［原著］Mechanical Stress regulates Chondrocyte Proliferation and Differentiation during Endochondral Bone Formation

Mechanical Stress regulates Chondrocyte Proliferation and Differentiation during Endochondral Bone Formation

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ABSTRACT

The effects of mechanical stress on the proliferation and differentiation of mesenchymal progenitor cells toward chondrogenesis are not well addressed. The purpose of this study was to assess how mechanical stress exerts the influence on chondrogenic proliferation and differentiation during endochondral bone formation. ATDC5 mouse chondroprogenitor cells, which undergo a reproducible multistep chondrogenic differentiation in response to insulin, were used in this study. Mechanical loading was applied to ATDC5 cells grown on plastic plates in monolayer cultures at a magnitude of 2800 μstrain and a frequency of 0.5 Hz for 24 hr at day 4, 7, 14, 21 and 28. The chondrogenesis pathway in ATDC5 cells was studied by analyzing differentiation stage-specific gene expression using a Northern blot analysis. ATDC5 cells responded to the addition of insulin with a significant increase in the expression of mRNA for histone H4, type II collagen, and aggrecan, but not type X collagen during a 28-day culture period. These inductive responses were time-dependent, and therefore, differentiation-dependent. Applying mechanical loading in the presence of insulin further up-regulated the mRNA levels of these proteins including type X collagen. Moreover, mechanical loading increased the histone H4 mRNA expression in immature cells, and promoted the mRNA expression of cartilage-type extracellular matrix in insulin-induced relatively mature cells, which also indicated differentiation-dependent. The expression of histone H4, type II and X collagen, and aggrecan mRNA levels were also increased with mechanical loading alone. These results demonstrated the adjunctive effects of mechanical loading on insulin-induced chondrogenesis and the peculiar effects of mechanical loading on the induction of chondrogenesis, thus suggesting that mechanical loading can promote endochondral bone formation. *Ryukyu Med. J., 26(1,2) 57~67, 2007*

Key words: mechanical stress, chondrocyte, chondrogenesis endochondral bone formation

INTRODUCTION

It is becoming increasingly evident that skeletal tissue, including bone, cartilage, skeletal muscles and ligaments are highly responsive to the mechanical environment[1]. Endochondral bone formation, which is presumed to occur during embryonic development[2,3], longitudinal bone growth, fracture repair, and ectopic bone formation are also modulated by mechanical forces[4,5]. For instance, physiologic strains on the growth plate are necessary for proper development of longitudinal bone growth[6], and fracture healing can be accelerated by the application of an optimized mechanical environment[7,8]. Several in vitro studies also indicate that chondrocytes respond to mechanical forces by altering the synthesis of the extracellular matrix, the rate of proliferation, and their state of differentiation[9-12].

During endochondral bone formation, undifferentiated mesenchymal cells condense at a site where

bone is newly formed, and these cells become chondrocytes, thereby forming a cartilage anlage. The cartilage anlage enlarges through chondrocyte proliferation. These proliferative chondrocytes actively secrete an extracellular matrix, such as type II collagen and proteoglycan aggrecan. The proliferative cells then lose their ability to divide and terminally differentiate into hypertrophic chondrocytes which synthesize type X collagen. Hypertrophic chondrocytes eventually undergo apoptotic cell death, and then the cartilage matrix is finally replaced by bone[13, 14].

ATDC5 chondroprogenitor cells derived from mouse embryonic teratocarcinoma cell line AT805 exhibit the multistep chondrogenic differentiation observed during endochondral bone formation[15,16]. The process of differentiation is started with 5% fetal bovine serum, transferrin, and selenium, followed by the addition of 10 μg/ml insulin to promote the differentiation into type II collagen-expressing chondrocytes with formation of cartilage nodules through cellular condensation. When the formation of cartilage nodules is completed, the cells are then converted to type X collagen-expressing hypertrophic chondrocytes followed by mineralization process encompassing the stages from mesenchymal condensation to calcification *in vitro*. ATDC5 cells provide an excellent model system for the study of endochondral bone formation *in vitro*.

