

Insulin/IGF-1 signals in Zucker fatty rats

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Abstract

【Background】 It is known that the ossification of spinal ligament amalgamates abnormal glucose tolerance at higher rate. In the present study, the involvement of insulin/IGF-1 signals and leptin signals in spinal ligament cells was investigated using Zucker fatty rats (fa/fa) that carry mutation of the leptin receptor gene (fa) and monosodium glutamate-treated (MSG) rats that present obesity due to destruction of the hypothalamic ventromedial nucleus.

【Methods】 The following rats were used : (1) Zucker fatty rats (ZFR group), (2) monosodium glutamate-treated Fa/Fa rats (MSG group), and Fa/Fa rats (control group). Each group consisted of 20 male rats aged 10-12 months. Thoracotomy was performed under general anesthesia. Then the blood was collected, and was subjected to measurement of fasting blood levels of glucose, insulin, IGF-1, and leptin. The thoracic vertebrae were excised, and were embedded in paraffin, stained with hematoxylin eosin (H.E.) and also immunohistologically stained with insulin receptor substrates (IRS)-1 and -2. Immunohistological staining was performed using the LsAB method. The amount of protein was quantified by the Western blot hybridization.

【Results and conclusions】 Rats of the ZFR and MSG groups developed hyperleptinemia and hyperinsulinemia. Histological staining showed that in the ZFR group bulging of the cartilage endplate and destruction of the fibrous ring were accompanied by an increase in the number of chondrocyte-like cells at the ligament attachment site, accompanied by marked hyperplasia of the fibrocartilage. IRS-1-positive cells, IRS-1 protein were eminent by detected in the cartilage endplate and the enthesis region in ZFR group, On the other hand, IRS-2-positive cells were slightly less in the ZFR group than in the MSG and control groups. The results suggest that IRS-1-mediated signaling for cell proliferation was enhanced in ZFR, which may explain the ossification of the posterior longitudinal ligament.

1. Introduction

Ossification of the posterior longitudinal ligament (OPLL) is an intractable disorder in which the ossified spinal ligament compresses the spinal cord and causes serious spinal palsy in the trunk and extremities. It has been reported that OPLL is complicated by many factors such as abnormal glucose tolerance¹⁾, and concomitant obesity²⁾. Furthermore a tendency toward a systemically high bone mass³⁾ has been noted, suggesting the involvement of a certain systemic predisposing factors in

the development of OPLL⁴⁾.

Our laboratory has performed basic studies to elucidate the ossification mechanism of OPLL using Zucker fatty rats (ZFR), as a spinal ligament ossification model with aberration of the leptin receptor gene. The ossification pattern in ZFR is mainly enchondral ossification, in what chondrocyte proliferation and cartilage matrix formation are noted mainly in the spinal ligament attachment site⁵⁾. From the clinical point of view, it has been shown that many patients with OPLL are clinically complicated by abnormal glucose toler-

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ance, while ZFR develop clinical symptoms similar to those of type 2 diabetes. The promotion of cell proliferation and osteogenesis are caused by insulin/insulin-like growth factor-1 (IGF-1) signals, in ZFR which may be associated with ossification of the spinal ligament⁶⁾. Akune et al. also reported that an increase or decrease in the contribution of insulin/IGF-1 signals to osteoblasts is mediated by insulin receptor substrates 1 and 2 (IRS-1 and -2) and an effect on bone-forming ability, clarifying that insulin/IGF-1 signals for osteogenesis are mainly transmitted via IRS-1 and an involved in ossification in OPLL⁷⁾.

Elucidation of the metabolic actions of leptin has also been progressing. Leptin has been reported to be responsible for energy metabolism, such as glucose and lipid metabolism, by increasing insulin sensitivity, and to act on the epiphyseal plate of long bones and vertebral bodies, and its cascade common to insulin has been attracting attention⁸⁾. Moreover leptin actions have been found to be mediated by IRS-2, and the presence or absence of leptin resistance and local differences in insulin/IGF-1 signaling sensitivity in OPLL⁹⁾, which is common among Japanese, are associated with ectopic ossification in the spinal ligaments in a high-insulin environment, therefore we conducted this experiment.

The ZFR have an internal environment that resembles that of so-called obesity syndrome in humans and of type 2 diabetes patients¹⁰⁾, and they are very suitable as experimental animals to use to elucidate OPLL, obesity, and insulin sensitivity. Because many humoral factors may have an ossification-enhancing action, we selectively destroyed the hypothalamic arcuate nucleus of Wistar rats with monosodium-glutamate (MSG)¹¹⁾ to induce hyperleptinemia and hyperinsulinemia, like ZFR because of a postnatal absence of feeding inhibition, and we examined the spinal ligaments using MSG-treated Wistar rats as controls but found no clear evidence of cell proliferation. It suggests that ectopic ossification does not develop because of hyperinsulinemia conditions alone¹²⁾.

Based on these findings, in this study, we investigated the involvement of insulin/IGF-1 and leptin signals in the posterior longitudinal ligament under condition a hyperinsulinemia using ZFR (fa/fa) with the recessive homozygous leptin receptor gene, non-fatty rats (NFR) with dominant homozygous gene (Fa/Fa), and Fa/Fa rats treated with MSG.

2. Materials and Methods

Three groups of animals were used: (1) the ZFR group, which exhibits ligament ossification due to the recessive homozygous leptin receptor gene (fa), ZFR (fa/fa), (2) the NFR group, which carries the dominant homozygous gene, NFR (Fa/Fa), and (3) the MSG

group prepared by subcutaneous administration of MSG (monosodium glutamate, Wako Pure Chemicals, Tokyo) to newborn NFR rats at 4 mg/g body weight for 5 days from the day of birth to destroy the feeding center in the arcuate nucleus of the hypothalamus. All rats were weaned 1 month after birth, and maintained under specified temperature, humidity, and lighting conditions. Twenty males at 10-12 months of age were used in each group. The recessive homozygote (fa/fa) was sterile due to gonadal dysfunction, and was produced by mating with a heterozygote (Fa/fa).

Under inhalation anesthesia with diethyl ether, thoracotomy was performed. Blood was collected from the left ventricle using a syringe, and fasting serum glucose, insulin/IGF-1 and leptin in the blood were measured as described below. After perfusion fixation with 4% paraformaldehyde, the upper thoracic vertebrae were excised, and fixed in 4% paraformaldehyde for 24 hours. The fixed vertebrae were decalcified with 20% EDTA for 3 days and embedded in paraffin. Then the paraffin-embedded thin sagittal cross sections of the spinal ligament attachment site were prepared. The sections were subjected to histopathological examination by hematoxylin eosin (HE) staining and immunohistological staining by the LsAB method using a Histofine SAB-PO kit (Nichirei Corp, Tokyo) and antibodies against insulin receptor substrate (IRS)-1 and -2 as the primary antibody (Santa Cruz Inc. U.S.A.). IRS-1 and -2 positive cells in the spinal ligament attachment site were counted under specified conditions using NIH/Image (National Institute of Health, U.S.A.), and between-group comparison was performed using Student's unpaired t-test. Separately, 20 animals in each group were similarly treated, and the sites of attachment the upper thoracic spinal ligament and intervertebral discs were excised and frozen with liquid nitrogen. After frozen tissues were homogenized in a homogenizer, protein was extracted, and the protein concentration was measured using Bio-Rad Protein Assay Staining Solution (Bio-Rad Laboratories) and standard bovine serum albumin solution. The protein extract was electrophoresed on SDS polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (PVDF membrane, Millipore Corp, U.S.A.) by Western blotting. Using antibodies against IRS-1 and -2 (Santa Cruz Inc, U.S.A.), P13 kinase p85, and ERK42/44 MAP kinase (Cell Signaling Technology Inc, U.S.A.) as the primary antibodies, IRS-1 and IRS-2 were quantified.

The present experiments were performed in accordance with the Tokyo Medical University guidelines for animal experiments (2006).

3. Results

3.1 Phenotypes and blood findings

Obesity was noted from about 3 weeks of age in ZFR,

while a state of being overweight was noted from 2 months of age in the MSG-treated rats, and similar severe obesity was noted at 6 months of age in the 2 groups (Fig. 1). The fasting blood glucose level was

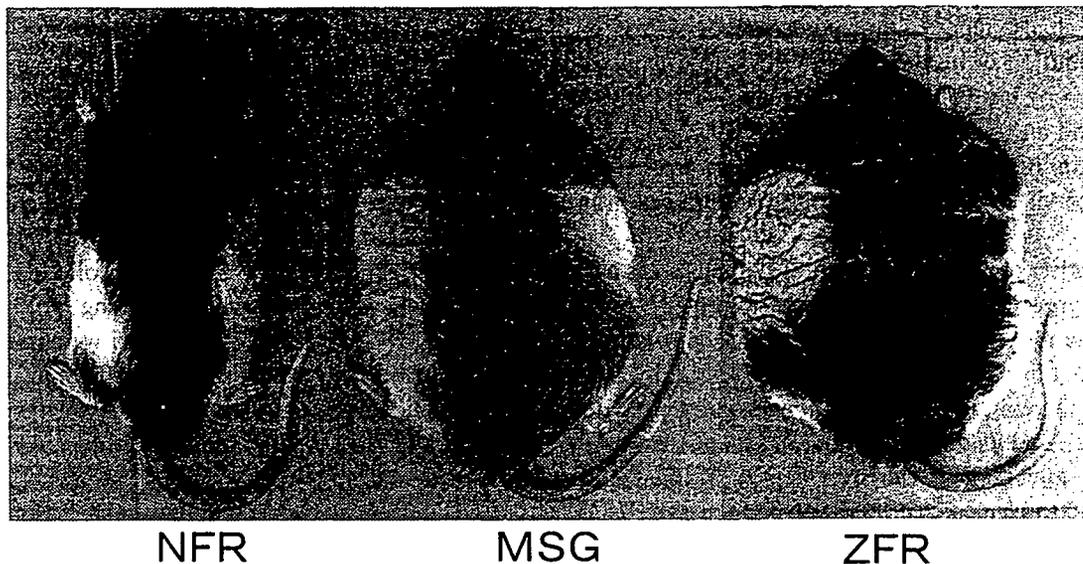


Fig. 1 Outlook of animals used for the present experiments
The phenotypes of the 3 groups including non-fatty rats (NFR group), MSG-treated rats (MSG group), Zucker fatty rat (ZFR group) at 11-12 months as shown. Similar severe obesity was noted at 6 months of age in the ZFR group and the MSG group

