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INTERVERTEBRAL DISC CELL APOPTOSIS BY NITRIC OXIDE: BIOLOGICAL UNDERSTANDING OF INTERVERTEBRAL DISC DEGENERATION

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KEY WORDS

intervertebral disc; apoptosis; nitric oxide; human

ABSTRACT

While the unphysiological mechanical load is a central etiologic factor of disc degeneration, biologic factors including nitric oxide (NO) seems to play an important role in this condition. It is, therefore, investigated whether NO is related to the degeneration of intervertebral disc by way of inducing the disc cell apoptosis. Twelve herniated lumbar disc and eight control specimens were obtained from the patients underwent surgery. Apoptotic cells were identified by TUNEL procedure and the percent apoptotic cell index (ACI%) of each sample was calculated. Detection of iNOS expression was performed by immunohistochemical analysis. Disc cell monolayer culture was prepared from the surgical specimen of the patients with lumbar disc herniation. NO generation of the disc cells was measured by Griess reaction. Cell proliferation was detected by measuring the incorporation of ^{3}H-Thymidine. The extent of fragmented DNA induced by NO donor, NOC-18, was also measured by an enzyme-linked immunosorbent assay. The incidence of apoptotic cell death (ACI%) was greater in the herniated group (61.3 ± 24.5%) than that of control group (5.6 ± 6.8%; $P<0.001$). iNOS positive disc cells were detected in all the samples. NO production of disc cells was enhanced by the stimulation of IL-1α. Suppression of ^{3}H-Thymidine incorporation and DNA fragmentation in the disc cells were promoted by treatment of 100 μM NOC-18. These results suggest that disc cells are able to release NO and NO may play an important role in the pathogenesis of disc degeneration through the induction of apoptosis of disc cells in situ.

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INTRODUCTION

Low back pain continues to be a significant clinical entity causing major disability in patients who come to see the orthopaedic doctors. Though the cause of low back pain is still discussed, degeneration of the intervertebral disc is one of the major pathological findings observed clinically. While the unphysiological mechanical load is thought to be a central etiologic factor of disc degeneration because of the epidemiological association between physical activities and disc degeneration, various biologic factors also seem to play a key role in this pathological condition.

Recently, high incidence of apoptosis in the intervertebral disc is found and probably it is related to aging and degeneration.[8, 11] In a mouse model, increase in the number of apoptosis with increasing compressive load is also reported.[14,15] Then, it is suggested that nitric oxide (NO) induces the apoptosis of the mesenchymal cells such as chondrocytes.[3, 5, 16] Promoted apoptosis of chondrocyte in articular cartilage resulted in the lower turnover of the extracellular matrix for maintaining the mechanical property and this may cause the deterioration of the articular cartilage function observed in the osteoarthritis patients. Therefore, we hypothesize that NO also may play an important role in the pathogenesis of the intervertebral disc degeneration through the induction of apoptosis of disc cells in situ. To study the role of NO in disc degeneration, we have, therefore, investigated in this paper that the effect of NO on both cell proliferation and DNA fragmentation of the intervertebral disc cell in vitro.

MATERIALS AND METHODS

1. Tissue sampling and preparation for histological examinations

As degenerative disc samples, twelve herniated lumbar disc specimens were obtained from the patients underwent the posterior surgery for lumbar disc herniation. The patients were 9 men and 3 women, with a mean age of 34.7 years (18-50 years). Eight control specimens as healthy disc samples were obtained from the patients of idiopathic scoliosis or traumatic burst fracture during undergoing the anterior surgery. The patients were 2 men and 6 women, with a mean age of 15.1 years (11-20 years). The control samples were classified 5 in annulus fibrosus and 3 in nucleus pulposus. Each tissue sample was prepared in 4 % paraformaldehyde, embedded in paraffin and serial sections of each sample were used for the following stains.

2. Preparation for detecting cell apoptosis and evaluation of percent apoptotic cell index (ACI%)

Morphological findings with hematoxylin and eosin staining were indicating the condensation of nucleus, which is characteristic of cell apoptosis. However, it is difficult to distinguish the apoptosis from the necrosis based on the histological observation under lightmicroscope. Therefore, tissue sample was labeled for fragmented DNA with an apoptosis detection kit, according to the manufacturer's instructions (ApopTag in Situ Apoptosis Detection Kit-Peroxidase, Oncor, Gaithersburg, MD, USA). This system contains terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase, which incorporates digoxigenin-labeled deoxyuridine triphosphate (dUTP) at 3'-OH ends of double-stranded DNA breaks. This technique recently called TUNEL (TdT-mediated dUTP nick end labeling) procedure were employed for identifying the cell apoptosis.[7] This procedure with horseradish peroxidase enhancement and hematoxylin counterstain; positive and negative controls were included with each procedure.

For quantitative assessment of incidence of apoptotic cells, an average of 139 cells were scored for each control or degenerative disc specimen. The percent apoptotic cell index (ACI %) was determined by the following formula: ACI % = (A1/A2) x 100 %, where A1 is the sum of all apoptotic cells, and A2 is the sum total of all cells in the specified area.

