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Research Article

Increased Apoptosis, Expression of Matrix Degrading Enzymes and Inflammatory Cytokines of Annulus Fibrosus Cells in Genetically Engineered Diabetic Rats: Implication for Intervertebral Disc Degeneration

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Summary

Objective: To investigate the effect of diabetes mellitus (DM) on apoptosis, expression of matrix degrading enzymes and inflammatory cytokines of intervertebral disc cells in genetically engineered OLETF (diabetic) and LETO (control) rats.
Methods: Lumbar disc tissues were obtained from 6-month old OLETF and LETO rats (10 each). We examined the annulus fibrosus (AF) using TUNEL, Western blotting, reverse transcription polymerase chain reaction, and histological analysis. The expression of matrix degrading enzymes, Fas (apoptosis-related protein) and inflammatory cytokines of AF cells were evaluated by semiquantitative analysis of densitometry.
Results: OLETF rats showed increased body weight and abnormal 2-hour glucose tolerance tests compared to LETO rats. The apoptosis index and the degree of expression of Fas of AF cells were statistically higher in the OLETF rats. The degree of expression of matrix metalloproteinase-1, -2, -3 and -13 and tissue inhibitor of metalloproteinase-1 and -2 was statistically higher in the OLETF rats. The expression of interleukin-1 and -6 and tumor necrosis factor-α was statistically higher in the OLETF rats. Finally, histological analysis showed more severe fibrosis and loss of lamellar pattern in AF tissues of OLETF rats.
Conclusion: Our results suggest that diabetes mellitus is associated with increased apoptosis and expression of matrix degrading enzymes and inflammatory cytokines in AF cells. This results in more severe fibrosis and loss of lamellar pattern of AF, which leads to intervertebral disc degeneration. Strict blood glucose control might be important to delay or prevent early intervertebral disc degeneration in patients with DM.

Key words: Diabetes mellitus, Apoptosis, Matrix degrading enzymes, Inflammatory cytokines, Intervertebral disc degeneration

Genetik Olarak Değiştirilmiş Diyabetik Sıçanların Annulus Fibrozis Hücrelerinde Artmış Apoptoz, Matriks Azaltıcı Enzim Ekspresyonu ve İnflamatuvar Sitokinler: İntervertebral Disk Dejenerasyonu için Göstergeler

Özet

Amaç: Diyabetes Mellitus' un (DM) genetik olarak değiştirilmiş OLETF (diyabetik) ve LETO (kontrol) sıçanlarının intervertebral disk hücrelerinde apoptoza, matriks azaltıcı enzimlerin ve inflamatuvar sitokinlerin ekspresyonuna etkisini araştırmak.
Yöntem: Altı aylık OLETF ve LETO sıçanlarından lomber disk dokusu elde edildi (her birinden 10 adet). TUNEL, Western blot, zıt transkriptaz polimeraz zincir reaksiyonu ve histolojik analiz kullanarak anulus fibrozisi (AF) değerlendirdik. Yarı kantitatif densitometre analizi ile AF hücrelerindeki matriks azaltıcı enzimler, Fas (apoptoz ilişkili protein) ve inflamatuvar sitokinler değerlendirildi.

Bulgular: OLETF sıçanları LETO sıçanlarına göre artmış vücut ağırlığı ve anormal 2 saatlik glukoz tolerans testi gösterdiler. Apopotoz indeksi ve AF hücrelerinde Fas ekspresyon derecesi OLETF sıçanlarında istatistiksel olarak daha fazlaydı. Matriks metaloproteinaz 1-2-3-13 ve doku metaloproteinaz 1-2 inhibitörü OLETF sıçanlarında istatistiksel olarak daha fazlaydı. OLETF sıçanlarında interlökin 1-6 ve tümör nekrotizan faktör alfa istatistiksel olarak daha fazlaydı. Son olarak histolojik analiz OLETF sıçanlarının AF dokularında daha fazla fibroz ve lamellar kayıp gösterdi.
Sonuç: Sonuçlarımız diyabetes mellitusun AF hücrelerinde apoptoz, matriks azaltıcı enzimlerin ekspresyonu ve inflamatuar sitokinler ile artma ile ilişkili olduğunu düşündürmüştür. Bu durum intervertebral disk dejenerasyonuna yol açan artmış ciddi fibroz ve AF'de lamellar paternin kaybı ile ilişkildir. Ciddi kan glukoz kontrolü DM tanılı hastalarda erken intervertebral disk dejenerasyonunun önlenmesinde ya da geciktirilmesinde önemli olabilir.