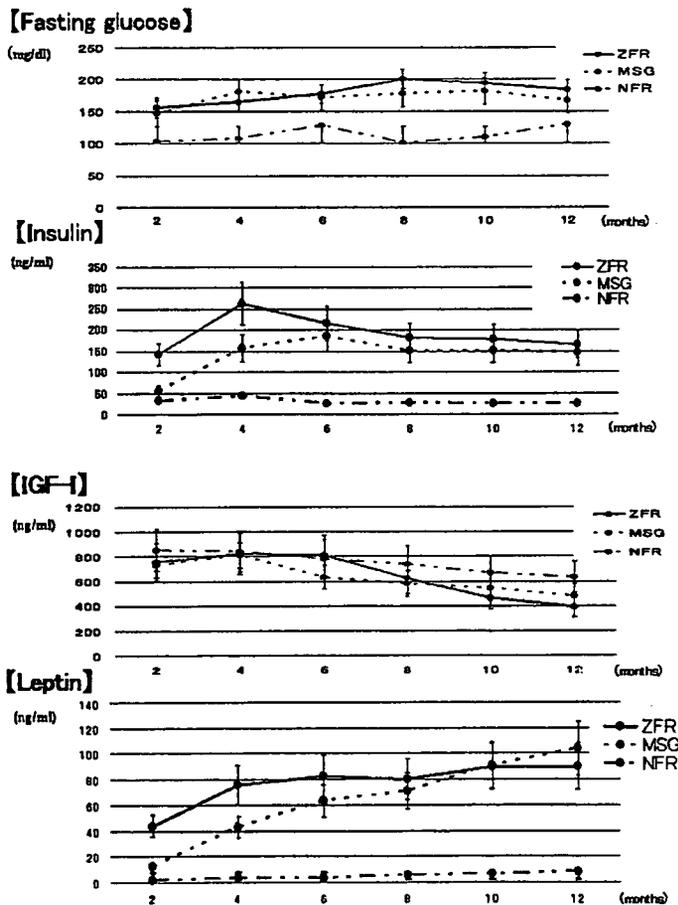


Fig. 2 Blood test results
The fasting blood glucose level was slightly higher in the ZFR and MSG groups than in the non-fatty rats (NFR) group. Both the ZFR group and MSG group exhibited hyperinsulinemia from 6 months age.

slightly higher in the ZFR and MSG groups than in the NFR group. The blood insulin level was about 5 times higher in the ZFR and MSG groups than in the NFR group after 8 months of age. No significant difference was noted in the blood IGF-1 level among the 3 groups, but the blood leptin level was about 10 times higher in the ZFR and MSG groups than in the NFR group after 8 months of age (Fig. 2).

3.2 Pathological and immunohistological findings

The HE-stained sagittal sections of the upper thoracic intervertebral discs in the 3 groups are shown in Fig. 3. In the ZFR group, destruction of the fibrous ring,

degeneration of elastic fibers, and appearance of osteocytes in and calcification of the cartilage end-plate and ligament attachment site were noted. The boundary of the ligament attachment site was unclear, and hypertrophy of the cartilage end-plate and an increased number of enlarged chondrocytes were noted. The fibrous ring structure was maintained in the other 2 groups (Fig. 3).

On immunostaining using anti-IRS-1 antibody, positive cells were noted in the destroyed fibrous ring, ligament attachment site, and enlarged chondrocytes in the cartilage end-plate in the ZFR group (Fig. 4). On

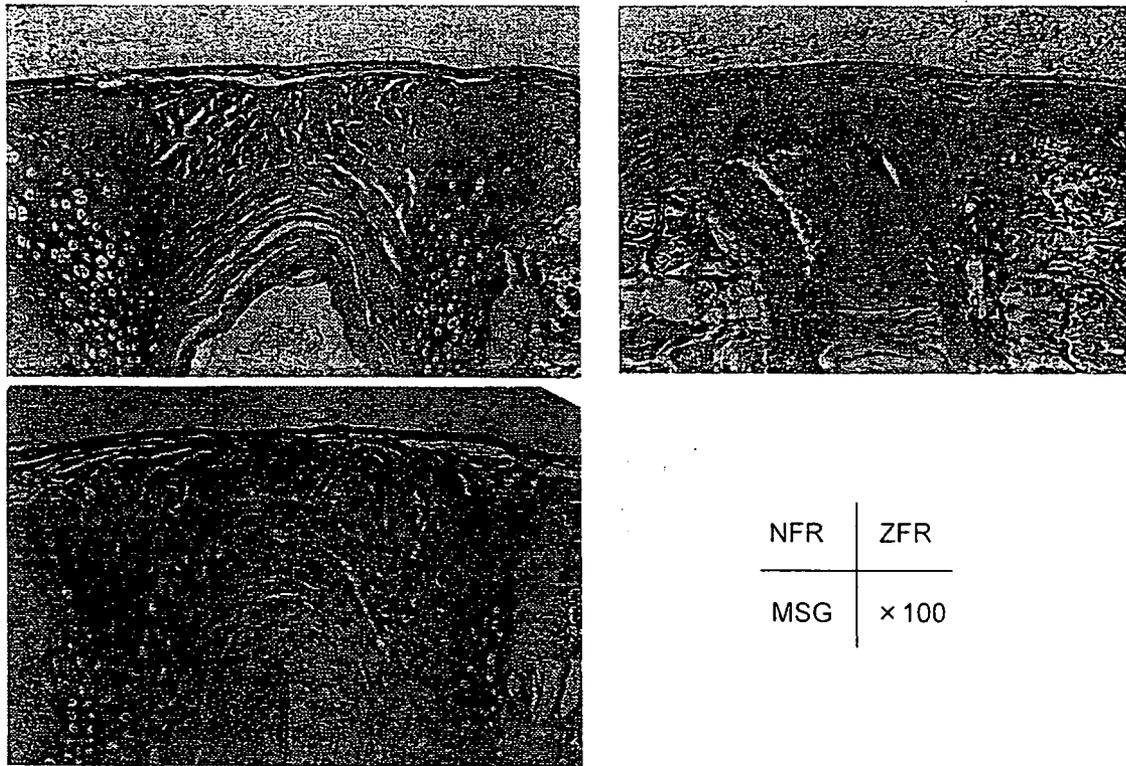


Fig. 3 The hematoxylin eodin (HE) staining sagittal sections of the intervertebral discs in the 3 groups at the 11-12 months. (×100)

In the ZFR group, appearance of osteocytes and calcification of the cartilage end-plate and ligament attachment site were noted.

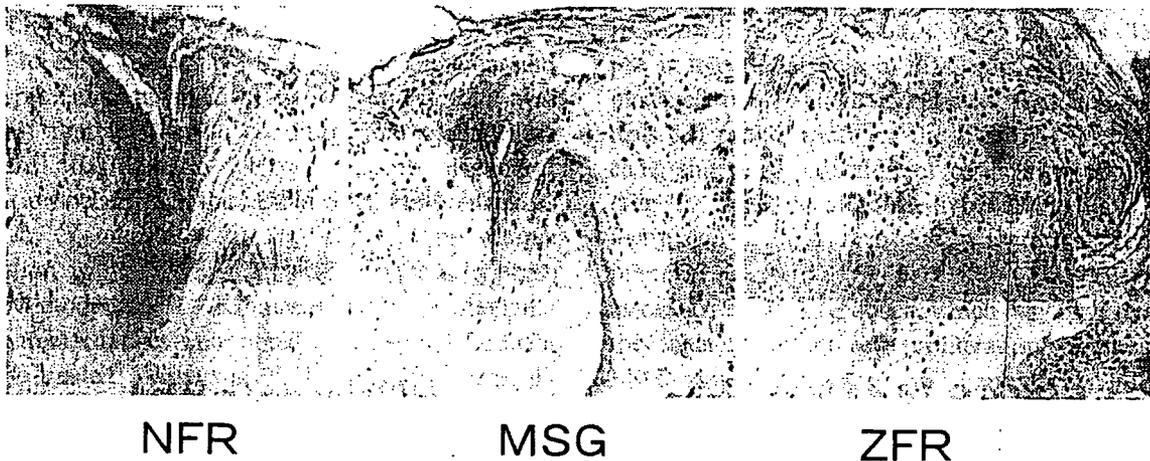


Fig. 4 Immunohistochemical staining with anti-IRS-1 antibody. (×100)

In the ZFR group, positive cells were noted in the destroyed fibrous ring, ligament attachment site.

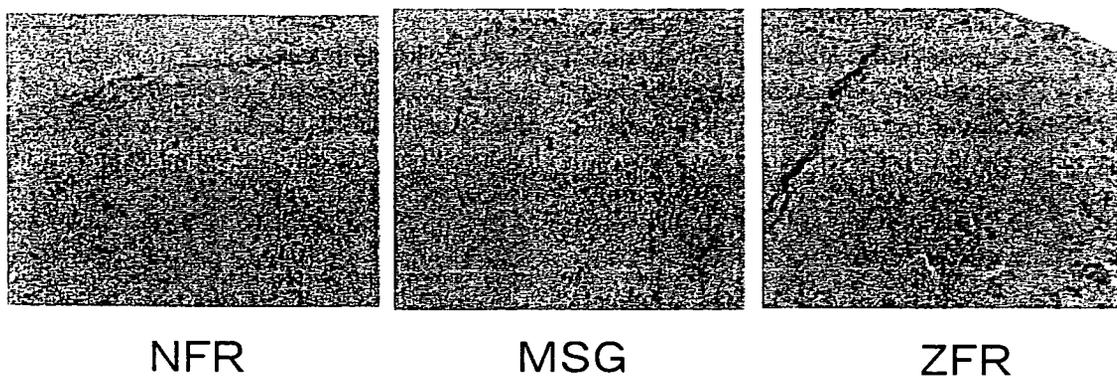


Fig. 5 Immunohistochemical staining with anti-IRS-2 antibody. (×100)
Positive cells were noted in the fibrous ring in the control NFR groups, but decreased in the ZFR group.

