Journal of Cellular Biochemistry 92:715–722 (2004)

Dexamethasone Induces Human Spinal Ligament Derived Cells Toward Osteogenic Differentiation

Hidenori Murata, Hiroshi Tanaka,* Toshihiko Taguchi, Eiichi Shiigi, Hiroshi Mizokami, Toshihiro Sugiyama, and Shinya Kawai

Department of Orthopedic Surgery, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Yamaguchi 755-8505, Japan

Abstract Ossification of spinal ligament is characterized by heterotopic bone formation in the spinal ligaments that are normally composed of fibrous tissues. The pathogenesis of ossification of spinal ligament has been suggested to be associated with osteogenic differentiation of the spinal ligament cells. In order to address this hypothesis, cells derived from human spinal ligament were investigated for their osteogenic potential by the treatment of dexamethasone in vitro. Yellow ligaments were obtained from patients with spinal disorders except ossification of spinal ligament during surgery, and the adhering tissues were removed completely. Most of the ligament cells treated with vehicle exhibited a fibroblast-like spindle shape, while the dexamethasone-treated cells acquired a polygonal morphology. Growth of the ligament cells was suppressed by dexamethasone at a high concentration. Some of the vehicle treated-cells were alkaline phosphatase-positive, and dexamethasone increased the alkaline phosphatase-positive cells and alkaline phosphatase activity in the cells. Northern blot analysis demonstrated that mRNAs expression of pro-α1(I) collagen and alkaline phosphatase were promoted by dexamethasone. Analysis by reverse transcription-polymerase chain reaction showed that expression of osteocalcin mRNA was detected in the dexamethasone-treated cells but not in the vehicle-treated cells, and dexamethasone-induced osteocalcin mRNA expression was promoted by 1,25-dihydroxyvitamin D_3. Finally, mineralization of extracellular matrix in the cells was induced by the presence of dexamethasone and 1,25-dihydroxyvitamin D_3. These results suggest for the first time that dexamethasone has a possible involvement in the osteoblastic differentiation of human spinal ligament cells. J. Cell. Biochem. 92: 715–722, 2004.

Key words: dexamethasone; 1,25-dihydroxyvitamin D_3; osteoblastic differentiation; ossification; spinal ligament

Ossification of spinal ligament occurs most frequently in Asian population. It often leads to the induction of compression of spinal cord and subsequent myelopathy or radiculopathy [Epstein, 2002]. Generally attempts to treat the ossification of spinal ligament using various types of surgical approaches have largely been unsuccessful due to the insufficient decompression of spinal cord. Currently very few therapeutic agents have satisfactory effect on the inhibition of the ectopic ossification. Etiological studies of the ossification of spinal ligament have shown possible links between ossification of spinal ligament and some genetic factors [Okawa et al., 1998; Sakou et al., 2000; Shirakura et al., 2000; Shiigi et al., 2001; Furushima et al., 2002]. In addition, bone morphogenetic proteins (BMPs) and BMP receptors have been indicated as the main factor to produce the pathologic ossification [Kon et al., 1997; Yonemori et al., 1997; Tanaka et al., 2001]. Furthermore, in the cultured human spinal ligament derived cells, several factors including transforming growth factor-β [Inaba et al., 1996], insulin-like growth factor I [Goto et al., 1998], CTGF/Hcs24 [Yamamoto et al., 2002], and mechanical stress [Ohishi et al., 2003; Tanno et al., 2003] have been reported to be involved in the osteogenic differentiation of these cells.

Osteoblastic differentiation is also closely regulated by hormones [Stein et al., 1990; Lian et al., 1999], and we previously demonstrated

Grant sponsor: Ministry of Health and Welfare's Research Grant for Specific Diseases, Japan.