Anahtar Kelimeler: Diyabetes mellitus, apoptoz, matriks azaltıcı enzimler, inflamatuvar sitokinler, intervertebral disk dejenerasyonu

INTRODUCTION

Diabetes mellitus (DM) is a chronic and lifelong condition that affects multiple human organs. If left untreated, DM causes many complications such as stroke, coronary artery disease, chronic kidney failure, and damage to the eyes and nervous system.[1,8] DM is considered a risk factor for development of intervertebral disc degeneration. Spinal stenosis and degenerative disc diseases are more prevalent in diabetic patients than in non-diabetic individuals.[2,7,9,18] To date, development of intervertebral disc degeneration has been reported to be associated with several biochemical factors such as apoptosis, matrix degrading enzymes and inflammatory cytokines. Excessive apoptosis of intervertebral disc cells has been suggested as a potential cause of intervertebral disc degeneration.[13,20,21] There are two kinds of matrix degrading enzymes: matrix metalloproteinases (MMPs) and their tissue inhibitor of metalloproteinases (TIMPs).[3,5,11,14,19] Increased expression of MMPs leads to extensive destruction of extracellular matrix of intervertebral disc. In addition, increased expression of TIMPs is implicated in fibrosis of destructed extracellular matrix of intervertebral disc, which results in intervertebral disc degeneration. Inflammatory cytokines, such as interleukin (IL)-1 and -6 and tumor necrosis factor-α (TNF-α), are known to be involved in catabolic processes of degenerative changes of human organs.[4,6,17] However, despite such potential association of DM and intervertebral disc degeneration, what is little known is whether or how DM affects apoptosis and expression of matrix degrading enzymes and inflammatory cytokines of intervertebral disc cells.

The intervertebral disc is a highly specialized structure that consists of inner gelatinous nucleus pulposus (NP) and outer lamellar annulus fibrosus (AF). In contrast to chondrocytic phenotype of NP cells, AF cells are fibroblastic and are phenotypically different. In addition, there is a significant difference in the composition of extracellular matrix produced by NP and AF cells.[15,16] Despite being from the same family of intervertebral disc cells, NP and AF cells are not biochemically the same nor are they morphologically similar. Most of the previous studies have focused on NP cells and tissues to investigate intervertebral disc degeneration and not on AF ones. From our literature review, little information is available about the effect of DM on apoptosis and expression of matrix

degrading enzymes and inflammatory cytokines of AF cells and tissues. Therefore, we performed the current study to investigate these issues.

MATERIAL AND METHODS

Twenty male 1-month-old rats (OLETF and LETO, 10 each) were kindly provided by the Tokushima Research Institute (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) and kept under controlled temperature (23 ± 3°C) and humidity (55± 5%) with a 12-hour light/12-hour dark cycle (light period, 7:00 am–7:00 pm) in the laboratory animal center. The rats were fed standard solid rat chow and had distilled and de-ionized drinking water ad libitum. The rats were treated in accordance with the ethical guidelines of our institution, and the Institutional Review Board (IRB) approved the experimental protocol.

The rats were weighed weekly, and glucose tolerance tests were performed monthly. The results were presented as mean ± SD (standard deviation). Twenty rats (10 OLETF and 10 LETO) were sacrificed at 6 months of age and lumbar disc tissues (L1-2 through L5-6), including cranial and caudal cartilaginous endplates, were obtained. Half of the specimens were fixed in paraformaldehyde, decalcified, dehydrated, and embedded in paraffin for histological analyses and the remaining specimens were used for Western blotting. For hematoxylin-eosin and Masson trichrome stains and in situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay, 4-μm-thick midsagittal sections of the lumbar disc tissues were cut on a microtome and mounted on poly-L-lysine-coated slides.

