学 位 論 文 の 要 旨

氏名　　井上　隆之

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

著 者 名　Takayuki Inoue, Ryuju Hashimoto, Akihiro Matsumoto, Esrat Jahan, Ashiq Mahmood Rafiq, Jun Udagawa, Toshihisa Hatta, and Hiroki Otani

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.

論文審査及び最終試験又は学力の確認の結果の要旨

㊙ ・ 乙	氏 名	井上　隆之
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	
学位論文審査委員	主 査	内尾　祐司
	副 査	北垣　一
	副 査	関根　浄治

論文審査の結果の要旨

軟骨内骨化は骨格形成において基本的なメカニズムであり、その過程において軟骨細胞は増殖・分化・肥大化後に細胞死を経て骨に置換される。その障害は軟骨無形成症や変形性関節症の発症に関与するともいわれている。軟骨内骨化の調節には細胞外基質に含まれるArg-Gly-Asp（RGD）配列の認識によるインテグリンα5β1を介したシグナルの関与が*in vitro*で示されているものの、*in vivo*による詳細な報告はない。申請者は上記シグナルの*in vivo*での役割を明らかにするためにマウス胎仔を用いて軟骨内骨化過程を組織学的、免疫組織化学的、および分子生物学的に検討した。マウス子宮外発生法により胎生15.5日マウス胎仔の上肢に細胞外基質接着阻害ペプチド（Arg-Gly-Asp-Ser; RGDS）（RGDS群）および抗インテグリンα5β1抗体（α5β1 ab群）を投与した。胎生16.5日に胎仔を得て対照群（対照ペプチド投与、マウスコントロールIgG投与、生理食塩水投与、無処置）と比較した。その結果、RGDS群では対照群に比べ、投与肢上腕骨長が有意に短縮した。また、RGDS群では軟骨細胞の増殖が有意に減少するとともに、増殖細胞層におけるII型コラーゲンの発現および肥大細胞層におけるX型コラーゲンの発現、ならびにその比はともに有意に減少した。さらにRGDS群においてTUNEL陽性死細胞はほとんど観察されず、pro-apoptotic factorsの発現も有意に減少していた。一方、α5β1 ab群における上記各実験の結果は、RGDS群と極めて類似していた。以上より、インテグリンα5β1の細胞外基質のRGD配列認識による細胞内へのシグナルが、軟骨内骨化における軟骨細胞の増殖・分化・肥大化および細胞死調節に関与することが*in vivo*で示された。本研究は軟骨内骨化過程におけるRGD配列の認識によるインテグリンα5β1を介したシグナルの役割を明らかにしただけでなく、骨関節疾患の病態解明や新たな治療手段ともなり得る可能性が示唆された。以上を総合的に評価して、本論文は学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者はマウス子宮外発生法を用いて*in vivo*での軟骨内骨化過程におけるインテグリンα5β1を介するシグナルの役割を組織学的、免疫組織化学的、および分子生物学的に明らかにした。これは骨関節疾患の病態解明や新たな治療法の確立にも貢献しうる研究といえ学位授与に値する。（主査　内尾祐司）

申請者はマウス胎仔を用い*in vivo*で初めて軟骨内骨化がインテグリンα5β1を介していることを証明した。軟骨内骨化のメカニズムを明らかにした意義のある研究である。周辺知識も豊富であり、学位授与に値すると判断した。（副査　北垣　一）

申請者は、マウスの軟骨内骨化過程におけるインテグリンα5β1の働きを分子生物学的に解明した。実験に用いた動物モデルは、子宮外発生法やマイクロインジェクションによるペプチドの局所投与等これまで申請者らが開発したエビデンスに基づく手技により作製されたもので、マウスの全身発育には影響を及ぼさずに、局所の変化のみを的確に評価することができた。結果、軟骨内骨化における軟骨細胞の増殖・分化・肥大化および細胞死調節に関与することが*in vivo*で示された。公開審査では、関連知識も豊富であり、学位の授与に相応しいと判断した。（副査　関根浄治）

（備考）要旨は、それぞれ４００字程度とする。