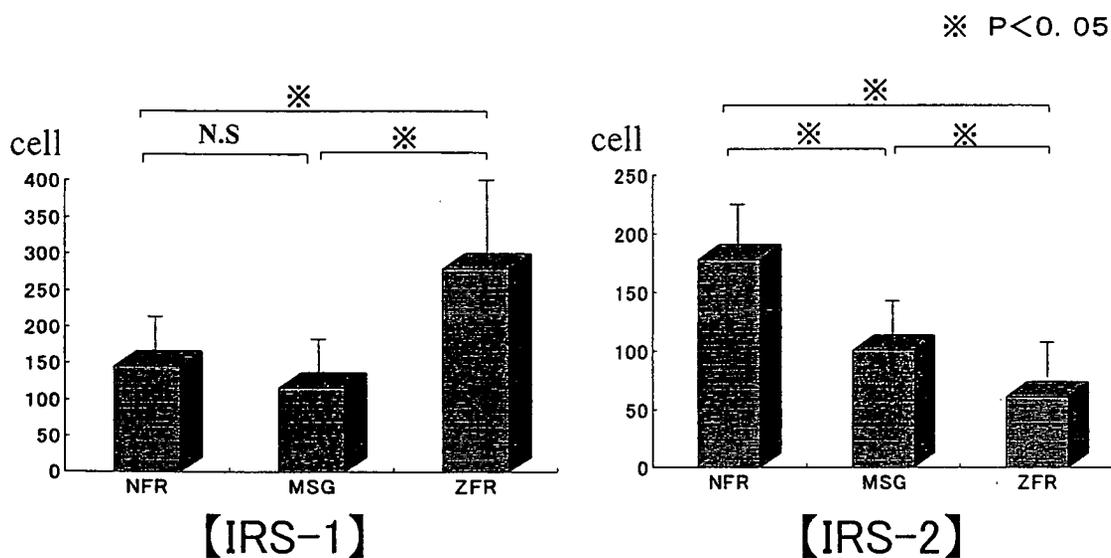


Fig. 6 The number of IRS-1 and IRS-2 Positive cells using NIH/Image.
The number of IRS-1-positive cells was 145.9 ± 43.1 (mean \pm SD) in the NFR group, 15.9 ± 24.9 in the MSG group, 79.8 ± 113.1 in the ZFR group, and significantly increased in the ZFR group compared to the NFR and MSG groups. The number of IRS-2-positive cells was 178.4 ± 48.6 in the NFR group, 0.04 ± 45.1 in the MSG group, 0.6 ± 31.4 in the ZFR group.

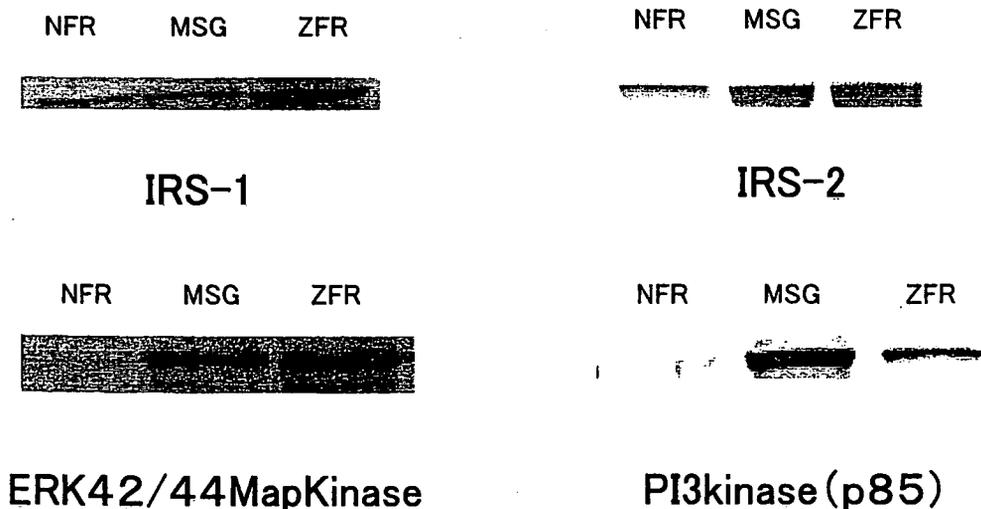


Fig. 7 Western Blot analysis
The IRS-1 and ERK42/44 MAP kinase protein expression level was higher in the ZFR group than in the other groups.

immunostaining using anti-IRS-2 antibody, many positive cells were present in the fibrous ring in the control NFR groups, and a few positive cells were noted partially in the fibrous ring and cartilage end-plate in the MSG group (Fig. 5). Positive cells at the specified location in the ligament attachment site were counted in the 3 groups using NIH/Image, and compared by Student's unpaired t-test. The number of IRS-1-positive cells was significantly increased in the ZFR group compared to the NFR and MSG groups. The number of IRS-2-positive cells was most markedly decreased in the ZFR, group followed by the MSG group, and the numbers in these groups were significantly lower than that in the NFR group. A significant decrease in the number of positive cells was also observed in the ZFR group compared to the MSG group (Fig. 6).

3.3 Western blotting

On Western blotting, the IRS-1 protein expression level was higher in the ZFR group than in the other groups. IRS-2 protein was expressed in all 3 groups, but no significant difference was noted among them. ERK42/44 MAP kinase protein was expressed in the ZFR and MSG groups, and the expression level was higher in the ZFR group. PI₃ kinase protein was expressed at a high level in the MSG group (Fig. 7).

4. Discussion

Insulin/IGF-1 signals then activate downstream signals by phosphorylation of the common IRS, and transmit the signals mainly to the PI₃ kinase system involved in glucose metabolism, such as gluconeogenesis, glycogen synthesis, and fat synthesis, and the MAP kinase system that promotes cell proliferation, playing important roles in blood glucose control and osteogenesis. Insulin/IGF-1 signals with this strong osteogenic action may be increased in hyperinsulinemia, and act on osteoblasts and enlarged chondrocytes in the spinal ligament attachment site, inducing ossification.

In this study, MSG-treated rats developed obesity similar to ZFR shown in Fig. 1, hyperinsulinemia and hyperleptinemia, as in ZFR shown in Fig. 2, it was predicted that it was the condition that could cause ligament ossification. However, no ligament ossification occurred in the MSG-treated rats prepared as in ZFR. Yamazaki et al. reported that no ectopic ossification was noted in the spinal ligament in obese NIDDM model rats¹³⁾. Kimura et al. also reported that no apparent changes were noted in the spinal ligament in MSG-treated Wistar rats¹²⁾, showing that hyperinsulinemia alone is insufficient to induce ectopic ossification. In our experiment, no ossification of spinal ligament cells was noted in MSG-treated rats, in which hyperinsulinemia was present but a leptin recep-

tor abnormality was absent, whereas fibrous ring destruction and an increased number of enlarged chondrocytes in the ligament attachment site, which may be the prestep to ligament ossification, were noted in ZFR, suggesting that the difference in the sensitivity of cells in the spinal ligament to leptin signals in ZFR had a certain influence on insulin/IGF-1 signals (Fig. 3).

ZFR maintained by our laboratory was a mutant discovered by Zucker¹⁴⁾. It develops hyperglycemia, hyperinsulinemia, and obesity due to abnormal leptin receptors, and is known as a ligament ossification model as well as a type 2 diabetes model. Leptin is a hormone secreted by mast cells, which controls food ingestion and energy consumption via leptin receptors in the hypothalamus. Leptin resistance induces obesity, and decreases insulin sensitivity. Araki et al. clarified using IRS-2 knockout mice that food ingestion was controlled by leptin in the hypothalamus via IRS-2, and leptin was also associated with the peripheral IRS-2-mediated insulin regulatory action¹⁵⁾, suggesting that IRS-1 and -2 are closely involved in ossification in OPLL, and leptin signals are also involved in the ossification through IRS.

The immunohistological investigation detected increases in the number of IRS-1-positive cells (Fig. 4, 6) and IRS-1 and ERK42/44 MAP kinase protein expression levels mainly in the spinal ligament in ZFR (Fig. 7), suggesting that insulin/IGF-1 signals were increased, particularly the cell proliferative action. In contrast, the number of IRS-2-positive cells (Fig. 5, 6) and PI₃ kinase protein expression level were increased in the MSG-treated rats (Fig. 7), suggesting that leptin signals were transmitted via IRS-2, and then acted on the downstream of the IRS-1 cascade involved in cell proliferation, regulating ossification.

In the spinal ligament in MSG-treated animals, insulin/IGF-1 signals are responsible mainly for the metabolic action after PI₃ kinase p85 and cell proliferation following MAP kinase via the IRS-1-mediated cascade. The signals tend to increase in hyperinsulinemia, and the IRS-2-mediated leptin action further activates PI3 kinase p85 in hyperleptinemia in MSG rats, which acts on the increased IRS-1 signals as negative feedback, and controls cell proliferation via the IRS-1/MAP kinase cascade, and thus, induction of ossification is less likely (Fig. 8). In ZFR, although hyperleptinemia is present, leptin signals are lacking due to abnormal leptin receptor function, reducing the PI₃ kinase p85 activation-associated negative feedback, which may have resulted in the enhancement of cell proliferation via the IRS-1/MAP kinase cascade and subsequent ligament ossification (Fig. 9). The difference in the ligament cell sensitivity through leptin receptors in the presence of similar degrees of hyperinsulinemia and hyperleptinemia may affect ossification.

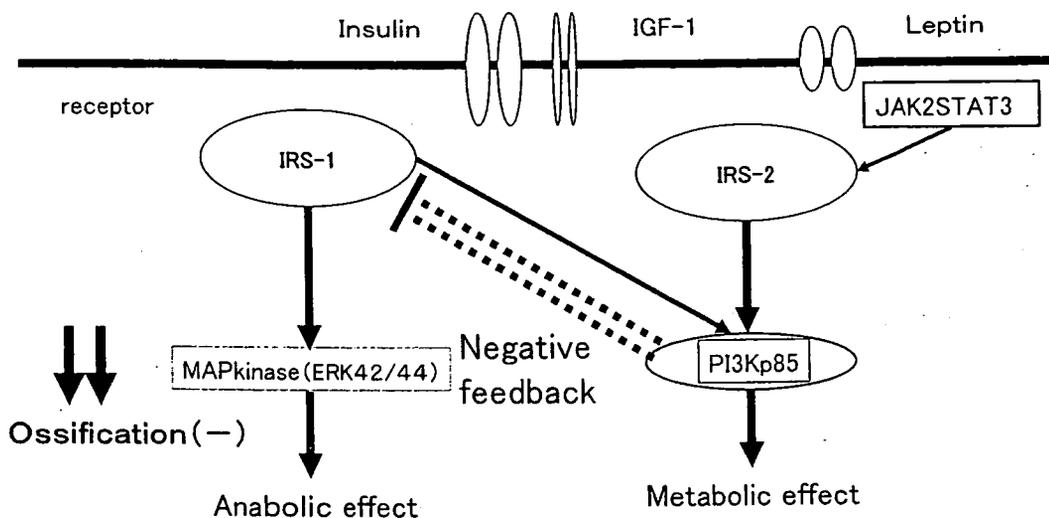


Fig. 8 Insulin-IGF-1 Signals in MSG Rat
 In MSG rats the signals control cell proliferation via the IRS-1/MAP kinase cascade, and thus, induction of ossification is less likely.