The TUNEL assay for apoptosis AF cells

To detect apoptosis of AF cells, TUNEL assays were performed using a TACS 2 TdT DAB in situ apoptosis detection kit (Trevigen, Gaithersburg, USA) according to the manufacturer's instructions. The lumbar discs paraffin sections were deparaffinized in xylene, rehydrated, and treated with proteinase K. Endogenous peroxidase was removed with 2% H2O2. Human tonsil tissue was used as a positive control. The total number of AF cells and the total number of TUNEL-positive AF cells were counted in 10 randomly selected high-power fields (x200). To reduce error, counting was performed twice by two pathologists, and the average from 4 countings was used as the final count. The apoptosis index of AF cells was the number of TUNEL-positive cells/total number of cells x 100% and was presented as mean ± SD (standard deviation).

Western blotting for expression of MMP-1, -2, -3, and -13, TIMP-1, -2, and as

AF tissues were homogenized and lysed. After centrifugation, the supernatants were obtained. Quantification of protein was performed with the Bradford method using a protein assay kit (Bio-Rad laboratories, Hercules, CA, USA) and reading was done at 595 nm by spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, United Kingdom). The protein was loaded onto gels for electrophoresis. The protein concentration of the lysed NP tissues was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Bio-Rad 500-0006, Pierce Chemical Company, Waltham, MA, USA). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, St. Quentin, France). Antibodies specific to MMP-1 (Lifespan Biosciences, Seattle, WA, USA), -2 (Oncogen, La Jolla, CA, USA), -3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and -13 (Lab Vision, Fremont, CA, USA) for intervertebral disc degeneration, TIMP-1 (Lab Vision) and -2 (Lab Vision) for inhibitors of MMPs, and Fas (Santa Cruz Biotechnology) for apoptosis were used. Antibody labeling was identified using horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Sciences,

Arlington Heights, IL, USA), and the results were visualized using enhanced chemiluminescence (Amersham Life Sciences). Beta-actin was used as an internal control for protein loading. Blots were quantified using Imaging Densitometer GF670 and Molecular Analyst software (Bio-Rad) 3 times in each sample, and the average of 3 densities was used as the final density. Value of density was presented as mean ± SD (standard deviation) (arbitrary units).

Reverser transcription polymerase chain reaction for expression of IL-1 and -6 and TNF-α

Measurement of IL-1 and-6 and TNF-α levels was performed by reverser transcription polymerase chain reaction. RNA of the cells was extracted with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were converted to cDNA using an oligo-dT primer (reverse transcription system; Promega, Fitchburg, WI, USA). Two μℓ of cDNA was used in 25 μℓ PCR reactions using the GoTaq Green Master NMix (Promega) according to the manufacturer's protocol. Thermocycling conditions included reverse transcription at 50℃ for 50 min (for RT-PCR) followed by an initial incubation at 95℃ for 5 min and 35 cycles of denaturation at 95℃ for 1 min, annealing at 48.3℃ for 1 min, and extension at 72℃ for 1 min. PCR products were analyzed on 2% agarose gels in the presence of ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. Gene expression analysis was performed with primers in the forward and reverse fashion. The primers used are given Table 1. Blots were quantified using Imaging Densitometer GF670 and Molecular Analyst software (Bio-Rad) three times in each sample and the average of three densities was used as the final density. Value of density was presented as mean ± SD (standard deviation) (arbitrary units).

Histological analysis of AF

Histological analysis was performed using hematoxylin-eosin and Masson trichrome stains. Masson's trichrome stain was used to evaluate the degree of fibrosis and hematoxylin-eosin stain was used to evaluate loss of lamellar pattern (loss, fragmentation and disorganization). Histologic analysis was independently performed by two pathologists on 10 randomly selected, high power fields (× 400) of each sample.

Statistical analysis

The Mann-Whitney U-test was used to assess the difference in apoptosis index, the degree of expression of MMP-1, -2, -3 and -13 and TIMP-1 and -2 and Fas, and the degree of expression of IL-1 and -6 and TNF-α between OLETF and LETO rats, respectively. Probability values < 0.05 were considered statistically significant.

