decreased by the shortened humerus length.

In the RGDS-injected limbs, the safranin-stained cartilage areas were smaller, and there were fewer 5-bromo-2'-deoxyuridine-positive cells per unit of cartilage area in both the proximal and distal cartilage areas, suggesting cartilage was shortened by the decrease in chondrocyte proliferation. Furthermore, the relative expression levels of protein and mRNA of Coll 2, the primary component of the ECM in articular cartilage tissue, were lower in the RGDS-injected limbs than in the control limbs. Cartilage ECM is constructed of networks of Coll 2, which plays an important role in cartilage tissue engineering and regulates the migration and proliferation of chondrocytes. Coll 2 was downregulated after integrin blockage by monoclonal antibodies in the embryonic mouse limb culture system in a previous report. Thus, the downregulated expression of Coll 2 in the present study is compatible with the decrease in chondrocyte proliferation in the RGDS-injected limbs.

In the present study, although the cartilage areas were smaller in the RGDS-injected

limbs, those limbs had an increased ratio of cartilage length to humerus length, as well as an increased ratio of safranin-stained cartilage areas to humerus areas. Furthermore, Coll X was particularly less densely distributed in the RGDS-injected limbs than in the control limbs. The relative expression levels of Coll X protein and mRNA were significantly decreased, and the Coll X / Coll 2 ratio was lower than in the control limbs. Previous experiments in cell culture have reported that, upon hypertrophy, chondrocytes undergo dramatic changes in their gene expression, including a switch from Coll 2 to Coll X as the major collagen type produced. In the present study, RGDS injection was suggested to decrease Coll X expression and to decrease both chondrocyte differentiation and proliferation. This suggested that replacement to bone was delayed in the RGDS-injected limbs.

TUNEL-positive cells were hardly observed in PHT and HT; relative expression for fractin (a marker of apoptosis-related events) was decreased, and the ratios of fractin to the Coll X / Coll 2 ratio were lower in the RGDS-injected limbs than in the control limbs. The expression levels of anti-apoptosis-related B-cell lymphoma 2 (Bcl2)-like 1 protein (BclX) and apoptotic acceleratory Bcl2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3) were significantly decreased in the RGDS-injected limbs. Whereas Bcl2 protects apoptosis, BclX binds to Bnip3 by inorganic phosphorus acid stimulation, and the balance between BclX and Bnip3 critically regulates the apoptosis of terminally differentiated chondrocytes both *in vitro* and *in vivo*. Thus, the present study suggested that RGDS injection inhibited the expression of Bnip3 as well as that of the BclX gene, and decreased apoptosis.

Several *in vitro* studies reported that integrin $\alpha5\beta1$ dimers are expressed in the Pe and Co. Some *in vitro* studies using human articular chondrocytes have shown that $\alpha5\beta1$ regulates various aspects of chondrocyte biology. In the present *in vivo* study, it was observed that fluorochrome-labeled RGDS peptides had accumulated especially in PHT and HT areas. Additionally, fluorochrome-labeled RGDS peptides showed colocalization with integrin $\alpha5\beta1$. Furthermore, the results of $\alpha5\beta1$ ab-injected limbs were very similar to those of RGDS-injected limbs. RGDS and $\alpha5\beta1$ ab injection decreased chondrocyte proliferation, differentiation, and apoptosis in enchondral ossification, respectively. The present findings thus suggested that RGDS injection inhibited integrin $\alpha5\beta1$-mediated binding with RGD-containing ECM proteins.

CONCLUSION

Our *in vivo* study by an *exo utero* development system suggested that the integrin $\alpha5\beta1$-mediated ECM signal through the RGD sequence is involved in enchondral ossification.