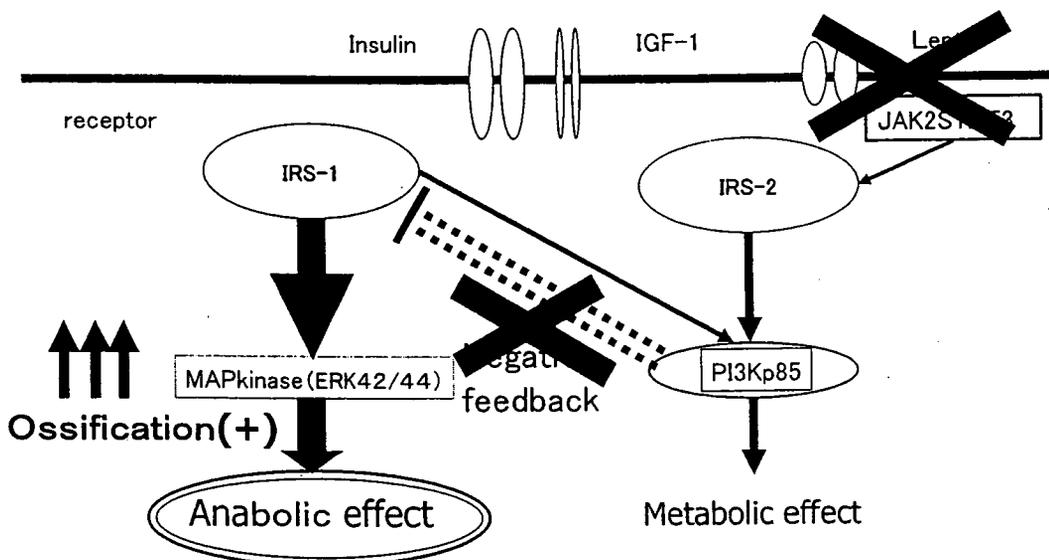


Fig. 9 Insulin-IGF-1 Signals in ZFR
 In the spinal ligament in ZFR, leptin signals are lacking due to abnormal leptin receptor function, reducing negative feedback, which may have resulted in the enhancement of cell proliferation via the IRS-1/MAP kinase cascade and subsequent ligament ossification.

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References

- 1) Shingyouchi Y, Nagahama A, Niida M: Ligamentous ossification of the cervical spine in the late middle-aged Japanese men; its relation to body mass index and glucose metabolism. *Spine* 21: 2474-2478, 1996
- 2) Takeuchi Y, Matumoto T, Takuwa Y, Hoshino Y, Kurokawa T, Shibuya N et al.: High incidence of obesity and elevated serum immunoreactive insulin level in patients with paravertebral ligamentous

- ossification ; a relationship to the development of ectopic ossification. *J Bone Miner Metab* 7: 17-21, 1989
- 3) Mamada T, Nakamura K, Hoshino Y, Saito K, Kurokawa T : Bone mineral density in patients with ossification of the posterior longitudinal ligament ; Minimal decrease of bone mineral density with aging. *Spine* 22 : 2388-2392, 1997
 - 4) Kawaguchi H, Kurokawa T, Kodama Y, Matsumoto T : Metabolic background of ossification of the posterior longitudinal ligament.in *OPLL ; Ossification of Posterior Longitudinal Ligament*. (Eds) Yonenobu K, Sakou T and Ono O. Springer-Verag, Tokyo, 73-77, 1997
 - 5) Miura Y, Tanaka S, Hikone R : Ossification of spinal ligament in Zucker fatty rats (in Japanese with English abstract). *Orthopedics* 44: 1107-1113, 1993
 - 6) Yamamoto K, Imakiire A, Nemoto T, Machida H : Ossification of spinal ligament in Zucker fatty rats (in Japanese with English abstract). *THE BONE* 16: 225-233, 2002
 - 7) Akune T, Ogata N, Hoshi K, Kubota N, Terauchi Y, Tobe K et al. : Insulin receptor substrate-2 maintains predominance of anabolic function over catabolic function of osteoblasts. *J Cell Biol* 159 : 147-156, 2002
 - 8) Zong CS, Chan J, Levy DE, Horvath C, Sadowski HB, Wang LH : Mechanism of STAT 3 activation by insulin-like growth factor I receptor. *J Biol Chem* 275 : 15099-15105, 2000
 - 9) Hirashima Y, Tsuruzoe K, Kodama S, Igata M, Toyonaga T, Ueki K : Insulin down-regulates insulin receptor substrate-2 expression through the phosphatidylinositol 3-kinase/Akt pathway. *J Endocrinol* 179 : 253-266, 2003
 - 10) Ahmad I, Steggle AW, Carrillo AJ, Finkelstein JA : Developmental changes in levels of growth hormone mRNA in Zucker rats. *J Cell Biochem* 43 : 59-66, 1990
 - 11) Olney, JW : Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* 164 : 719-721, 1969
 - 12) Kimura D, Yamamoto K, Machida H : Expression of ossification-related factors in Zucker hereditary obese rats and monosodium glutamate-induced obese rats (in Japanese with English abstract). *Tokyo Medical University Journal* 62 : 253-260, 2004
 - 13) Yamazaki M, Ogiwara Y, Aida Y, Nakajima A, Shimizu S, Ookawa A et al. : Analysis of spinal ligament tissue in a rat osteogenic (bone resorption factors) obese NIDDM model (OLETF) (in Japanese with English abstract). 2000 Report of the Ministry of Health, Labour and Welfare Specified Disease Countermeasure Study. Survey concerning ossification of posterior longitudinal ligament : 92-94, 2001
 - 14) Zucker LM, Antoniades HN : Insulin and obesity in the Zucker genetically obese rat 'fatty'. *Endocrinology* 90 : 1320-1330, 1972
 - 15) Araki E, Yoshimi K, Tsuruzoe S : Energy equilibration and control of female reproductive function by IRS-2 (in Japanese with English abstract). *Endocrinology/Diabetes* 13 : 269-272, 2001

Zucker fatty rat 脊柱靱帯骨化に対するレプチンの影響

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【要旨と結論】 レプチン受容体機能異常を認める Zucker fatty rat (以下 ZFR) は交感神経活動低下を呈する脊柱靱帯骨化自然発生モデルである。レプチンは骨形成・骨吸収の制御機能を有し骨量の制御を行っているが、レプチンの脊柱靱帯骨化に対する影響は明らかではない。今回、ZFR 脊柱靱帯を中心にレプチン受容体 (以下 Ob-R) および $\beta 2$ アドレナリン受容体 (以下 $\beta 2AR$) の発現を調べ、レプチンの脊柱靱帯骨化に対する影響を検討した。ZFR の脊柱靱帯骨化前段階において脊柱靱帯付着部や椎間板辺縁を中心に Ob-R の発現を有意に認めた。また、同部位を中心に $\beta 2AR$ の発現も認めた。ZFR の Ob-R は機能異常によりその役割を果たしていないことから、ZFR 靱帯骨化にはレプチンによる直接作用の関与は少ないと考えられた。また、ZFR は視床下部の Ob-R 機能異常により交感神経活動が低下しているが、 $\beta 2AR$ の発現を認めたことから ZFR は交感神経刺激により脊柱靱帯骨化が抑制できることが示唆された。

はじめに

後縦靱帯骨化症 (ossification of the posterior longitudinal ligament、以下 OPLL) は脊椎の後縦靱帯が骨化する疾患であり、1975年に厚生省より難病に指定され、厚生労働省特定疾患調査研究班により調査・研究が進められている。OPLLは脊柱における力学的負荷などの局所因子や、糖脂質代謝異常・肥満などの環境的因子が原因となる多因子疾患であると考えられている¹⁾。最近では Collagen 6A1 遺伝子異常が日本人の OPLL 患者に認められることが報告され²⁾、OPLLは遺伝的背景に局所因子・環境因子などの影響が加わることによって発生する疾患であるとも考えられている。

当教室ではレプチン受容体機能異常を有する ZFR を用いて OPLL の発生原因解明などを目的とし継続的に研究を行っている。これまでの当教室の研究により ZFR は肥満・過食・性腺機能異常・交感神経活動低

下を呈し、生後 14 か月齢で 23.8% に脊柱靱帯の骨化が発生することが明らかになっている³⁾。ZFR の靱帯骨化にレプチン受容体機能異常が影響していることが考えられるがその詳細は明らかでない。靱帯骨化のメカニズムを明らかにするために、レプチンに着目し実験を行うこととした。

レプチンは、著明な肥満を呈する ob/ob mouse の原因遺伝子として 1994 年に positional cloning により同定された 167 個のアミノ酸からなる分泌タンパクである⁴⁾。レプチンは主に脂肪細胞から生産され、視床下部において神経ペプチドを介して食欲を抑制すると同時に、末梢組織に直接作用してエネルギー産生を増加させることで、体内のエネルギー蓄積を減少させる肥満や体重増加の制御に関与する抗肥満ホルモンである⁵⁾⁶⁾。近年、肥満の抑制という作用以外にも造血、胎児の成長、生殖機能の調節などの多彩な生理的作用を有していることが明らかになっている。その中で、ob/ob mouse に性差なく骨量の増加を認めることが報告

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されてから、レプチンの骨代謝調節作用が注目されている⁷⁾。レプチンは視床下部に豊富に存在する Ob-R へ結合することで交感神経活動を調節することが明らかになり、交感神経系は主に骨芽細胞上の $\beta 2AR$ に作用して、Receptor activator NF- κ B Ligand (以下 RANKL) の発現を誘導することで破骨細胞形成を調節することなどで骨形成・骨吸収を制御していると言われている⁸⁾。ZFR における交換神経活動の低下は視床下部における Ob-R の機能異常によるものと考えられており⁹⁾、レプチンの視床下部を介した間接作用が ZFR の靱帯骨化に何らかの影響を与えていると考えられる。