RESULTS

At 6 months of age, OLETF rats weighed significantly more than LETO rats (565 ± 51 vs. 448 ± 16 g, $p < 0.05$). In addition, OLETF rats had abnormal 2-hour glucose tolerance test results at 6 months of age compared with LETO rats (225 ± 34 vs. 119 ± 16 mg/dl, $p < 0.01$) (Fig. 1).

Apoptosis index of AF cells

The apoptosis index of AF cells was significantly increased in OLETF rats compared with LETO rats (22.9 ± 3.5% vs. 15.2 ± 2.7%, $p < 0.05$) (Fig. 2).

Expression of MMPs, TIMPs, and Fas

The results are summarized in Figure 3. The degree of expression of MMP-1, -2, -3, and -13 was statistically higher in OLETF rats compared with LETO rats ($p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.05$, respectively). The degree of expression of TIMP-1 ($p < 0.01$) and -2 ($p < 0.05$) was statistically higher in OLETF rats compared with LETO rats ($p < 0.01$ and $p < 0.05$, respectively). In addition, expression of Fas was significantly

increased in OLETF rats compared with LETO rats ($p < 0.01$).

Expression of IL-1 and -6 and TNF-α

The results are summarized in Figure 4. Expression of IL-1 ($p < 0.05$) and -6 ($p < 0.001$) and TNF-α ($p < 0.05$) was significantly increased in OLETF rats compared with LETO rats (p , 0.05, $p < 0.001$, $p < 0.05$, respectively).

Fibrosis and loss of lamellar pattern in AF

Hematoxylin-eosin stain showed more severe loss of lamellar pattern in AF tissues of OLETF rats compared to LETO rats. In addition, the residual lamellar of AF tissues were fragmented and disorderly in OLETF rats (Fig. 5A). Masson trichrome stain showed that most of the area of AF tissues was stained a blue color, indicating that most of the area was fibrotic, in OLETF rats compared to LETO rats (Fig. 5B).

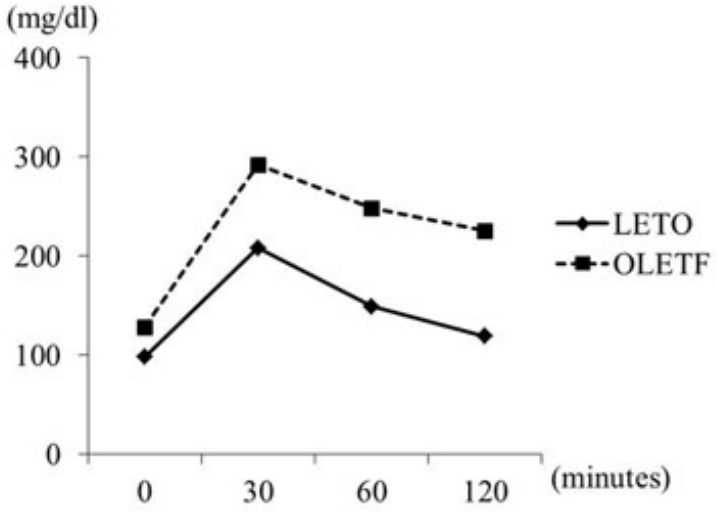

Figure 1: *OLETF rats s had abnormal 2-hour glucose tolerance test results at 6 months of age compared with LETO rats (225 ± 34 vs. 119 ± 16 mg/dl, p < 0.01).*

Figure 2: *TUNEL showed significantly increased apoptosis of annulus fibrosus cells in OLETF rats compared with LETO rats (22.9 ± 3.5% vs. 15.2 ± 2.7%, p < 0.05).*