The process of endochondral bone formation is intricately regulated by various growth, differentiation and transcription factors. For example, parathyroid-hormone-related peptide (PTHrP) stimulates chondrocyte proliferation and inhibits hypertrophic differentiation of chondrocytes[17-19]. Fibroblast growth factors (FGFs) increase chondrocyte proliferation and inhibit the terminal differentiation of hypertrophic chondrocytes[20-23]. Bone morphogenetic proteins (BMPs) induce both the proliferation of chondrocytes and chondrogenic differentiation, hypertrophy and mineralization[20, 21, 24]. These locally produced factors coordinated with systemic factors such as growth hormone, thyroid hormone, vitamin D_3, estrogen, and glucocorticoids regulate chondrocyte cellular activities[25]. Therefore, endochondral bone formation is a complex process orchestrated by a complex interaction between genetic and epigenetic factors.

Mechanical force is one of the important epigenetic factors. Available evidence indicates that mechanical stress has an important function in chondrocytes while also playing a key role in endochondral bone formation[26]. For instance, intermittent hydrostatic pressure increases extracellular matrix production in chondrocytes[10,27,28], and stimulation with low intensity pulsed ultrasound in the murine bone culture model has been shown to promote bone cell differentiation, leading to an elongation of the bone collar[29,30]. However, there have been few studies on how mechanical stress regulates chondrocyte proliferation and differentiation in the process of endochondral bone formation. The present study examines the effects of mechanical stress on ATDC5 cells. These results suggested that mechanical stress promotes the proliferation and differentiation of ATDC5 cells in a differentiation-dependent manner.

MATERIALS and METHODS

Cell culture

ATDC5 cells (Riken cell bank, Tsukuba, Japan) were plated onto 42×75-mm plastic plates at 1×10^6 cells/plate in a maintenance medium consisting of a 1 : 1 mixture of DME and Ham's F-12 medium (ICN Biomedicals Inc, Ohio, USA) containing 5% fetal bovine serum (FBS : Invitrogen, California., USA), 10 μg/ml human transferrin (T: Boehringer Mannheim GmbH, Mannheim, Germany), and 3×10^{-8} M sodium selenite (S: Wako Pure Chemical, Osaka, Japan). Cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day. Three days after plating, on day 0, when the cultures reached 90% confluence, chondrogenesis was induced in half of the cultures by supplementing the maintenance medium with 10 μg/ml bovine insulin (I: Wako, differentiation medium). On day 21, the culture medium was switched to alpha modified essential medium (ICN Biomedicals Inc) containing 5% FBS plus TS in the insulin-untreated cultures or ITS in the insulin-treated cultures for the facilitation of cellular hypertrophy and mineralization in culture.

The plates were transferred to bending dishes that were filled with fresh medium. Loading was started 24 hr later. Control cell cultures were prepared in parallel with loaded cell cultures, using the same procedures without loading.

Mechanical loading

An in vitro cell loading system was utilized with 4-point bending, in which mechanical deformation and fluid flow effects are created. Culture plates were loaded using a Vitrodyne 1000 Universal Materials Tester (Catillon, N.C., USA), which consists of a computer-controlled, servomotor-driven linear actuator assembly with an interface controller that controls vertical displacement and actuator ram speed (displacement rate). The actuator assembly was placed in a 5% CO_2 incubator to maintain an optimum environment for the cell cultures during the loading experiments. The cells were subjected to compressive strain, and the magnitude of this strain was determined by the displacement and thickness of the culture plate (Fig. 1), as previously described[31]. In the present study, loading was applied for 24 hr at day 4, 7, 14, 21, and 28 at the magnitude of 2800 μstrain, and the frequency of 0.5 Hz.

Histological procedures

A Histological analysis was performed using Alcian blue or Toluidine blue staining. ATDC5 cells were stained with Alcian blue in order to determine the presence of cartilage-specific proteoglycans in the extracellular matrix on day 7, 14, 21, and 28. In brief, ATDC5 cells were plated in a 12-multiwell plate and cultured under the conditions described above. The cells were washed twice with distilled water, fixed with 1% acetic acid in ethanol for 10 min, rinsed with 3% acetic acid, stained with 0.1% Alcian blue 8GX (Sigma-Aldrich, MO, USA) in 3% acetic acid for 15 min, and rinsed three times with 3% acetic acid and distilled water.