*Correspondence to: Hiroshi Tanaka, Department of Orthopedic Surgery, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Yamaguchi 755-8505, Japan. E-mail: htanaka@yamaguchi-u.ac.jp

Received 20 November 2003; Accepted 17 February 2004

DOI 10.1002/jcb.20090

that parathyroid hormone, calcitonin, prostaglandin E_2, and 1,25-dihydroxyvitamin D_3 could be associated with the osteogenic differentiation of cultured human spinal ligament derived cells [Ishida and Kawai, 1993]. In contrast, dexamethasone, a synthetic glucocorticoid, is one of the potent stimulants that could induce expression of osteoblastic phenotypic markers in both immature osteoblasts and in less-committed cells [Rickard et al., 1994]. It has been demonstrated that dexamethasone induced alkaline phosphatase (ALP) activity and cAMP responses to parathyroid hormone and prostaglandin E_2, and mineralization of human bone marrow derived stromal cells [Cheng et al., 1994]. Dexamethasone has also been reported to exert modulatory effects on osteogenesis of various bone-forming cells in organ cultures with or without other glucocorticoids such as 1,25-dihydroxyvitamin D_3 and retinoic acid [Nishimoto et al., 1987; Kim and Chen, 1989; Ng et al., 1989].

Given the pathogenesis of ossification of spinal ligament has been suggested to be associated with osteogenic differentiation of spinal ligament cells, we examined the ability of ligament cells derived from human spinal ligaments to develop osteoblast phenotypic markers in the presence of dexamethasone. Here, we suggest for the first time that dexamethasone could be involved in the osteoblastic differentiation of human spinal ligament cells.

MATERIALS AND METHODS

Cell Isolation and Culture

Yellow ligaments of the lumbar spine were obtained from 23 adult patients with spinal disorders except ossification of spinal ligament during surgery, and the adhering tissues were removed completely under aseptic conditions. The fresh ligaments were cut into pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Life technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The outgrown cells were harvested before confluence and subcultured after trypsinization with 0.05% trypsin and 0.53 mM EDTA. The first passaged cells were cultured in DMEM containing 10% fetal bovine serum with vehicle or various concentrations (10^{-10}–10^{-6} M) of dexamethasone, and used for each assay. Dexamethasone (Sigma Chemical Co., St. Louis, MO) were dissolved in ethanol, and the final concentration of ethanol in the medium was 0.01%.

The diagnosis of ossification of spinal ligament was performed on the basis of clinical symptons and radiologic examinations using X-ray photograph and computed tomograph of the spine. All subjects were Japanese, and were excluded if they had diseases known to affect bone metabolism. The study was approved by the Institutional Review Board at Yamaguchi University Hospital, and all patients gave informed consent.

MTT Assay

The first passaged cells were plated at a density of 10^2/well in 96-well plates and cultured in DMEM containing 10% fetal bovine serum with vehicle or various concentrations (10^{-10}–10^{-6} M) of dexamethasone for 48 h. Cell growth was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Chemicon International, Inc., Temecula, CA) according to the manufactural procedure. In brief, the cells were tapped after the addition of 10 μl of MTT and incubated at 37°C for 4 h followed by the addition of 0.1 ml of isopropanol/HCl solution to each well. The absorbence at 570 nm was measured using a plate reader [Mosmann, 1983].

ALP Staining and Activity Assay

The first passaged cells were plated at a density of 5×10^3 cells/cm^2 in 6-well plates for ALP staining and in 10 cm dishes for ALP activity assay, and cultured in DMEM containing 10% fetal bovine serum with vehicle or various concentrations (10^{-10}–10^{-6} M) of dexamethasone for 8 days.

ALP staining was performed according to the manufactural procedure (Sigma diagnostics, St. Louis, MO). In brief, the cells were rinsed with phosphate buffered saline and fixed in citrate buffered acetone. Alkaline-dye solution was added and stained for 30 min at room temperature in dark. The cells were then rinsed with water and air-dried [Howlett et al., 1986].

ALP activity was measured as descried preciously [Leboy et al., 1989]. In brief, the cells were washed three times with ice-cold phosphate buffered saline. They were then homogenized and the optical density was determined at 405 nm using a spectrophotometer. The

amount of protein in each cell extracts was also measured as described by Bradford [1979], and each ALP activity was normalized by the amount of protein.

Northern Blot and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

The first passaged cells were plated at a density of 5×10^3 cells/cm^2 in 10 cm dishes and cultured in DMEM containing 10% fetal bovine serum with vehicle or 10^{-8} M dexamethasone for 8 days. In RT-PCR analysis, the cells were also cultured with combination treatment of 10^{-8} M dexamethasone and 10^{-8} M 1,25-dihydroxyvitamin D_3 in the same condition. 1,25-dihydroxyvitamin D_3 was dissolved in ethanol, and the final concentration of ethanol in the medium was 0.01%. Total cellular RNA was extracted by a single step method using TRIzol reagent according to the manufactural protocol (Gibco BRL).