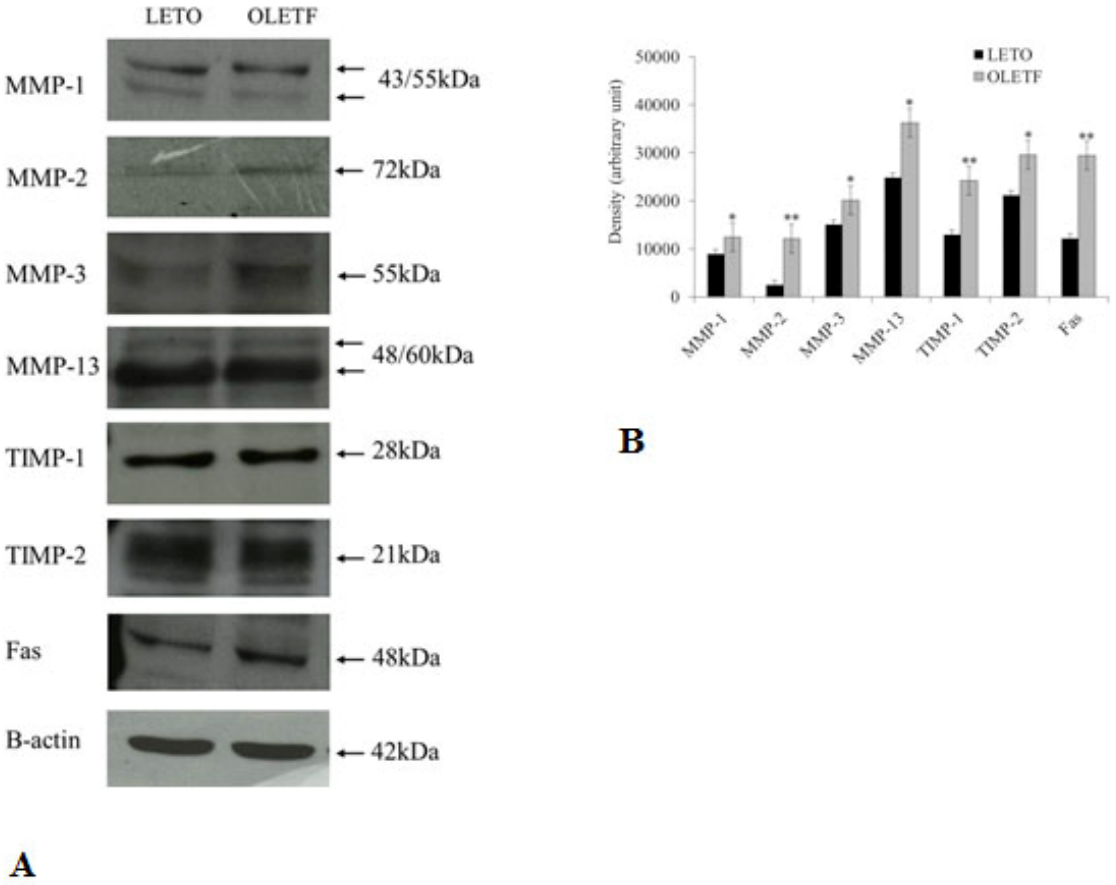

Figure 3: *Western blotting (A) showed that the degree of expression of MMP-1, -2, -3, -13, TIMP-1, -2 and Fas was statistically higher in OLETF rats compared with LETO rats (B).*