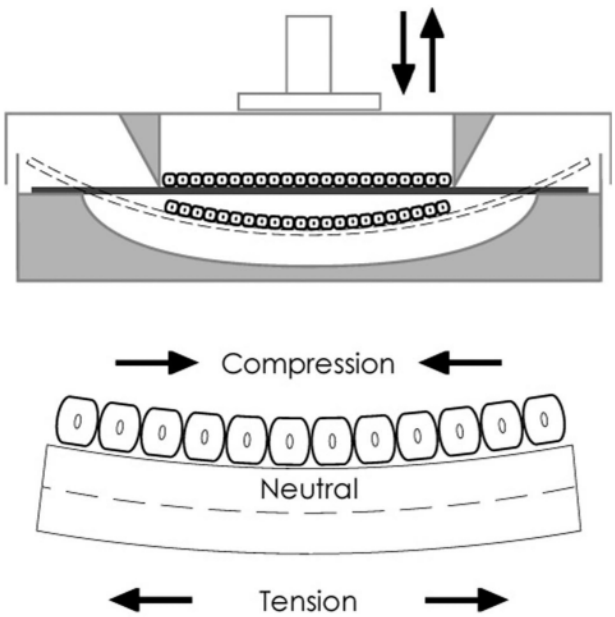

Fig. 1 Cell loading system
Diagram of the cell loading system. The plates are bent by applying 4-point loading, causing compressive strains on the cells. As the plate is pushed through the culture medium, fluid shear forces and pressures are imposed on the cells.

Toluidine blue staining was done to examine the effects of insulin and mechanical loading on the morphology of the cells. ATDC5 cells were cultured on the loading plates in the presence or absence of insulin, and loaded for 24 hr on day 4, 7, 14, 21 and 28. Immediately after loading, the cells were washed twice with distilled water, fixed with 98% ethanol for 20 min, rinsed with distilled water twice, stained with 0.05% Toluidine blue O (Sigma) with pH adjusted to 2.5.

Northern blot analysis

Immediately after loading, total RNA was extracted using the TRIzol reagent (Invitrogen). Isolated RNA (10 μg/lane) were separated on a 1% agarose-0.44 M formaldehyde gel, and transferred to Hybond nylon membrane (Amersham Biosciences Corp., NJ, USA) by capillary blotting, and fixed to the membrane by ultraviolet irradiation. After 1 hr of prehybridization in a rapid hybridization buffer (Amersham) at 65℃, filters were hybridized for 2 hr at 65℃ in the same solution containing radiolabeled cDNA probes. Hybridization probes were labeled with [^{32}P]dCTP (specific activity of>3000 Ci/mmol, Amersham), and prepared by the random-primer method with a High prime DNA Labeling Kit (Roche, Mannheim, Germany) using the appropriate cDNA fragments: 1.4 kb EcoRI fragment of pKT1180[32] as a probe for α1(II) collagen mRNA; 0.65 kb HindIII fragment of pSAm10h[33] as a probe for α1(X) collagen mRNA; 0.87 kb PstI fragment of p1355[34] as a probe for aggrecan mRNA. These fragments were generous gifts from Dr. Yuji Hiraki (Kyoto University, Kyoto). Histone H4 cDNA was purchased from Open Biosystems, AL, USA.

The filters were washed once in 2× saline-sodium citrate (SSC)-0.1% sodium dodecyl sulfate (SDS) solution at room temperature and twice in

0.2 × SSC-0.1% SDS solutions at 65℃. After they were washed, the filters were exposed to BAS-SR (Fuji Photo Film Ltd., Tokyo, Japan), at room temperature. The relative band intensities were quantified by densitometric scanning of the autoradiographs using a BAS 1500 (Fuji Photo Film Ltd.), and normalized to the housekeeping gene, β-actin mRNA expression in each sample. The mean values of the molecule in the non-loaded cell, in the absence of insulin on day 4 were set as 100%. The mean ± SD values of the cultures were presented as a percent of the means values for each molecule from the non-loaded cells in the absence of insulin on day 4. Experiments were done at least in triplicate.

Statistical analysis

The results were expressed as the means ± SD of 3 cultures. Comparisons between multiple groups were performed using a one-way ANOVA followed by Tukey-Kramer's test. P values of less than 0.05 were considered to be significant.