Expression of mRNAs of pro-α1(I) collagen and ALP was determined by Northern blot analysis. Aliquots of 20 μg of total were electrophoresed in 1.2% agarose-formaldehyde gels and transferred onto nylon membranes. The total RNA bound to the membranes was then hybridized with cDNA probes for rat pro-α1(I) collagen [Genovese et al., 1984], human ALP [Weiss et al., 1986], and human glyceraldehydes-6-phosphate dehydrogenase (GAPDH). Labeling of probes was performed by using random primers and Klenow fragment according to the method described previously [Feinberg and Vogelstein, 1983]. Hybridization was performed in the solution containing 5× SSC, 10× Denharts' solution, 10 mM Na_2PO_4, 0.5% SDS, 50% formamide, and 0.1 mg/ml ssDNA. The extent of hybridization of probe to membranes was determined by autoradiography and quantitated densitometrically using NIH image.

Expression of osteocalcin mRNA was analyzed by RT-PCR. Aliquots of 2 μg of total RNA were reverse-transcripted to cDNA according to the manufactural procedure (Gibco BRL). PCR was performed with 20 ng of cDNA using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Buckinghamshire, England) using a Thermal Cycler. The primer sequences for amplification were as follows: human osteocalcin foward primer, 5′-ATGAGAGCCCTCACACTCCTC-3′; human osteocalcin reverse primer, 5′-CGGGCCGTAGAAGCGCCGATA-3′; human GAPDH foward primer, 5′-GGTGAAGGTCGGAGTCAACGG-3′; human GAPDH reverse primer, 5′-GGTCATGAGTCCTTCCACGAT-3′. PCR conditions for each primer couple were 95°C for 30 s, 58°C for 30 s, 72° for 60 s at 35 cycles for osteocalcin and at 25 cycles for GAPDH. The PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide and quantitated using NIH image.

von Kossa Staining

The first passaged cells were plated at a density of 5×10^3 cells/cm^2 in 10 cm dishes and cultured with 10^{-8} M dexamethasone for 4 weeks in DMEM containing 10% fetal bovine serum, 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10^{-8} M 1,25-dihydroxyvitamin D_3. We used the 2 mM β-glyceropohsphate, because, in the long-term cell culture, 10 mM β-glycerophosphate might cause abnormal mineralization. The cells were fixed with ethanol for 5 min and rinsed with water, and exposed to light for 1 h following to the addition of 5% silver nitrate solution. The dishes were rinsed with water and the residual silver nitrate was neutralized by 5% sodium thiosulfate.

Statistics

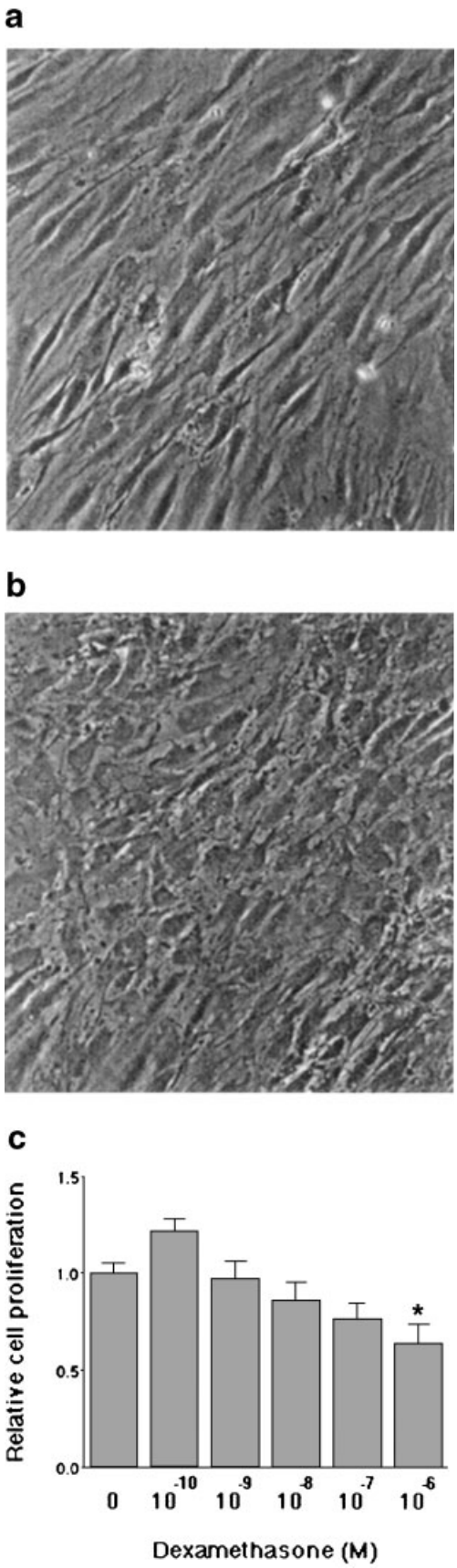
Data were expressed as mean ± SE. Analysis using the one way ANOVA was performed, and P value of smaller than 0.05 was taken to indicate significant differences.