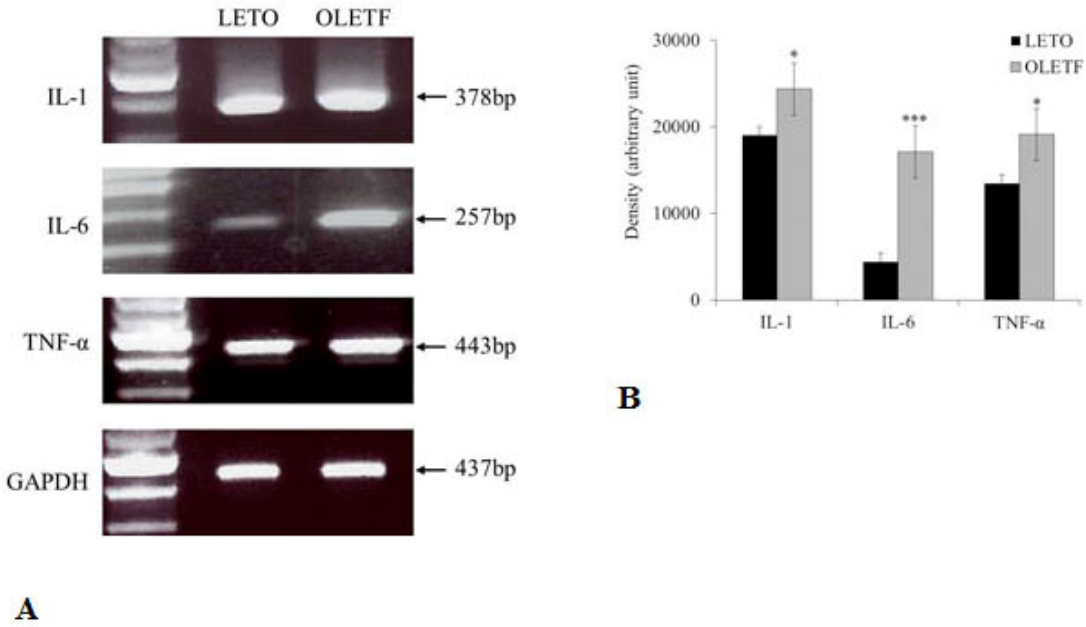

Figure 4: *Reverser transcription polymerase chain reaction (A) showed that the degree of expression of IL-1, -6, and TNF-α was statistically higher in OLETF rats compared with LETO rats (B).*

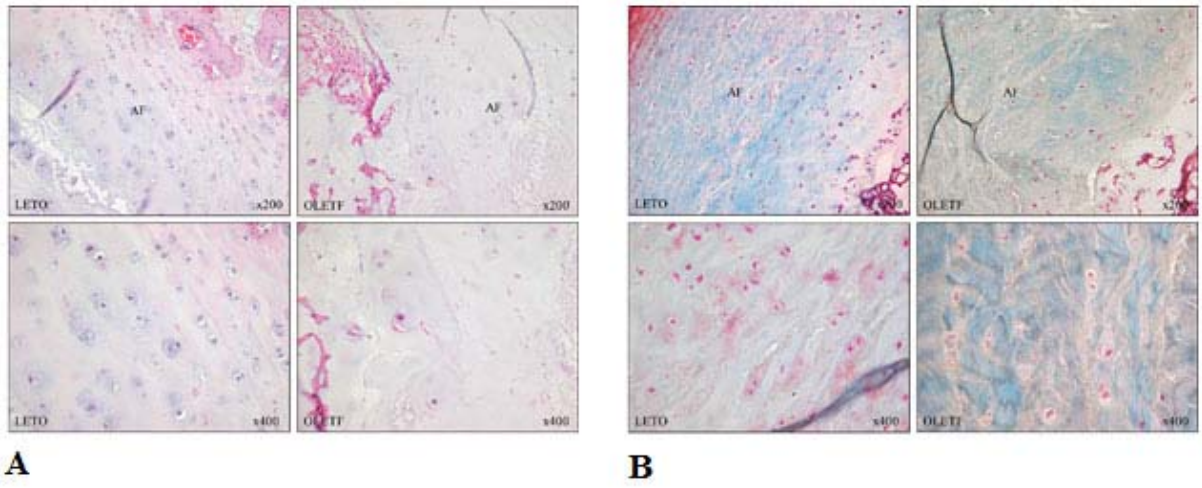

Figure 5: *Hematoxylin-eosin stain showed more severe loss of lamellar pattern in AF tissues of OLETF rats compared to LETO rats. In addition, the residual lamellar of AF tissues were fragmented and disorderly in OLETF rats (A). Masson trichrome stain showed that most of the area of AF tissues was stained a blue color, indicating that most of the area was fibrotic, in OLETF rats compared to LETO rats (B).*

DISCUSSION

The Otsuka-Long-Evans-Tokushima fatty (OLETF) rats are genetically engineered diabetic rats and are characterized by mild obesity and late-onset hyperglycemia (after 4-5 months of age), bearing resemblance to human type 2 DM.[10,12] Long-Evans Tokushima Otsuka (LETO) rats are the non-diabetic genetic controls for OLETF rats. One hundred percent penetrance of DM is observed in male OLETF rats by 6 months of age compared with age-matched control LETO rats. Therefore, OLETF and LETO rats have been used as suitable animal models to investigate the effect of diabetes mellitus on human organs and tissues[19-21]. The advantage of genetically-engineered animal studies is that one can completely control all of the variables to isolate the variable of interest. Therefore, in the current study, we used OLETF and LETO rats to investigate the cellular effects of DM on intervertebral disc degeneration.