RESULTS

Histological analysis

ATDC5 cells which displayed a flattened appearance in monolayer cultures reached confluence at day 4 (Fig. 3a). When the cells were grown in the presence of insulin, cells piled up, with no sign of contact inhibition (Fig. 3c), and exhibited the formation of discrete cartilaginous nodules at day 7 (Fig. 3f). These cartilaginous nodules were intensely stained with Alcian blue and Toluidine blue. In contrast, no cartilage nodule was found in the absence of insulin on day 7 (Fig. 3e) In the presence of insulin, the number and size of these nodules increased during culture (Fig. 3g). Eventually, hypertrophic cells which displayed a polygonal morphology appeared on day 21, thus indicating chondrogenesis induction (Fig. 3h). As

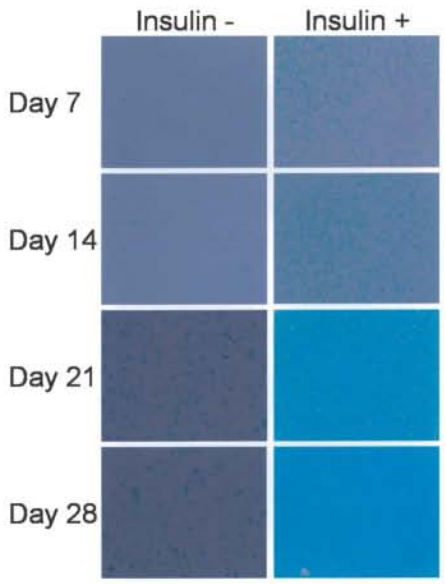

Fig. 2 Chondrogenic differentiation of ATDC5 cells.
ATDC5 cells were induced to undergo chondrogenic differentiation by insulin, and the proteoglycans were stained with 0.1% Alcian blue. The images were obtained macroscopically.

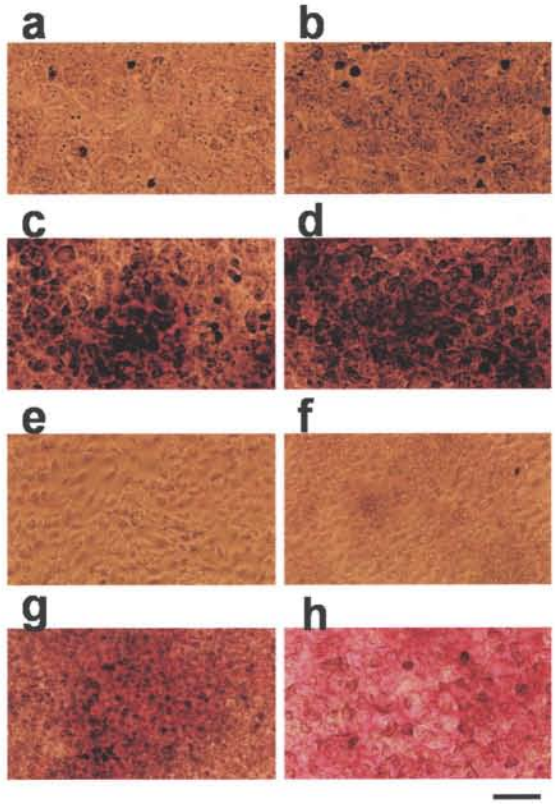

Fig. 3 Phase-contrast micrographs of ATDC5 cells, loaded and non-loaded cells in the presence and absence of insulin stained with Toluidine blue.
Cells on day 4 are shown in (a-d). Cells were plated in the absence of insulin without loading (a) or with loading (b), in the presence of insulin without loading (c) or with loading (d). Cultures were incubated for 7days in the absence of insulin on day 7(e) or in the presence of insulin (f). Cells were grown to form cartilage nodules on day 14 with insulin (g). Hypertrophic cells appeared on day 21 with insulin (h). Bar represents 50 μm for a, b, c, d and h and 100 μm for e, f and g.

shown in Figure 2, a progressive increase of Alcian blue positive extracellular matrix was observed, suggesting the accumulation of cartilage-type proteoglycans in cultures grown in the presence of insulin, but not in control cultures except for a few spotted areas at day 21 and 28. No morphological changes have been observed in response to 24 hr of mechanical loading (Fig. 3b, d).