今回、レプチンの骨形成・骨吸収の制御機能が骨量の制御だけでなく、ZFR 脊柱靱帯骨化にも影響を与えている可能性が高いと考え、ZFR 脊柱靱帯におけるレプチンの影響を病理組織学的・免疫組織化学的に検討を行った。ZFR は月齢 10 か月から 12 か月の間に骨化前段階様の変化を脊柱靱帯に認めることが報告されている⁹⁾ことから、月齢 10 か月から 12 か月のラットを対象として実験を行った。

脊柱靱帯骨化を認める ZFR における脊柱靱帯の Ob-R の発現を明らかにすることで、Ob-R を介したレプチンの靱帯骨化への直接作用を検討した。また、レプチンの交感神経系を介した骨代謝調節に $\beta 2AR$ が重要な役割を果たしていることから⁸⁾、脊柱靱帯における $\beta 2AR$ の発現を明らかにし、 $\beta 2AR$ を介した骨形成調節作用が靱帯骨化に与える影響を検討した。

研究材料および方法

1. 実験動物

本実験では表現系に異常を有さない Fa/Fa Rat・Non fatty rat (以下 NFR)、NFR に生後 5 日間、体重 1 kg 当たり 4 mg の Monosodium glutamate を皮下投与した Monosodium glutamate 処置ラット (以下 MSG)、およびレプチン受容体のミスセンス変異によりレプチン受容体機能異常を有する fa/fa rat・Zucker fatty rat (ZFR) をそれぞれ使用した。

各群、月齢 2 か月から 12 か月のラットをそれぞれ 20 匹使用して実験を行った。

ZFR はレプチン受容体機能異常から過食・肥満・耐糖能異常および交感神経活動低下を認める脊柱靱帯骨化自然発生モデルラットであるため、対象として表現系が正常である NFR と、視床下部弓状核を破壊し摂食中枢異常を呈する MSG を作製した。

2. 検討項目

1) 血中レプチン値の測定

月齢 2 か月から 2 か月ごとに 12 か月まで、全身麻酔後に開胸右心室から採血を施行した。血中のレプチン濃度を測定した。

2) 体重測定

月齢 2 か月から 12 か月まで 1 か月毎に体重測定を施行した。

3) 血中レプチン濃度を測定した後、全身麻酔下に 4%パラホルム還流固定施行し、上位胸椎を摘出した。48 時間固定後エタノールを用い 48 時間脱脂を行い、20%EDTA で 2 週間脱灰施行した。上位胸椎矢状断パラフィン薄切片を作製し、ヘマトキシリン・エオジン染色 (以下 HE 染色) を施行した。

4) 免疫組織化学染色

Labeled streptavidin-biotin (LSAB) 法にて抗レプチン受容体 (Ob-R) 抗体【ObRb、Santa cruz 社】、抗 $\beta 2$ アドレナリン受容体 ($\beta 2AR$) 抗体【 $\beta 2AR$ 、Santa cruz 社】を使用し免疫組織化学染色を施行した。第 5 胸椎を中心とした上位胸椎矢状断パラフィン切片を使用した。脱パラフィン及び親水化を施行の後、抗原賦活処理は Microwave method を用い 95°C にて 12 分間施行した。内因性ペルオキシダーゼのブロッキングにはニチレイ社ブロッキング試薬を用いた。1 次抗体・2 次抗体には同社 Max-PO set を使用し、Ob-R 抗体は 200 倍希釈、 $\beta 2AR$ 抗体は 100 倍希釈と設定し、反応時間は 8 時間 (Over-night) とした。発色基質溶液は DAB Tris を使用し、封入剤を用い封入・固定を施行した。

月齢 10 か月から 12 か月の矢状断パラフィン切片を使用し、第 5 胸椎後方の脊柱靱帯および靱帯付着部・椎間板辺縁における陽性細胞の発現とその局在を検討し、陽性細胞数を椎体内陽性細胞を除外して測定した。測定には NIH imaging を用いた。陽性細胞数の平均値はマンホイットニー U 検定を用いて統計処理を施行した。

結 果

1) 血中レプチン値は ZFR が 2 か月より他の 2 群より高くなったが、12 か月にて NFR 5.7 ± 3.5 ng/ml、MSG 70.9 ± 9.2 ng/ml、ZFR 80.2 ± 8.1 ng/ml と MSG、ZFR が近似した血中濃度となった (Fig. 1)。

2) 体重は月齢 12 か月にて MSG 789.5 ± 17.5 g、ZFR 808.1 ± 28.2 g と明らかな高値となり、NFR

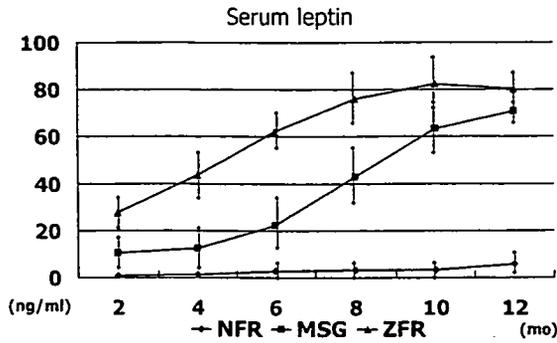


Fig. 1 Body weight measurement
The body weight markedly increased in ZFR and MSG compared to NFR with no phenotypic abnormality. At 10-12 months of age, at which the experiments were performed, the body weights of ZFR and MSG were similar.

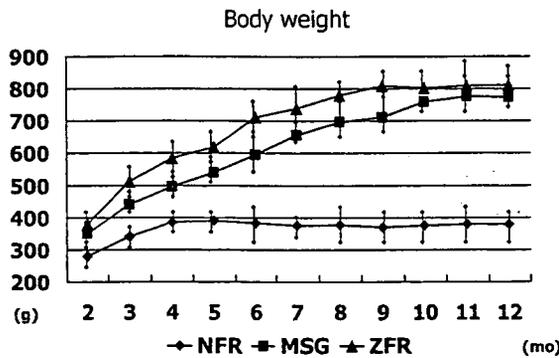


Fig. 2 Blood leptin level
The blood leptin levels at 12 months of age in ZFR and MSG were similar, but significantly higher than that in NFR.

382.5±25.2 g と比べて体重増加が著明であった (Fig. 2)。

3) HE 染色 (第5胸椎)

第5胸椎後方にて NFR では明らかな異常所見を認めないが、MSG は椎体隅角部に軽度の後方膨隆と靭帯成分の変性を認めた。ZFR は椎体隅角部における骨性隆起と、椎間板の後方への膨隆を認めた。その膨隆は他の2群と比較して明らかであった。また、同部位の靭帯の変性は著明であり、その付着部を中心に骨芽細胞様細胞の増殖を認めた (Fig. 3)。

4) 免疫組織化学染色

〈Ob-R〉

NFR ではほとんど陽性細胞の発現を認めないが、MSG では椎間板辺縁から靭帯付着部にかけて少数の陽性細胞の発現を認めた。ZFR は椎間板辺縁から椎体隅角部の靭帯付着部にかけて陽性細胞の発現を多く認めた (Fig. 4)。

椎体内を除外して陽性細胞数を測定すると ZFR は NFR および MSG と比べ陽性細胞数が有意 ($P < 0.01$) に多かった (Fig. 5)。

〈β2AR〉

NFR ではほとんど陽性細胞の発現を認めない。MSG では椎体隅角部に軽度の陽性細胞の発現を認めるのみであったが、ZFR では椎間板辺縁から靭帯付着部を中心に陽性細胞の発現を著明に認めた (Fig. 6)。

同様に陽性細胞数を測定すると ZFR が NFR、MSG と比べ陽性細胞数が有意 ($P < 0.01$) に多かった

NFR MSG ZFR

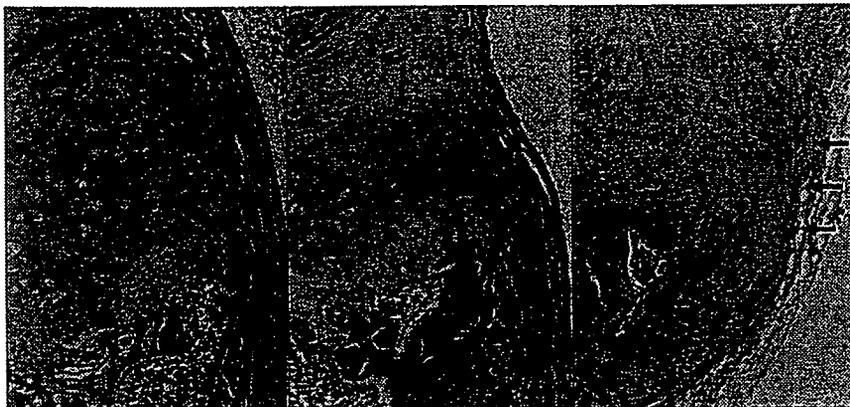


Fig. 3 HE staining (Th5) 12 months ×100
In ZFR, the posterior intervertebral disc margins were bulged (arrow), and proliferation of osteoblast-like cells was noted, mainly in the posterior margin of the vertebral body over the ligament attachment site (arrowhead). Mild cell proliferation was noted in the same region in MSG, but not in NFR.

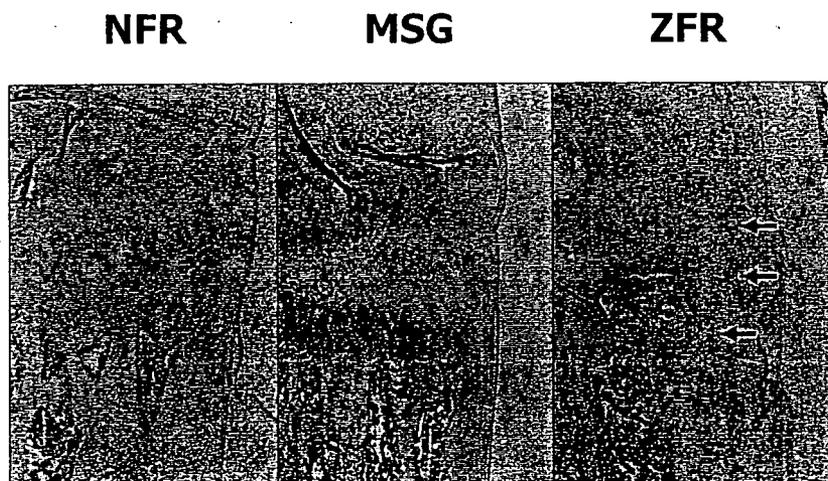


Fig. 4 Ob-R immunohistochemical staining 12 months ×50
 Many positive cells with the stained cytoplasm (arrow) were noted in the intervertebral disc margins over the ligament attachment site in ZFR. Mild cell proliferation was noted in the same region in MSG.