Our findings demonstrated that the apoptosis index of AF cells was significantly increased by 1.5 fold in OLETF rats compared with LETO rats. The degree of Fas (apoptosis-related protein) expression was also significantly increased by 2.4 fold in AF tissue of OLETF rats compared with LETO rats. Previous study reported that AF cells undergo apoptosis through the death-inducing signaling complex of Fas apoptotic pathway.[15] The current results suggest that Fas-mediated apoptosis of AF cells occurs more extensively in the AF cells of diabetic rats than in the AF of nondiabetic rats, which results in significant decrease in number of functioning and surviving AF cells. This results in disequilibrium between synthesis and destruction of extracellular matrix in AF tissues of diabetic rats, leading to intervertebral disc degeneration.

MMPs are known to be the major proteolytic enzymes responsible for extracellular matrix degradation in the intervertebral disc. It is generally believed that upregulation of expression and activity of MMPs is responsible for intervertebral disc degeneration and disc herniation. MMPs are traditionally categorized into 3 groups based on their substrate specificity:

collagenases (MMP-1, 8, and -13), gelatinases (MMP-2 and-9), and stromelysins (MMP-3, -7, and -10).[5,11] The catabolic activity of MMPs is balanced by the inhibitory actions of TIMPs, such as TIMP-1 and -2. The TIMPs bind MMPs in 1:1 stoichiometry. TIMPs are co-expressed with MMPs and contribute to the regulation of their activity so that increases in TIMP levels reduce MMP activity. However, contradictory results regarding the delicate balance between MMPs and TIMPs expressions have been reported in several pathological conditions.[3,14,19] Our findings demonstrated that the expression of MMP-1, -2, -3, and -13 was significantly increased in the AF of OLETF rats compared with LETO rats. The expression of TIMP-1 and -2 was also upregulated in the AF of OLETF rats more than in LETO rats. These results suggest that TIMPs are unregulated to counter the extensive catabolic effect of MMPs in AF of diabetic rats, which in turn generate more fibrotic, denatured extracellular matrix components of AF. This results in more severe and more rapid intervertebral disc degeneration.

There is increasing evidence implicating important role of inflammatory cytokines, such as IL-1 and -6 and TNF-α in the development of intervertebral disc degeneration.[4,6,17] Previous studies have reported the increased expression of IL-1α and -1β, -6, -8 and TNF-α in degenerated and/or aging discs of human and animals. In addition, these inflammatory cytokines are known to upregulate expression of MMPs and TIMPs and catabolic mediators, which results in intervertebral disc degeneration. In the current study, we found that the degree of expression of IL-1 and -6 and TNF-α was significantly higher in OLETF rats by 1.3, 3.9, and 1.4 fold, respectively, compared to LETO rats. We speculate that such higher degree of expression of inflammatory cytokines is attributed to increased expression of matrix degrading enzymes in diabetic rats compared to normal control rats.

Intervertebral disc degeneration is known to be associated with increased proteolytic degradation and fibrosis of extracellular matrix, resulting in structural changes. However, little information is available about histologic changes of AF tissues in intervertebral disc degeneration. In addition, to the best our knowledge, there has been no study that has investigated the biochemical factors such as matrix degrading enzymes and inflammatory cytokines associated with these histologic changes. In the current study, hematoxylin-eosin stain clearly demonstrated more severe destructive changes, loss of lamellar pattern, fragmentation, and disorganization, in AF tissues of OLETF rats compared to LETO rats. In addition, Masson trichrome stain showed that most of the area of AF tissues was fibrotic in OLETF rats compared to LETO rats. We speculate that DM conditions cause increased apoptosis of AF cells and expression of matrix degrading enzymes and inflammatory cytokines, which lead to severe histologic changes of AF degeneration in diabetic rats.