Histone H4 mRNA Expression

The regulatory effect of mechanical stress on the markers of proliferation as well as on specific markers of the chondrogenic phenotype in ATDC5 cells was observed at different time points. Histone H4 synthesis occurs mainly in S phase and therefore it was used as a marker for proliferation[35, 36]. The histone H4 mRNA expression remained almost constant during all of the tested stages (Fig. 4). Despite the stimulatory effect of insulin on proliferation, no significant changes were detected in the histone H4 mRNA expression between insulin treated and non-treated cells except on day 4. The histone H4 mRNA levels in mechanically loaded cells showed a small but significant increase over the control levels regardless of insulin treatment on day 4. The effect of mechanical stress on the histone H4 expression disappeared thereafter.

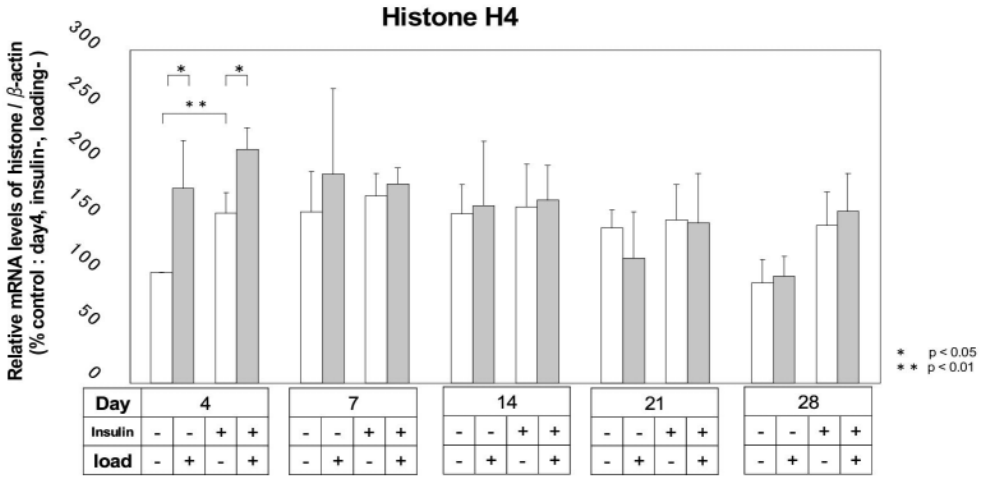

Fig. 4 Time course of changes of Histone H4 mRNA expression in ATDC5 cells, loaded and non-loaded cells in the presence and absence of insulin were compared by a Northern blot analysis.

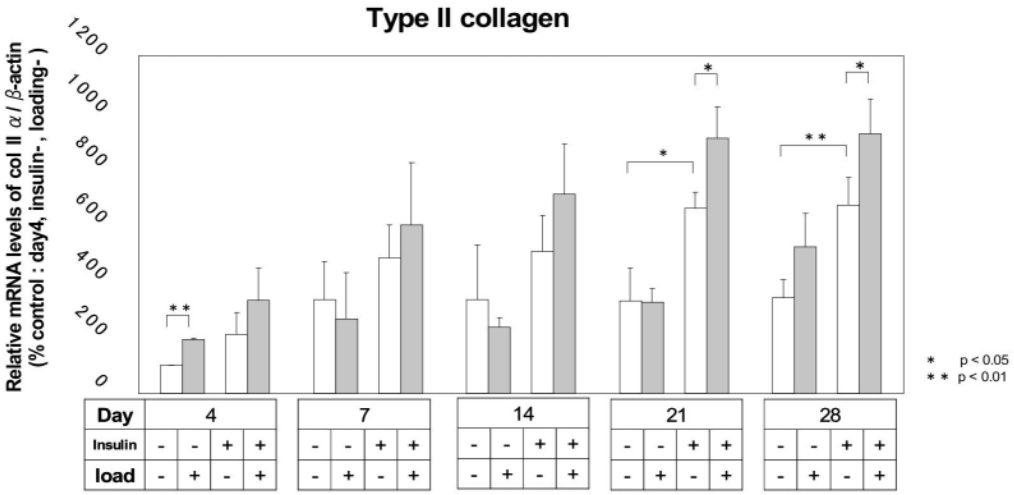

Fig. 5 Time course of changes of type II collagen mRNA expression in ATDC5 cells, loaded and non-loaded cells in the presence and absence of insulin were compared by a Northern blot analysis.