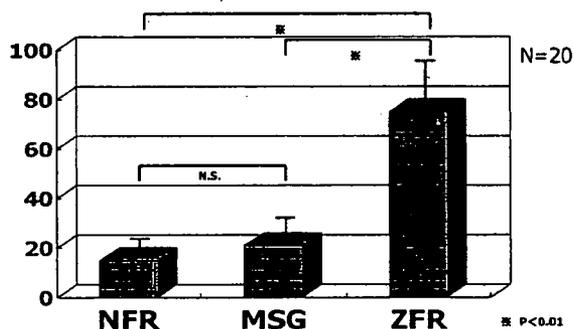


Fig. 5 Ob-R positive cell count
 Ob-R positive cells were counted using NIH-Imaging. The positive cell count was significantly higher in ZFR than in the other 2 groups. No significant difference was noted between NFR and MSG.

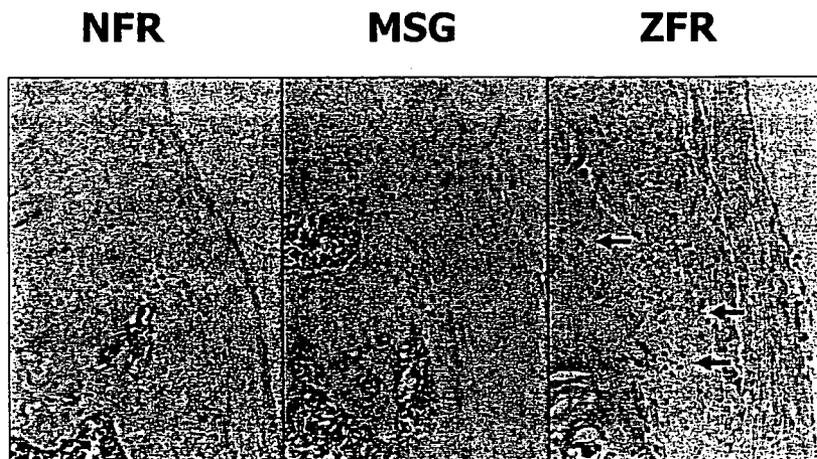


Fig. 6 β 2AR immunohistochemical staining 12 months ×100
 In ZFR, many positive cells with the stained cytoplasm (arrow) were noted mainly in the intervertebral disc margins over the ligament attachment site. In MSG, fewer positive cells were noted in the same region.

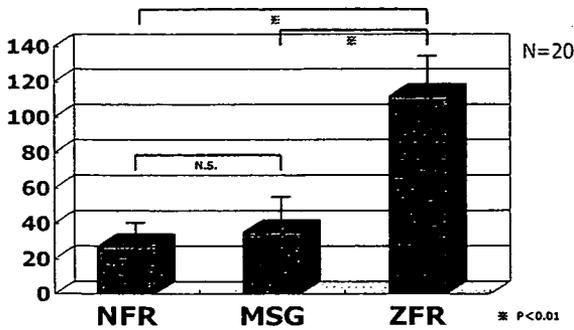


Fig. 7 β 2AR positive cell count
The β 2AR positive cell count was significantly higher in ZFR than in the other 2 groups. No significant difference was noted between NFR and MSG. The tendency was similar to that of the Ob-R positive cell count.

(Fig. 7).

考 察

レプチンは視床下部弓状核のレプチン受容体に作用して摂食抑制の調節を行うとともに、視床下部腹内側核に作用して交感神経系の活動を亢進させることによって骨形成を調節していることが報告されている⁷⁾。また、交感神経系の活動により骨芽細胞上の β 2ARを介した骨形成・骨吸収の制御が行われていることから、交感神経系の影響を検討するには肥満や高レプチン血症が骨代謝に与える影響を除外しなければならない。

今回、対象動物として表現系に異常を示さない Fa/Fa rat と、Fa/Fa rat に Monosodium glutamate 処置を施行し視床下部弓状核を破壊し、摂食中枢抑制機能が低下し肥満を呈する MSG rat を作製した。MSG rat は fa/fa rat に類似した肥満や高血糖を呈するため、交感神経を介した骨代謝作用の影響をより明確に検討することが可能であると考えた⁹⁾。実験結果にて MSG は ZFR とほぼ同等の体重を示し、血中レプチン濃度も月齢 12 か月にてほぼ同等の数値であったため、MSG は対象動物としては適していたと考えられた。ZFR の HE 染色で認めた椎体隅角部の骨形成や椎間板の後方膨隆、靭帯付着部を中心とした骨芽細胞様細胞の増殖は靭帯骨化発生の前段階であると考えられている³⁾。過去の報告にて MSG 処置ラットは ZFR にみられる脊柱靭帯骨化を認めておらず¹⁰⁾、今回の実験においても MSG には脊柱靭帯・靭帯付着部の変化や骨芽細胞様細胞の増殖をほとんど認めなかった。しかし、NFR と比較すると脊柱靭帯・靭帯付着部に軽度の変性を認めている。これは体重増加によるメカニカル

ストレスの増大によるものが主であると考えられた¹¹⁾¹²⁾。しかし、ヒトにおいて女性肥満患者に限り高レプチン血症と OPLL の相関があることが報告されていることから¹³⁾¹⁴⁾ 高レプチン血症が影響も関与している可能性も考えられた。

免疫組織化学染色にて ZFR の椎間板辺縁や脊柱靭帯に Ob-R の発現を有意に認めているが、レプチン受容体機能異常を認める ZFR において Ob-R の発現が有意に多かったのは、靭帯付着部や変性した椎間板辺縁の増殖した細胞がレプチン受容体をもつ骨芽細胞や肥大軟骨細胞様の性質⁷⁾ をもっていたためであると考えられる。レプチンの骨組織への直接作用は in vitro で骨芽細胞の分化を亢進させることや、in vivo では破骨細胞の形成を抑制すること¹⁵⁾ など骨化促進に働く可能性が高いとされている。しかし今回の結果から Ob-R の発現はあるものの ZFR の Ob-R は機能異常によりその役割を十分に果たしていないことから、ZFR の靭帯骨化にはレプチンによる直接作用が多く関与していないことが示唆された。近年、レプチンの骨組織への直接作用は中枢神経を介したレプチンの間接作用と比較すると影響は少ないと考えられており¹⁶⁾、Ob-R を介したレプチンの直接作用は強力な作用がないと考えられた。当教室の研究からもレプチンが Insulin receptor protein substrate (IRS)1・2 を介した細胞増殖に間接的に関与していることが報告されており¹⁷⁾、今回の結果はレプチンによる作用は間接的なものが主であることを支持するものであった。

ZFR の脊柱靭帯や椎間板辺縁における β 2AR の発現は Ob-R 同様に著明に多く認めていた。ZFR は交感神経活動が低下しているが、Ob-R とは異なり β 2AR の機能異常は認めない¹⁸⁾。脊柱靭帯および付着部における受容体の発現は交感神経活動低下による反応性の増加とも考えられ¹⁹⁾²⁰⁾、靭帯骨化前段階において β 2AR が発現していることが確認できた。 β 2AR は骨芽細胞上でイソプロテノール刺激により Protein kinase A を介して骨芽細胞特異的な転写因子である ATF4²¹⁾ をリン酸化させ RANKL の転写を促進させることが報告されており、交感神経系が骨芽細胞に作用し RANKL の発現を誘導することで破骨細胞形成を促進しているとされている⁸⁾ (Fig. 8)。ZFR では交感神経活動が低下していることから、破骨細胞からの RANKL の発現が誘導されず骨形成が骨吸収を上回ること骨化が進行していることが推測された。今回の結果にて交感神経活動が低下している ZFR 脊柱靭

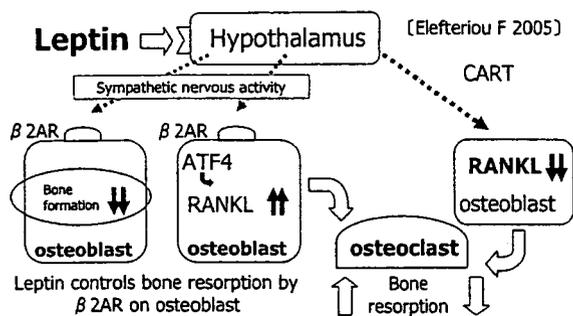


Fig. 8 Elevation of sympathetic nerve activity by the action of leptin on the hypothalamus induced expression of receptor activator NF- κ B ligand (RANKL) via β 2 adrenergic receptors, by which the differentiation and action of osteoclasts were controlled.