In conclusion, our study suggests that DM is associated with excessive apoptosis of AF cells in the AF of diabetic rats, which results in disequilibrium of homeostasis of extracellular matrix. In addition, DM causes a vicious circle of accelerated expression of MMPs, TIMPs, IL-1 and -6, and TNF-α, leading to more severe histologic changes of AF degeneration in diabetic rats. This is the first study to investigate the mechanism underlying the association of DM, apoptosis, matrix degrading enzymes, inflammatory cytokines, and AF degeneration.

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REFERENCES

1. *Alberti KG, Zimmet P, Shaw J. Metabolic syndrome-a new world-wide definition. A consensus statement from the International Diabetes Federation. Diabet Med 2006:23;469-480.*
2. *Anekstein Y, Smorgick Y, Lotan R, Agar G, Shalmon E, Floman Y, et al. Diabetes mellitus as a risk factor for the development of lumbar spinal stenosis. Isr Med Assoc J 2010:12;16-20.*
3. *Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. Matrix Biol 2015:44-46;247-254.*
4. *Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nat Immunol 2015:16;448-457.*
5. *Khokha R, Murthy A, Weiss A. Metalloproteinases and their natural inhibitors in inflammation and immunity. Nat Rev Immunol 2013:13;649-665.*
6. *Krishnan SM, Sobey CG, Latz E, Mansell A, Drummond GR. IL-1â and IL-18: inflammatory markers or mediators of hypertension? Br J Pharmacol 2014:171;5589-5602.*
7. *Lotan R, Oron A, Anekstein Y, Shalmon E, Mirovsky Y. Lumbar stenosis and systemic diseases: is there any relevance? J Spinal Disord Tech 2008:21;47-51.*
8. *McClelland AD, Kantharidis P. MicroRNA in the development of diabetic complications. Clin Sci (Lond) 2014:126;95-110.*
9. *Mobbs RJ, Newcombe RL, Chandran KN. Lumbar discectomy and the diabetic patient: incidence and outcome. J Clin Neurosci 2001:8;10-13.*
10. *Moran TH. Unraveling the obesity of OLETF rats. Physiol Behav 2008:94;71-78.*
11. *Mroczko B, Groblewska M, Barcikowska M. The role of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the pathophysiology of neurodegeneration: a literature study. J Alzheimers Dis 2013:37;273-283.*
12. *Nagai N, Ito Y. Therapeutic effects of sericin on diabetic keratopathy in Otsuka Long-Evans Tokushima Fatty rats. World J Diabetes 2013:4:282-289.*
13. *Park EY, Park JB. Dose- and time-dependent effect of high glucose concentration on viability of notochordal cells and expression of matrix degrading and fibrotic enzymes. Int Orthop 2013:37;1179-1186.*
14. *Park JB, Kong CG, Suhl KH, Chang ED, Riew KD. The increased expression of matrix metalloproteinases associated with elastin degradation and fibrosis of the ligamentum flavum in patients with lumbar spinal stenosis. Clin Orthop Surg 2009:1;81-89.*
15. *Park JB, Park IC, Park SJ, Jin HO, Lee JK, Riew KD. Anti-apoptotic effects of caspase inhibitors on rat intervertebral disc cells. J Bone Joint Surg Am 2006:88;771-779.*
16. *Park JS, Park JB, Park IJ, Park EY. Accelerated premature stress-induced senescence of young annulus fibrosus cells of rats by high glucose-induced oxidative stress. Int Orthop 2014:38;1311-1320.*
17. *Probert L. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience 2015:302:2-22.*
18. *Sakellaridis N, Androulis A. Influence of diabetes mellitus on cervical intervertebral disc herniation. Clin Neurol Neurosurg 2008:110;810-812.*
19. *Vo NV, Hartman RA, Yurube T, Jacobs LJ, Sowa GA, Kang JD. Expression and regulation of metalloproteinases and their inhibitors in intervertebral disc aging and degeneration. Spine J 2013:13;331-341.*
20. *Won HY, Park JB, Park EY, Riew KD. Effect of hyperglycemia on apoptosis of notochordal cells and intervertebral disc degeneration in diabetic rats. J Neurosurg Spine 2009:11;741-748.*
21. *Zhao CQ, Jiang LS, Dai LY. Programmed cell death in intervertebral disc degeneeation. Apoptosis 2006:11;2079-2088.*