RESULTS

Cell Morphology and Proliferation

Most of the cells treated with vehicle exhibited a fibroblast-like spindle shape, while in the presence of dexamethasone the cells acquired a polygonal morphology (Fig. 1a,b). The morphological transformation in dexamethasone-treated cells was observed at day 4, and the colony constituted of polygonal cells increased until the cells reached confluence at day 8.

Treatment with dexamethasone exhibited a slight inhibition of cell proliferation in a dose dependent manner. Significant inhibition was observed in the 10^{-6} M dexamethasone (Fig. 1c). These results were within the linear range of absorbance for a standard curve for increasing cell number.



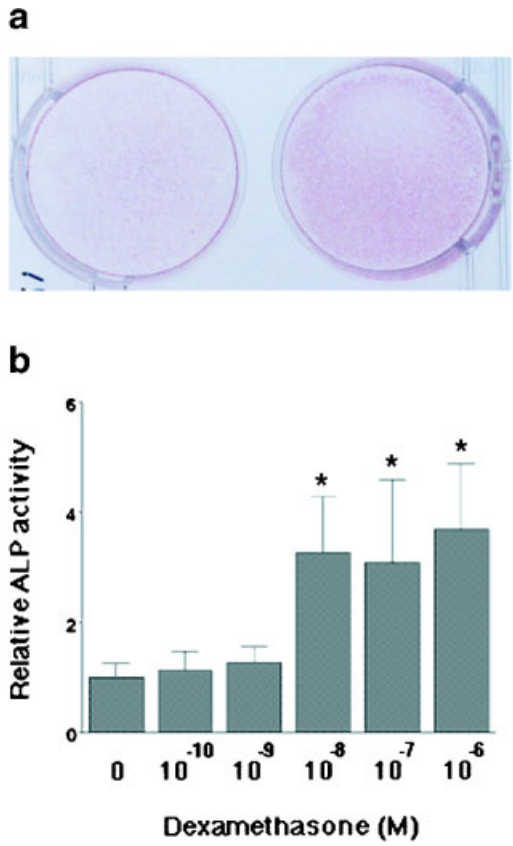


Fig. 2. Effects of dexamethasone on alkaline phosphatase (ALP) stain and activity of human spinal ligament cells. **a**: The cells were cultured with vehicle (left) or 10^{-8} M dexamethasone (right) for 8 days as described in the ''Materials and Methods.'' **b**: The cells were cultured with vehicle or dexamethasone (10^{-10}–10^{-6} M) for 8 days as described in the ''Materials and Methods.'' Bars represents mean ± SE (n = 8). *$P < 0.05$ versus vehicle (one way ANOVA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ALP Staining and Activity

The positive ALP activity in the cells was characterized by a violet coloration. The results showed that there are many colonies demonstrated positive reactions in the dexamethasone-treated cells (Fig. 2a). The staining was often localized in cells with polygonal or cuboidal appearance in their cytoplasmic area. Some of the vehicle treated-cells were also ALP positive.