Type II collagen mRNA expression

As mesenchymal cells differentiate into chondrocytes, they initiate the synthesis of type II collagen. Type II collagen mRNA expression was detectable on day 4 in the absence of insulin, and slightly increased and reached plateau on day 7 (Fig. 5). In contrast, a continuous and progressive increase in the expression of type II collagen mRNA was observed in the presence of insulin, which was significantly higher than that of untreated cells on days 21 and 28. The effects of mechanical loading were detected in undifferentiated cells on day 4 and cells in the late stage of differentiation on days 21 and 28.

Aggrecan mRNA expression

In the presence of insulin, the aggrecan mRNA expression dramatically increased and reached a plateau on day 14 (Fig. 6). Exposure of mechanical loading to these cells resulted in slight increase of aggrecan mRNA level in comparison to the non-loaded cells, but the difference was significant only on day 7 partly because the expression induced by insulin was too high to recognize the additive effect of loading. The expression was also up-regulated in the absence of insulin to a lesser degree, in comparison with insulin treated cells. Interestingly, the effects of mechanical loading were observed in the absence of insulin on days 21 and 28.

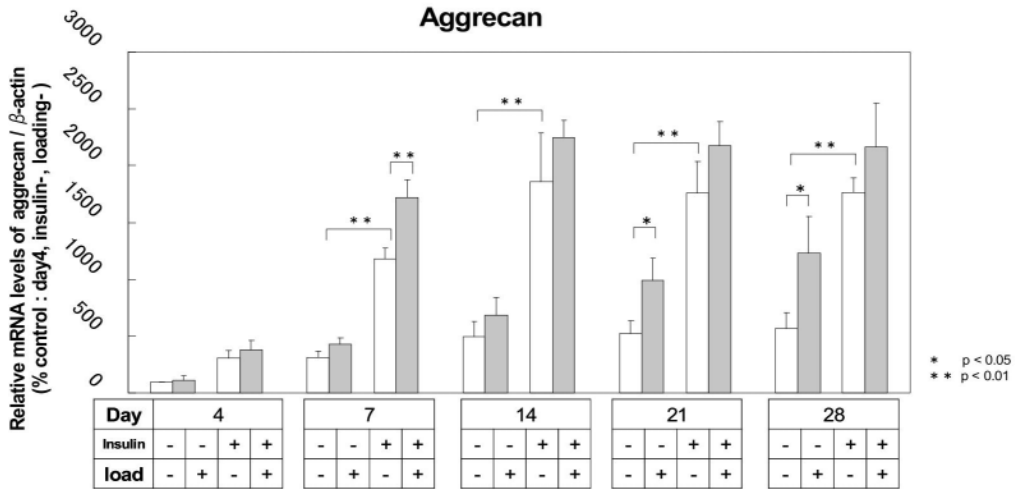

Fig. 6 Time course of changes of aggrecan mRNA expression in ATDC5 cells, loaded and non-loaded cells in the presence and absence of insulin were compared by a Northern blot analysis.