帯における β 2AR の発現を確認できたことで、交感神経刺激により、ZFR における脊柱靱帯骨化は抑制できる可能性があると考えられた。

しかし、ZFR の靱帯骨化は IRS1-2 を介した細胞増殖作用やメカニカルストレスにより発生しているとの報告もあり¹⁷⁾、骨化発生・進展のメカニズムは交感神経系の活動低下のみでなく多因子の影響を受けていると考えられる。この交感神経活動の低下が骨化進展に与える影響の程度は不明であり、今後検討する必要がある。また、骨化過程における破骨細胞形成の抑制により靱帯骨化が進展する可能性は大きいですが、靱帯骨化の発生初期には関わっていないため、ZFR のレプチン受容体機能異常による交感神経活動低下は骨化の進展のみに関与していると考えられる。

β アドレナリン受容体刺激薬の投与が靱帯骨化の進展を予防することが可能であれば、OPLL の進展防止薬として使用できることが期待される。今後、ZFR において β アドレナリン刺激により靱帯骨化の進展を防止できるか検討すべきである。また、 β 2 アドレナリン受容体拮抗薬がヒトの骨量を増加させ、骨折の危険率を 30% 減少させることが報告されており²²⁾²³⁾、骨粗鬆症に対し治療薬として使用されることが予想される。しかし、OPLL などの骨増殖病変を持つ患者に対する β アドレナリン受容体拮抗薬の投与は骨化を進展させる危険性があり注意が必要と思われる。今後、交感神経活動低下が靱帯骨化に与える影響のメカニズムとその程度を明らかにしていく必要がある。

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文 献

- 1) Sakou T, Matsunaga S, Koga H: Recent progress in the study of pathogenesis of ossification of the posterior longitudinal ligament. *J Orthop Sci* 5: 310-315, 2000
- 2) Tsukahara S, Miyazawa N, Akagawa H, Forejtova S, Pavelka K, Tanaka T, Toh S, Tajima A, Akiyama I, Inoue I: COL6A1, the candidate gene for ossification of the posterior longitudinal ligament, is associated with diffuse idiopathic skeletal hyperostosis in Japanese. *Spine* 30(20): 2321-2324, 2005
- 3) Yamamoto K, Imakiire A, Shishido T, Masaoka T: Effects of ethane-1-hydroxy-1, 1-diphosphonate on ossification of the posterior longitudinal ligament in Zucker fatty rats. *J Orthop Surg* 12(1): 45-54, 2004
- 4) Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372(6505): 425-432, 1994
- 5) Halaas JL, Gajiwara KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269(5223): 543-546, 1995
- 6) Friedman JM, Halaas JL: Leptin and the regulation of the body weight in mammals. *Nature* 395(6704): 763-770, 1998
- 7) Ducey P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G: Leptin inhibits bone formation through a hypothalamic relay: central control of bone mass. *Cell* 100(2): 197-207, 2000
- 8) Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, Noda M, Clement K, Vaisse C, Karsenty G: Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* 434(7302): 514-520, 2005
- 9) Onley JW: Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* 164: 719-721, 1969
- 10) 木村 大、山本謙吾、町田英明: Zucker 遺伝性肥満ラットおよび Monosodium Glutamate 投与肥満ラットにおける骨化関連因子の発現 脊柱靱帯における免疫組織学的検討。東京医大誌 62: 253-260, 2004
- 11) Furukawa K: Current topics in pharmacological research on bone metabolism: molecular basis of ectopic bone formation induced by mechanical stress. *J Pharmacol Sci* 100(3): 201-204, 2006
- 12) Tanno M, Furukawa K, Ueyama K, Harata S, Motomura S: Uniaxial cyclic stretch induced osteogenic differentiation and synthesis of bone

- morphogenetic proteins of spinal ligament cells derived from patients with ossification of posterior longitudinal ligaments. *Bone* **33**(4): 475-484, 2003
- 13) Shirakura Y, Sigiya T, Tanaka H, Taguchi T, Kawai S: Hyperleptinemia in female patients with ossification of spinal ligaments. *Biochem Biophys Res Commun* **267**(3): 752-755, 2000
- 14) Tahara M, Aida A, Yamazaki M, Ikeda Y, Goto S, Moriya H, Okawa A: The extent of ossification of posterior longitudinal ligament of the spine associated with nucleotide pyrophosphatase gene and leptin receptor gene polymorphisms. *Spine* **30**(8): 877-880, 2005
- 15) Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL: Leptin acts on human marrow stromal cell to enhance. *Endocrinology* **140**(4): 1630-1638, 1999
- 16) Burguera B, Hofbauer LC, Thomas T, Gori F, Evans GL, Khosla S, Riggs BL, Turner RT: Leptin reduces ovariectomy-induced bone loss in rats. *Endocrinology* **142**(8): 3546-3553, 2001
- 17) Kubo K, Yamamoto K, Watanabe A, Kimura D: Ossification in leptin-resistant rat. *東京医大誌* **64**: 395-406, 2006
- 18) Mory G, Wiel M, Adli H, Duppy FD, Ferre P, Bazin R: Impaired beta-adrenergic signaling pathway in white adipocytes of suckling fa/fa Zucker rats: a defect in receptor coupling. *Int J Obes Relat Metab Disord* **25**(11): 1592-1598, 2001
- 19) Lipworth BJ: Antagonism of long-acting beta2-adrenoceptor agonism. *Br J Clin Pharmacol* **54**(3): 231-245, 2002
- 20) Jockers R, Angers S, Silva AD, Benaroch P, Strosberg D, Bouvier M, Marullo S: Beta2-adrenergic receptor down-regulation. Evidence for pathway that does not require endocytosis. *J Biol Chem* **274**(41): 28900-28908, 1999
- 21) Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-corsi P, Townes TM, Hanauer A, Karsenty G: ATF4 is a substrate of RSK2 and essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117**(3): 387-398, 2004
- 22) Pasco JA, Henry MJ, Sanders KM, Kotowicz MA, Seeman E, Nicholson GC: Beta-adrenergic blocker reduce the risk of fracture partly by increasing bone mineral density: Geelong Osteoporosis Study. *J Bone Miner Res* **19**(1): 19-24, 2004
- 23) Shlienger RG, Kraenzlin ME, Jick SS, Meier CR: Use of beta-blocker and risk of fracture. *JAMA* **292**(11): 1326-1332, 2004

Effect of leptin on ossification of the spinal ligament in Zucker fatty rats

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Abstract

[Background] Zucker fatty rats (ZFR), that have a with functional abnormality of leptin receptors are a spontaneous model of ossification of the posterior longitudinal ligament that develops sympathetic nerve hypoactivity. Leptin has recently been reported to control bone mass by regulating bone formation and resorption, suggesting that it affects not only bone mass but also ossification of the spinal ligament. Thus, we investigated the effects of leptin on the spinal ligament in ZFR histopathologically and immunohistochemically.

[Materials and Methods] ZFR with functional abnormality of leptin receptors and rats treated with monosodium glutamate (MSG), which selectively destroys the arcuate nucleus of the hypothalamus, were used. Thin spinal sections were prepared, and immunohistochemically stained using antibodies against leptin receptor (Ob-R) and β 2-adrenergic receptor (β 2AR).

[Results] Significant expression of Ob-R was noted in the central region of the attachment site of the spinal ligament in ZFR. β 2AR was also expressed as markedly as Ob-R in the spinal ligament in ZFR, confirming that β 2AR was expressed in the stage preceding ligament ossification.

[Conclusions] Since Ob-R does not play any role due to functional abnormality in ZFR, the direct involvement of leptin in ligament ossification may be slight in ZFR. β 2AR expression in the stage preceding ligament ossification was confirmed, suggesting that ossification of the spinal ligament may be inhibited by sympathetic nerve stimulation in ZFR. If administration of β -stimulators prevents the advancement of ligament ossification, they can be expected as OPLL-preventive drugs.

<Key words> OPLL: Ossification of the posterior longitudinal ligament, Leptin, β 2-adrenergic receptor, Zucker fatty-rat, Spinal ossification

Immunohistochemical Demonstration of Advanced Glycation End Products and the Effects of Advanced Glycation End Products in Ossified Ligament Tissues *In Vitro*

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Study Design. This study correlates advanced glycation end products with ossified ligament tissues of the cervical spine *in vitro*.

Objective. To investigate the effect of advanced glycation end products on ossification of the spinal ligaments *in vitro*.

Summary of Background Data. We have hypothesized that an accumulation of advanced glycation end products in the spinal ligament might result in some observable change in specific growth factors responsible for ossification in the spinal ligaments.

Methods. Samples of the posterior longitudinal and yellow ligaments were harvested from patients (n = 5) with ossification of the posterior longitudinal ligament, and analyzed for the presence of advanced glycation end products and their receptor advanced glycation end product receptor by immunohistochemistry. Real-time polymerase chain reaction (PCR) was used to quantify the messenger ribonucleic acid (mRNA) levels of bone morphogenetic protein (BMP)-2, BMP-7, alkaline phosphatase, an osteoblast-specific transcription factor 1 (Cbfa1), and osteocalcin from yellow ligament cells treated with advanced glycation end products.

Results. Immunohistochemical analysis revealed that advanced glycation end products and advanced glycation end product receptor were localized to within the posterior longitudinal and yellow ligaments. Advanced glycation end products were found to increase significantly the expression of BMP-2, BMP-7, Cbfa1, and osteocalcin at the mRNA levels after treatment with advanced glycation end products (1 µg/mL).

Conclusions. This is the first report to investigate the correlation, if any, between the ossified spinal ligament and advanced glycation end products. These results suggested that accumulation in advanced glycation end products and

their interaction with advanced glycation end product receptor were 1 of the important risk factors in the process of ossification in the spinal ligaments.

Key words: advanced glycation end products, advanced glycation end product receptor, glucose intolerance, ossification of posterior longitudinal ligament, yellow ligament, bone morphogenetic protein, Cbfa1, osteocalcin. *Spine* 2007;32:E337-E339

Advanced glycation end products result from nonenzymatic glycation or the spontaneous reaction of reducing sugars with proteins (the Maillard reaction).^{1,2} Studies have reported that high accumulation in advanced glycation end products leads to dystrophic mineralization, calcification, and osteoblastic differentiation^{3,4} after advanced glycation end products reacted with advanced glycation end product receptor.⁵

One hypothesis is that the accumulation of advanced glycation end products in the spinal ligaments (posterior longitudinal and yellow ligaments) may result in an increase in specific growth factors and, thus, contribute to ossification of the spinal ligaments. Here, the authors evaluated how advanced glycation end product accumulation influenced the biologic function of spinal ligaments in an *in vitro* setting.

Materials and Methods

Cervical spine specimens of yellow ligament were obtained from 5 patients with ossification of the posterior longitudinal ligament during spinal surgery with the informed consent of family members and approval of the Kurume University Ethics Committee (Table 1). Yellow ligament cells were extracted from the yellow ligament and used for cell cultures and PCR. Both yellow and posterior longitudinal ligament specimens underwent histologic evaluations.

Cell Harvest and Culture. Yellow ligament tissues were isolated and cultured using Dulbecco modified Eagle medium⁶⁻⁸; the presence of chondrocyte-like cells was confirmed with routine microscopy.

Histologic Study. Histologic specimens were embedded in paraffin. Immunohistochemical analysis was used to detect human advanced glycation end product and advanced glycation end product receptor. The Envision method was used for anti-advanced glycation end products (code No. KH001; 1:125 dilution; Transgenic Corp., Kumamoto, Japan) and anti-

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The device(s)/drug(s) that is/are the subject of the manuscript is/are not intended for human use.