Fig. 1. Effects of dexamethasone on cell morphology and proliferation of human spinal ligament cells. **a**, **b**: The cells were cultured with vehicle (a) or 10^{-8} M dexamethasone (b) for 8 days as described in the ''Materials and Methods.'' **c**: The cells were cultured with vehicle or dexamethasone (10^{-10}–10^{-6} M) for 48 h and the proliferation was determined by MTT assay as described in the ''Materials and Methods.'' Bars represents mean ± SE (n = 8). *$P < 0.05$ versus vehicle (one way ANOVA).

In order to determine the optimal concentration of dexamethasone for the induction of ALP activity of the cells, ALP activities were measured. Dexamethasone enhanced the ALP activity of the cells, with almost maximum activity achieved at 10^{-8} M (Fig. 2b). In the following experiments, we chose 10^{-8} M dexamethasone on the basis of the fact that its concentration in human is very low.

Gene Expressions of Osteoblast Related Markers

Northern blot analysis demonstrated that mRNA expressions of pro-α1(I) collagen and alkaline phosphatase were promoted by dexamethasone (Fig. 3a,b). Analysis by RT-PCR showed that expression of osteocalcin mRNA was detected in the dexamethasone-treated cells but not in the vehicle-treated cells, and dexamethasone-induced osteocalcin mRNA expression was promoted by 1,25-dihydroxyvitamin D_3 (Fig. 3c).

Mineralization

For analysis of mineralization potential, the cells were cultured in medium supplemented with β-glycerophosphate, ascorbic acid, and 1,25-dihydroxyvitamin D_3. The adherent layers of the cells treated with dexamethasone demonstrated mineralized material that stained positively with the von Kossa technique (Fig. 4), and the number of mineralization nodule was 0.12 ± 0.8 per cm^2. The extracellular matrix, possibly consisted of collagen, deposited in a lamella fashion at day 8, and the matrix acquired thickness with multilayer during the 4-week culture period.

DISCUSSION

This is the first report investigating the effects of dexamethasone on human spinal ligament cells. Cells of ligament tissue differentiation into osteoblastic cells may result in the ectopic ossified tissue formation. In the present study, we demonstrated that dexamethasone induced human spinal ligament cells toward differentiation of osteoblastic phenotypes. These include apparent morphological transformation from spindle-like into polygonal shapes, increase of ALP activity, and mineralization of extracellular matrix. Since dexamethasone is a potent osteoinductive substance, it can be a causative factor in ossification of spinal ligament.