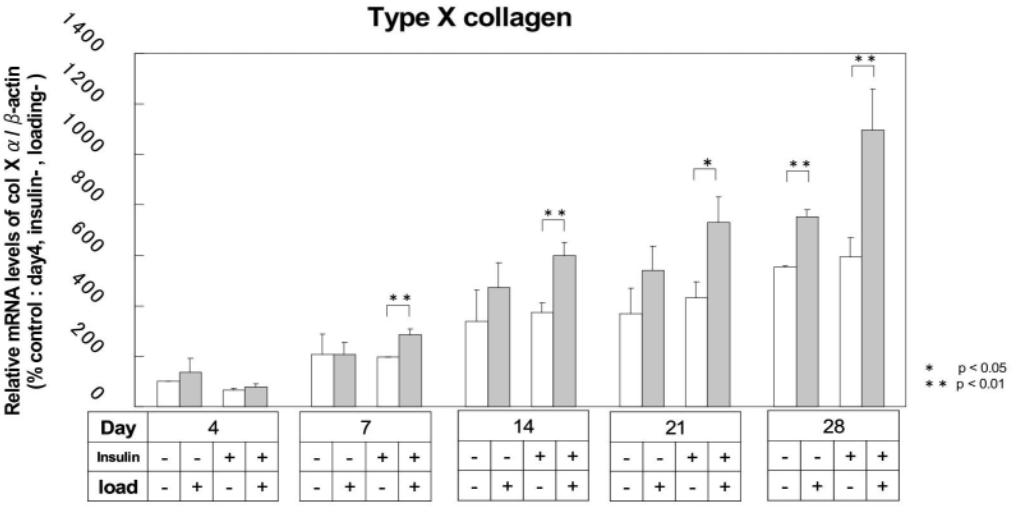

Fig. 7 Time course of changes of type X collagen mRNA expression in ATDC5 cells, loaded and non-loaded cells in the presence and absence of insulin were compared by a Northern blot analysis.

Type X collagen mRNA expression

The production of type X collagen is thought to be confined to the hypertrophic chondrocytes and it facilitates new bone formation by regulating matrix mineralization. It is therefore accepted that type X collagen is a reliable marker for endochondral bone formation. Unexpectedly, type X collagen mRNA expression gradually increased in both insulin treated and non-treated cells similarly from day 4 to day 28 (Fig. 7). However, the effects of mechanical loading on type X collagen mRNA expression were much higher in insulin treated cells than in non-treated cells, thus suggesting that mechanical stress induced hypertrophic differentiation in cooperation with insulin.

DISCUSSION

The effects of mechanical stress on the proliferation and differentiation of mesenchymal progenitor cells toward chondrogenesis have not yet been fully addressed. The purpose of this study was to assess how mechanical stress exerts the influence on chondrogenic proliferation and differentiation during endochondral bone formation. The results of this study indicate that mechanical stress regulates proliferation and differentiation during chondrogenesis in a differentiation-dependent manner, since mechanical loading induced proliferation in immature ATDC5 cells, and promoted the production of cartilage-type extracellular matrix in insulin-induced mature ATDC5 cells.

ATDC5 cells were a useful cell line for the study of endochondral bone formation. ATDC5 cells cultured in the presence of insulin undergo chondrogenic differentiation[15,16]. The current results indicated that cartilage nodules, which formed in the presence of insulin at day 7, were very weakly stained by Alcian blue in contrast to the strong Alcian blue staining observed in/around nodules on days 21 and 28, thus indicating a significant production of cartilage-type proteoglycans in the later stage of differentiation. Furthermore, mRNA expression of chondrocytes specific genes such as type II collagen, and aggrecan were markedly upregulated from day 14 to day 21 in the presence of insulin, although there was little increase in type II collagen and aggrecan mRNA expressions in the absence of insulin. These data demonstrate that insulin induces ATDC5 cells to differentiate into mature chondrocytes, as reported earlier[15].

To investigate the effect of mechanical stress on proliferation of ATDC5 cells, mRNA expression of histone H4 was assessed as a marker of proliferation, because histone H4 is up-regulated during the G1/S phase transition of the cell cycle. Mechanical stress had a small stimulatory effect on the expression of histone H4 mRNA, suggesting up-regulation of cell proliferation in growing phase of ATDC5 cells. However, the result of this experiment was not fully adequate to assess cell proliferation. Because we have not assessed for mitogenic activity by measuring [3H] thymidine incorporation into the DNA to confirm this phenomenon.

It is well known that insulin plays an important role in the growth and metabolism of developing cartilage. In the absence of insulin, ATDC5 cells ceased the proliferation when they became confluent. Insulin supported the proliferation of cells in a postconfluent phase and promoted the formation of cartilage nodule-like aggregates with chondrocyte-characteristic phenotype expressions[21]. The stimulatory effect of insulin on chondrogenic differentiation seems to be indirect, probably via increasing cell proliferation, because chondrogenic differentiation of ATDC5 cells only occurred in a postconfluent phase. Atsumi *et al.* reported that insulin enhanced the cell proliferation in a dose-dependent manner[37]. It is unlikely that insulin can be substituted by mechanical stress, although mechanical loading alone increased mRNA expressions of histone H4, type II collagen, and type X collagen. Further studies are needed to address this issue.