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Table 1. Profile of Patients With Ossification of the Posterior Longitudinal Ligament

Patient No.	Sex/Age (yrs)	Diagnosis	Region of Yellow Ligament Tissue
1	F/60	Th-OPLL	C5/6
2	F/48	C-OPLL	C5/6
3	M/50	C-OPLL	C5/6
4	F/71	C-OPLL	C5/6
5	M/51	C-OPLL	C5/6

C indicates cervical spine; F, female; M, male; OPLL = ossification of posterior longitudinal ligament; Th, thoracic spine.

advanced glycation end product receptor (lot No. HKH02; 1:500 dilution; Techno Corp., Minneapolis, MN). Mouse immunoglobulin G (Dako, Carpinteria, CA) was adjusted to the concentrations of the primary antibodies and used as the negative control.^{6,8}

Preparation of Advanced Glycation End Products. We used glyceraldehyde-derived advanced glycation end products that had previously shown various biologic activities through interaction with the advanced glycation end product receptor.⁹

Quantitative Assay for mRNAs. Yellow ligament cells were stimulated in serum-free medium with 1 $\mu\text{g}/\text{mL}$ advanced glycation end products, or nonglycated bovine serum albumin (control) for 6 days at 37°C, changing the medium every 2 days. RNAs were isolated (conditions of reverse transcription were 25°C for 10 minutes, 45°C for 30 minutes, and 95°C for 5 minutes) and quantified by real-time PCR (Table 2), as described previously.^{6,7} The volume of mRNA was measured and normalized by β -actin using an internal standard according to the δ -delta-computed tomography method.¹⁰

Statistical Analysis. The data were compared using the Student *t* test. The results were recognized as statistically significant at a probability (*P*) level <0.05.

■ Results

Immunohistochemical Localization

Yellow ligament and posterior longitudinal ligament tissue from ossification of the posterior longitudinal ligament patients showed diffused staining for advanced glycation end product receptor, with strongly labeled focal areas around the circumference of the cell surface and

nuclear membrane. In addition, these tissues showed staining for advanced glycation end products with strongly labeled focal areas in the extracellular matrix and at the margins of the tissue cleft. No staining was apparent in the negative control. Similar results were seen in the tissues from all donors.

Expression Levels of BMP-2, BMP-7, Alkaline Phosphatase, Cbfa1, and Osteocalcin mRNAs

The mRNA expression levels in cultured yellow ligament cells were determined after 6 days of treatment with 1 $\mu\text{g}/\text{mL}$ advanced glycation end products. In the presence of 1 $\mu\text{g}/\text{mL}$ advanced glycation end products, the mRNA expression level in BMP-2, BMP-7, Cbfa1, and osteocalcin, was increased significantly to 137.1% ($P < 0.01$), 172.7% ($P < 0.01$), 135.3% ($P < 0.01$), and 172.4% ($P < 0.05$), respectively (compared to the untreated control). The expression levels of alkaline phosphatase did not differ significantly from the untreated control levels.

■ Discussion

This study documented the presence of advanced glycation end products and advanced glycation end product receptor in ossified spinal ligaments. Additionally demonstrated was that advanced glycation end products significantly increased the expressions of BMP-2, BMP-7, osteocalcin, and Cbfa1 in the yellow ligament cells from ossification of the posterior longitudinal ligament patients at mRNA levels. We have postulated that advanced glycation end products contribute to the development of ossification in the spinal ligaments. Yamagishi *et al*³ have reported that advanced glycation end products showed an ability to induce osteoblastic differentiation in pericytes. Moreover, Kume *et al*⁴ have reported that dystrophic mineralization may occur in advanced glycation end product-treated human mesenchymal stem cells.

The pathogenesis of ossification of the posterior longitudinal ligament includes mechanical stress, genetic factors, glucose intolerance, and obesity.^{11,12} Ossification of the posterior longitudinal ligament cells have a greater potential to differentiate into osteogenic cells than ligament cells from non-ossification of the posterior

Table 2. Primers Used for Amplification

Gene	Primer Sequence (5'→3')	Product Size (bp)	Reference
BMP-2	Sense: GTGGAATTGACTGGATTGTGGCT Antisense: GGACACAGCATGCCTTAGGAAT	161	Accession No. NM001200
BMP-7	Sense: CACGCTACCACCATCGAGAGTT Antisense: TGGAGCACCTGATAAACGCTG	151	Accession No. NM001719
Osteocalcin	Sense: AGCCTTTGTGTCCAAGCAGGA Antisense: TCACAGTCCGGATTGAGCTCA	141	Accession No. NM199173 NM000711
Alkaline phosphatase	Sense: ACCTCGTTGACACCTGGAAGA Antisense: ACGTTGTTCTGTTCCAGCTCG	160	Accession No. NM000478
Cbfa1	Sense: TCTTCCCAAAGCCAGAGTGGA Antisense: AATAGCGTGCTGCCATTCCGAG	164	Accession No. AF001450
β -actin	Sense: TCATCACCATTGGCAATGAG Antisense: CACTGTGTTGGCTACAGGT	125	JOR21(2003) No. 256–264

longitudinal ligament patients, and respond to various factors such as BMP-2, osteocalcin, and Cbfa1, which have the potential to induce osteogenic differentiation and/or osteoblast differentiation in spinal ligament cells.¹³⁻¹⁵ Accordingly, these results suggested that advanced glycation end products binding to advanced glycation end product receptor may be a causative factor for the onset and progression of ossification in the spinal ligaments.

■ Key Points

- This study correlates advanced glycation end products with ossified ligament tissues of the cervical spine *in vitro*.
- Advanced glycation end products were found to increase significantly the expression of BMP-2, BMP-7, Cbfa1, and osteocalcin.
- These results suggested that accumulation in advanced glycation end products and their interaction with advanced glycation end product receptor were 1 of the important risk factors in the process of ossification in the spinal ligaments.

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References

1. Thorpe SR, Baynes JW. Role of the Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* 1996;9:69-77.
2. DeGroot J. The AGE of the matrix: Chemistry, consequence and cure. *Curr Opin Pharmacol* 2004;4:301-5.
3. Yamagishi S, Fujimori H, Yonekura H, et al. Advanced glycation endproducts accelerate calcification in microvascular pericytes. *Biochem Biophys Res Commun* 1999;258:353-7.
4. Kume S, Kato S, Yamagishi S, et al. Advanced glycation end-products attenuate human mesenchymal stem cells and prevent cognate differentiation into adipose tissue, cartilage, and bone. *J Bone Miner Res* 2005;20:1647-58.
5. Loeser RF, Yammani RR, Carlson CS, et al. Articular chondrocytes express the receptor for advanced glycation end products: Potential role in osteoarthritis. *Arthritis Rheum* 2005;52:2376-85.
6. Yokosuka K, Park JS, Jimbo K, et al. Advanced glycation end-products downregulating intervertebral disc cell production of proteoglycans *in vitro*. *J Neurosurg Spine* 2006;5:324-9.
7. Jimbo K, Park JS, Yokosuka K, et al. Positive feedback loop of interleukin-1beta upregulating production of inflammatory mediators in human intervertebral disc cells *in vitro*. *J Neurosurg Spine* 2005;2:589-95.
8. Kanazawa T, Soejima T, Murakami H, et al. An immunohistological study of the integration at the bone-tendon interface after reconstruction of the anterior cruciate ligament in rabbits. *J Bone Joint Surg Br* 2006;88:682-7.
9. Takeuchi M, Yamagishi S. TAGE (toxic AGEs) hypothesis in various chronic diseases. *Med Hypotheses* 2004;63:449-52.
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.
11. Iwasaki K, Furukawa KI, Tanno M, et al. Uni-axial cyclic stretch induces Cbfa1 expression in spinal ligament cells derived from patients with ossification of the posterior longitudinal ligament. *Calcif Tissue Int* 2004;74:448-57.
12. Shingyouchi Y, Nagahama A, Niida M. Ligamentous ossification of the cervical spine in the late middle-aged Japanese men. Its relation to body mass index and glucose metabolism. *Spine* 1996;21:2474-8.
13. Song J, Mizuno J, Hashizume Y, et al. Immunohistochemistry of symptomatic hypertrophy of the posterior longitudinal ligament with special reference to ligamentous ossification. *Spinal Cord* 2006;44:576-81.
14. Kon T, Yamazaki M, Tagawa M, et al. Bone morphogenetic protein-2 stimulates differentiation of cultured spinal ligament cells from patients with ossification of the posterior longitudinal ligament. *Calcif Tissue Int* 1997;60:291-6.
15. Yonemori K, Imamura T, Ishidou Y, et al. 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* 1997;150:1335-47.

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後頭骨再建術の問題点の検討

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The clinical problem of the occipitocervical fixation.

by

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Key words: occipitocervical fixation (後頭頸椎固定術), atlantoaxial subluxation (環軸椎亜脱臼), complication (合併症)

はじめに

後頭頸椎再建術は上位頸椎の不安定性を要する病態に対し外固定の簡略化，早期離床を可能にするよい術式であるが問題点もある。今回われわれは過去5年間に経験した症例から問題点を検討したので報告する。

対象および方法

対象は2001年1月から2005年12月まで当科で後頭頸椎再建術を施行した11例（男7例，女4例）である。年齢は平均52歳であった。疾患は環軸椎亜脱臼が9例，頸椎腫瘍が1例，歯突起偽関節が1例であった。罹病期間は平均8.2か月，経過観察期間は19.1か月であった。

当科では朴ら¹⁾が報告したように整復不可能な不安定性を有する環軸椎亜脱臼には後頭頸椎再

建術を施行している。また環軸椎亜脱臼で整復可能であっても解剖学的に環軸椎固定術が困難な症例にも後頭頸椎再建術を施行した。

これら11例に対して出血量と手術時間，除圧範囲と固定範囲，頸椎アンカーの種類，術後の症状・神経学的所見の予後，術後合併症について調査した。

結 果

出血量，手術時間，除圧範囲

出血量は平均252.2g (25~495g)，手術時間は平均248.9分 (175~325分) であった。除圧範囲は後頭下減圧術のみ行ったものが1例，後頭下減圧術に中下位頸椎の椎弓の処置を加えたものが9例，前方から除圧を施行したものが1例あった (表1)。

表1 除圧範囲

・ 後頭下減圧術	:1例
・ 後頭下減圧術+C1後弓切除	:6例
・ 後頭下減圧術+C1C2椎弓切除	:1例
・ 後頭下減圧術+C1C3椎弓切除	
+C2C4椎弓形成術	:1例
・ 後頭下減圧術+C1, C4-7椎弓切除	:1例
・ C2/3部分椎弓切除+前方除圧(C2-4)	:1例