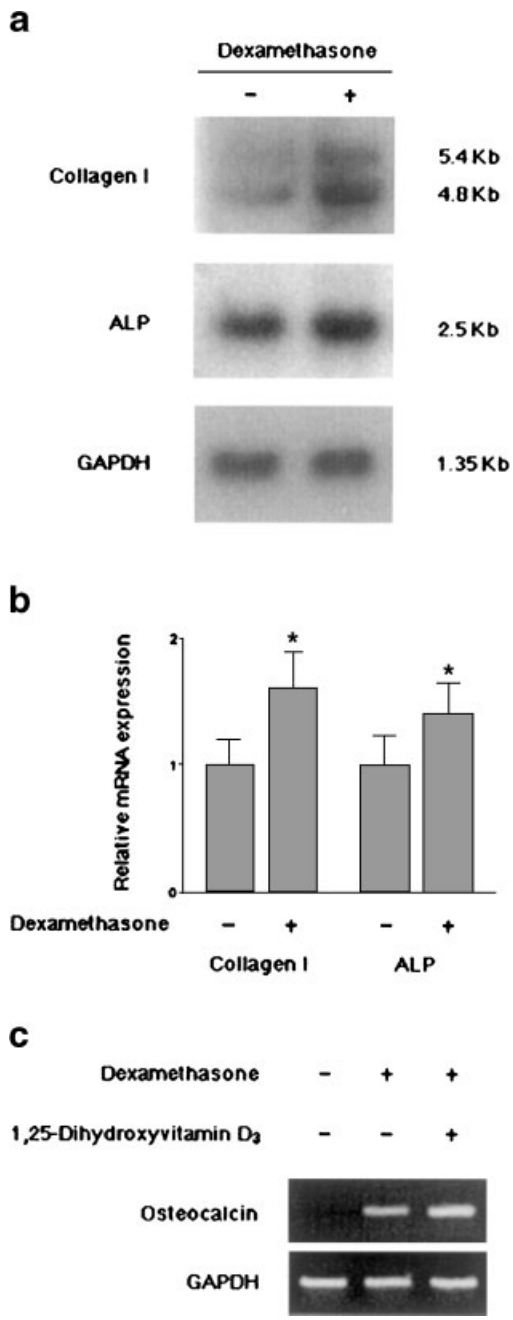


Fig. 3. Effects of dexamethasone on the mRNAs expression of human spinal ligament cells. The cells were cultured with vehicle or 10^{-8} M dexamethasone for 8 days and the total RNA was extracted from the cells as described in the ''Materials and Methods.'' **a**: Representative Northern blot photograph of mRNAs for pro-α1(I) collagen (collagen I), alkaline phosphatase (ALP) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH). **b**: Histogram of the relative mRNAs expression of collagen I and ALP standardized by GAPDH. Bars represents mean ± SE (n = 7). $*P < 0.05$ versus vehicle (one way ANOVA). **c**: Detection of osteocalcin mRNA expression by the RT-PCR analysis. The cells were also cultured with combination treatment of 10^{-8} M dexamethasone and 10^{-8} M 1,25-dihydroxyvitamin D_3 in the same condition.

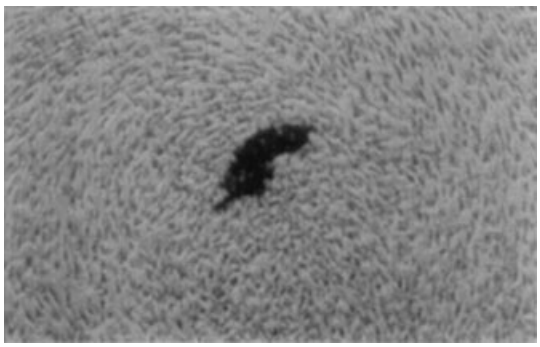


Fig. 4. Representative von Kossa staining of human spinal ligament cells. The cells were cultured with 10^{-8} M dexamethasone in the medium containing with β-glycerophosphate, ascorbic acid and 10^{-8} M 1,25-dihydroxyvitamin D_3 for 4 weeks as described in the "Materials and Methods."

Although relatively high concentration of dexamethasone was required for the suppression of the proliferation, the result from the MTT assay may support the concept that proliferation was down-regulated prior to the onset of differentiation toward osteoblastic cells [Owen et al., 1990]. Cells of osteogenic lineage express characteristically high levels of ALP which is a typical indicator of osteogenic differentiation [Leboy et al., 1991]. Thus, the measurement of ALP activity in cell extracts obtained from cultures was performed preliminary to determine the optimal conditions such as dose and timing of the addition for the treatment with osteotropic hormones and used for an evidence of enhanced differentiation of the ligament cells to an osteoblastic lineage.

Many investigators have been reported the ability of dexamethasone to induce osteogenic differentiation in various cell cultures. Leboy et al. [1991] demonstrated that growth of rat marrow stromal cells with dexamethasone in both primary and secondary cultures can yield a population of cells with mRNA patterns characteristic of osteoblasts: high levels of ALP mRNA as well as vitamin D-inducible osteocalcin mRNA. Extracellular matrix proteins in bone such as collagen type I, ALP, and osteocalcin were also shown to be increased by dexamethasone in a culture system using rat bone marrow cells [Kasugai et al., 1991]. In contrast with these data, several studies indicated inhibitory effects of glucocorticoids on the osteoblastic differentiation cell culture systems, that is glucocorticoids inhibit collagen synthesis [Wong, 1979], protein synthesis [Peck et al., 1967], and cell proliferation [Chen et al., 1977] in isolated osteoblastic cells in vitro. This discrepancy may be related to the difference in glucocorticoid treatment, such as the dose, duration, timing of the steroids added and the cell type studied [Wong et al., 1990]. In addition, the heterogeneity of the cell population may also be associated with the complexity of the effect of glucocorticoids.