Unlike previous reports, type X collagen mRNA expression, which is thought be confined to the hypertrophic chondrocytes, was gradually increased in both insulin-treated and non-treated cells although the level of hypertrophic differentiation of ATDC5 cells in the absence of insulin is very low based on the histological analysis. Several factors are reported to affect type X collagen mRNA expression. Chen *et al.* showed that hypoxic incubation of insulin-treated ATDC5 cells delays and suppresses hypertrophic differentiation[38], and Shukunami *et al.* reported that it is better to switch the medium from DMEM to α-MEM, and the CO_2 level from 5 % to 3 % around day 21 to induce hypertrophy[16]. We did not change the CO_2 level from 5 % to 3 %, and this may cause the suppressed type X collagen mRNA expression in in-

sulin-treated cells. The reason why type X collagen mRNA expression was increased in non-treated cells was unidentified. To confirm the hyper-trophic differentiation of the cells, we could also examine the expression patterns of other hypertrophic markers, such as Runx2 and matrix metalloproteinase-13.

The effect of mechanical stress on chondrocytes varies depending on the experiments. For example, mechanical stimulation has been found to increase chondrocyte proliferation in some studies[11,39], whereas other studies found decreased cell numbers[12]. The responses of chondrocytes to mechanical stimuli involves many factors, including the magnitude of the strain, type of strain, and fluid flow[26,40,41]. Chondrocytes are sensitive to the type of mechanical stress seen with cyclical applications of high frequency loads stimulating matrix synthesis and static loads depressing it[42,43]. Cyclic loading leads to rapid increases in hydrostatic pressure, matrix deformation, and fluid flow, and these changes to the extracellular environment of the chondrocytes tend to stimulate aggrecan and protein synthesis[44,45]. The magnitude of strain used in these experiments was relatively low compared to previous studies. However, it is likely that fluid effects are more important that mechanical strain. In addition, the present results clearly indicate that the nature of the response to mechanical stress in chondrocytes is dependent on the stage of differentiation of the cells used in the experiments.

Recently, several transcription factors including Sox and Runx family members have been shown to be essential for endochondral bone formation. The initial stage of chondrogenesis is characterized by the expression of Sox9 mRNA, which is a chondrogenic-related transcription factor, followed by the increased expression of chondrocyte specific genes, such as type II collagen and aggrecan, and the final hypertrophic stage is distinguished by increased expression of type X collagen[46,47]. Previous studies have identified the gene for type II collagen, Col2 α 1, as a target of Sox9 in mouse chondrocytes. Mechanical force was found to promote Sox9 expression, which in turn increases type II collagen and aggrecan expressions and inhibits IL-1beta expression resulting in chondrogenesis induction[48].

The cells within growth plate cartilage are highly organized into columns, which consist of differentiating chondrocytes that can be distinguished into four zones: a reserve zone, a proliferative zone, a hypertrophic zone, and a zone of calcification[13]. The longitudinal bone growth depends on the activities of individual chondrocytes. The present results also indicate that chondrocytes derived from a different zone exhibit a differential response to mechanical stimulation.

In chondrogenesis, cell differentiation is a multistep process starting from chondrogenic mesenchymal progenitor cells and eventually resulting in mature, hypertrophic chondrocytes. Growth plate and articular chondrocytes share several common features and extracellular matrix proteins such as type II collagen and aggrecan are among the cartilage markers produced by both cell types. However, during endochondral bone formation, the mature chondrocytes express type X collagen synthesis and mineralize the ECM with the regulation of various factors, such as vascular endothelial growth factor and chondromoodulin-I[49,50]. Usually, the stage of hypertrophy is reached only in cartilage undergoing endochondral ossification during embryonic limb development, growth, and fracture repair. All of these processes are influenced by the mechanical environment. The present study showed that ATDC5 cells differentiated into chondrocytes, which progressively developed into hypertrophic cells, and this process was regulated by mechanical stress in a differentiation-dependent manner. In conclusion, mechanical stress is an important epigenetic factor which controls bone development through the coordinated proliferation and differentiation of chondrocytes during endochondral bone formation.

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