Our findings showed that dexamethasone increases ALP activity, enhances mRNA expression of osteoblast markers and induces mineral deposition, although the relative change of ALP activity appears to be emphasized because that of vehicle-treated ligament cells was very low. These results suggest that cells derived from human ligament cells were capable of differentiation into osteoblastic lineage by glucocorticoids. In addition, it is noteworthy that some of the ligament cells were ALP-positive without dexamethasone treatment. This result suggest the heterogeneous population consisting human ligament tissues, that is cells of osteoblastic lineage, might exist in the ligament tissue but require extracellular stimulants for generation of ossified tissue in vivo.

The mechanisms of glucocorticoid induced bone formation in ligament cells, however, remain unclear. It has been shown that glucocorticoids induce the expression of BMP-6 whereas BMP-6 antisense oligonucleotide inhibited the bone nodule formation of glucocorticoids-treated osteoblasts [Boden et al., 1997], suggesting that BMP-6 may play a critical component of the osteoblast differentiation pathway. It is possible that dexamethasone-induced osteoblastic differentiation observed in the cultured ligament cells may be secondary regulated by such an intrinsic growth factor including BMP-6, though the expression of BMP-6 in human ligament cells has not been investigated.

In conclusion, the present findings suggest that human ligament tissue could have a cell population that responds to glucocorticoids and differentiate towards osteoblastic lineage. It is possible that spinal ligament tissue have the potential to induce heterotropic ossification, although whether glucocorticoid is involved in this process is still unclear.

ACKNOWLEDGMENTS

We thank Dr. T Noma (Tokushima University) for technical advices, Dr. MH Zheng

(University of Western Australia) for valuable discussions, and orthopedic surgeons in Yamaguchi University School of Medicine for collecting human spinal ligament tissues.

REFERENCES

Boden SD, Hair G, Titus L, Racine M, Mccuaig K, Wozney JM, Nanes MS. 1997. Glucocorticoid-induced differentiation of fetal rat calvarial osteoblasts is mediated by bone morphogenetic protein-6. Endocrinology 138:2820–2828.

Bradford MM. 1979. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Chen TL, Aronow L, Feldman D. 1977. Glucocorticoid receptors and inhibition of bone cell growth in primary culture. Endocrinology 100:619–628.

Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the oteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Epstein N. 2002. Diagnosis and surgical management of cervical ossification of the posteior longitudinal ligament. Spine J 2:436–449.

Feinberg AP, Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13.

Furushima K, Shimo-onoda K, Maeda S, Nobukuni T, Ikari K, Koga H, Komiya S, Nakajima T, Harata S, Inoue I. 2002. Large-scale screening for candidate genes of ossification of the posterior longitudinal ligament of the spine. J Bone Miner Res 17:128–137.

Genovese C, Rowe D, Kream B. 1984. Construction of DNA sequences complementary to rat α_1 and α_2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxy D. Biochemistry 23:6210–6216.

Goto K, Yamazaki M, Goto S, Kon T, Motiya H, Fujimura S. 1998. Involvement of insulin-like growth factor I in development of ossification of the posterior longitudinal ligament of the spine. Calcif Tissue Int 62:158–165.

Howlett CR, Cav'e J, Williamson M, Farmer J, Ali SY, Bab I, Owen ME. 1986. Mineralization in in vitro cultures of rabbit marrow stromal cells. Clin Orthop 213:251–263.

Inaba K, Matsunaga S, Ishidou Y, Imamura T, Yoshida H. 1996. Effect of transforming growth factor-beta on fibroblasts in ossification of the posterior longitudinal ligament. In Vivo 10:445–449.

Ishida Y, Kawai S. 1993. Effects of bone-seeking hormones on DNA synthesis, cyclic AMP level, and alkaline phosphatase activity in cultured cells from human posterior longitudinal ligament of the spine. J Bone Miner Res 8:1291–1300.

Kasugai S, Todescan R, Nagata T, Yao KL, Butler WT, Sodek J. 1991. Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: Inductive effects of dexamethasone on the osteoblastic phenotype. J Cell Physiol 147:111–120.

Kim HT, Chen TL. 1989. 1,25-dihydroxyvitamin D_3 interaction with dexamethasone and retinoic acid: Effects on procollagen messenger ribonucleic acid levels in rat osteoblast-like cells. Mol Endocrinol 3:97–104.

Kon T, Yamazaki M, Tagawa M, Goto S, Terakado A, Moriya H, Fujimura S. 1997. Bone morphogenetic protein-2 stimulates differentiation of cultured spinal ligament cells from patients with ossification of the posterior longitudinal ligament. Calcif Tissue Int 60: 291–296.

Leboy PS, Vaias L, Uschmann B, Golub E, Adams SL, Pacifici M. 1989. Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. J Biol Chem 264:17281–17286.

Leboy PS, Beresford JN, Devlin C, Owen ME. 1991. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.

Lian JB, Stein GS, Stein JL, van wijnen AJ. 1999. Regulated expression of the bone-specific osteocalcin gene by vitamins and hormones. Vitam Horm 55:443–509.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Ng KW, Manji SS, Young MF, Findlay DM. 1989. Opposing influences of glucocorticoid and retinoic acid on transcriptional control in preosteoblasts. Mol Endocrinol 3:2079–2085.

Nishimoto SK, Salka C, Nimni ME. 1987. Retinoic acid and glucocorticoids enhance the effect of 1,25-dihydroxyvitamin D_3 on bone γ-carboxyglutamic acid protein synthesis by rat osteosarcoma cells. J Bone Miner Res 2:571–577.

Ohishi H, Furukawa K, Iwasaki K, Ueyama K, Okada A, Motomura S, Harata S, Toh S. 2003. Role of prostaglandin I2 in the gene expression induced by mechanical stress in spinal ligament cells derived from patients with ossification of the posterior longitudinal ligament. J Pharmacol Exp Ther 305:818–824.

Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S. 1998. Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet 19:271–273.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. 1990. Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430.

Peck WA, Brandt J, Miller I. 1967. Hydrocortisone-induced inhibition of protein synthesis and asidine incorporation in isolated bone cells in vitro. Proc Natl Acad Sci USA 57:1599–1606.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I. 1994. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 161:218–228.

Sakou T, Matsunaga S, Koga H. 2000. Recent progress in the study of pathogenesis of ossification of the posterior longitudinal ligament. J Orthop Sci 5:310–315.

Shiigi E, Sugiyama T, Tanaka H, Murata H, Shirakura Y, Kawai S. 2001. Possible involvement of vitamin D receptor gene polymorphism in male patients with ossification of spinal ligaments. J Bone Miner Metab 19:308–311.

Shirakura Y, Sugiyama T, Tanaka H, Taguchi T, Kawai S. 2000. Hyperleptinemia in female patients with ossification of spinal ligaments. Biochem Biophys Res Commun 267:752–755.

Stein GS, Lian JB, Owen TA. 1990. Bone cell differentiation: A functionally coupled relationship between expression of cell-growth- and tissue-specific genes. Curr Opin Cell Biol 2:1018–1027.

Tanaka H, Nagai E, Murata H, Tsubone T, Shirakura Y, Sugiyama T, Taguchi T, Kawai S. 2001. Involvement of bone morphogenic protein-2 (BMP-2) in the pathological ossification process of the spinal ligament. Rheumatology 40:1163–1168.

Tanno M, Furukawa KI, Ueyama K, Harata S, Motomura S. 2003. Uniaxial cyclic stretch induces osteogenic differentiation and synthesis of bone morphogenetic proteins of spinal ligament cells derived from patients with ossification of the posterior longitudinal ligaments. Bone 33:475–484.

Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Raducha M, Harris H. 1986. Isolation and characterization of a cDNA encoding a human bone/liver/kidney-type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186.

Wong GL. 1979. Basal activities and hormone responsiveness of osteoblast-like and osteoclast-like bone cells are regulated by glucocorticoids. J Biol Chem 254:6337–6340.

Wong MM, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murray TM. 1990. Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. J Bone Miner Res 5:803–813.

Yamamoto Y, Furukawa K, Ueyama K, Nakanishi T, Takigawa M, Harata S. 2002. Possible roles of CTGF/Hcs24 in the initiation and development of ossification of the posterior longitudinal ligament. Spine 27:1852–1857.

Yonemori K, Imamura T, Ishidou Y, Okano T, Matsunaga S, Yoshida H, Kato M, Sampath TK, Miyazono K, Dijke PT, Sakou T. 1997. Bone morphogenetic protein receptors and activin receptors are highly expressed in ossified ligament tissues of patients with ossification of the posterior longitudinal ligament. Am J Pathol 150:1335–1347.