3. Preparation for detecting expression of inducible nitric oxide synthase (iNOS) by immunohistochemistry analysis

Each section was also incubated with polyclonal rabbit anti-iNOS antibody (Affinity BioReagents, Inc., Golden, CO, USA). The reacting antibodies were detected using indirect immunoperoxidase staining with Histofine SAB-PO Kit according to the manufacturer's instructions (Nichirei, Tokyo, Japan).

4. Cell culture

Intervertebral disc specimens were freshly obtained from the patients with lumbar disc herniation by posterior surgeries. The disc samples were initially stored in sterile normal saline solution. Cells were isolated from the diced tissue by 0.2 % collagenase (Sigma, St. Louis, MO.) digestion. Isolated disc derived cells were centrifuged at 1400 rpm for five minutes and collected. These cells were grown at 37 °C in Dulbecco's modified eagle's medium (DMEM; Gibco, Grand Island, NY.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Bioserum, Melbourne, Victoria, Australia) and antibiotics (20 units/ml penicillin and 20 μg/ml streptomycin) in humidified 5% CO_2 atmosphere. First or second passaged cells were allowed to attach in every well of either 24-mutiwell flat-bottomed culture plates (Corning Glass Works, Corning, NY) at the cell density of 1.0×10^5 cells or 96-well plates at 1.0×10^4 cells. Once the cells have attached to the culture well, they were used for following experiments.

5. Measurement of NO

Disc derived cells plated into 24-well plates were stimulated with inflammatory cytokines such as IL-1α (Genzyme, Boston, MA). Generation of NO was detected by nitrate accumulation, its oxidative product, using the Griess reaction. The concentration of NO in the supernatant of the cultured cells in 24-well plates was measured as nitrite concentration by spectrophotometric assay based on Griess reaction using a standard curve calibrated by sodium nitrite.

6. Proliferation assay

Disc derived cells plated into 96-well plates were cultured with various concentration of NO donor, NOC-18 (Wako Pure Chemical Industries, Osaka, Japan) for 72 hours. Fifteen hours before terminating the proliferation assay, 37KBq of ^{3}H-Thymidine (^{3}H-TdR; DuPont/NEN Research Products Boston, MA) was added to each well. At the end of assay, cells were washed three times and 100 µl of scintillation liquid was then added. Total radioactivity of cell associated ^{3}H-TdR was quantified by liquid scintillation counting (Packard Instrument Company, Meriden, CT) for measuring the extent of DNA synthesis.

7. DNA fragmentation assay

The cells were plated into 24-well plates at the cell density of 1.0×10^{5} cells/well. Once the cells have attached to the culture well, various concentration of NOC-18 was added. After 72 hours, cells were washed with 0.05 M phosphate buffered saline (PBS; pH 7.4) and lysed in PBS containing 1% sodium dodecyl sulfate and 1% Tween 20 (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 4 °C for 30min. The cell lysate were centrifuged at 200 g at 4°C for 10 minutes to remove cell debris and high molecular weight DNA. The amount of fragmented DNA was measured by quantitation of cytosolic oligonucleosome-bound DNA with cell death detection enzyme-linked immunosorbent assay kit (Boehringer Mannheim GmbH, Germany) according to the manufacture's instructions. This assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allowed the specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysate.

8. Statistical analysis

Statistical analysis was carried out on all data points with regard to control by an unpaired Student's *t*-test. *P* value under 0.05 was considered significant statistically.

RESULTS

1. Percent apoptotic cell index

When herniated disc tissue sample from a 41-year old male were stained by an end-labeling method, fragmented DNA was visualized as a brown color in the nucleus of apoptotic cells (Figure1-a). In contrast, non-apoptotic cell in annulus fibrosus tissue obtained from a 20-year old male suffering from thoracolumber burst fracture shows a violet color stained by hematoxyline which indicates that digoxygenin-labeled dUTP was not incorporated at ends of DNA breaks (Figure 1-b). Then, the number of cells positively stained by TUNEL method was compared between degenerative disc samples and non-degenerative control samples. The ACI % was calculated by formula stated in materials and methods. The incidence of apoptotic cell death expressed by ACI % was significantly greater in the herniated disc group (61.3 ± 24.5%) than that of control specimens (5.6 ± 6.8%; $P<0.001$) shown in figure 2. This was probably a result of a significantly greater average age of individuals in the herniated population (34.7 ± 11.4 years) compared with in the control (15.1 ± 3.6 years; $P<0.001$).

2. Expression of iNOS in intervertebral disc cells

The localization of iNOS was shown in some of intervertebral disc cells prepared for the histological examination. As shown in figure 3-a, the cytoplasm of herniated disc tissue sample from a 31-year old male stained with brown color indicates the strong localization of iNOS proteins. On the other hand, relatively weak staining to anti-iNOS antibody was detected in the nucleus pulposus tissue sample from a 12 year-old girl suffering from idiopathic scoliosis (Figure 3-b). These results suggested that iNOS proteins are expressed in site histologically though their enzyme activities were not detected in this study.

3. NO production of intervertebral disc cells in vitro

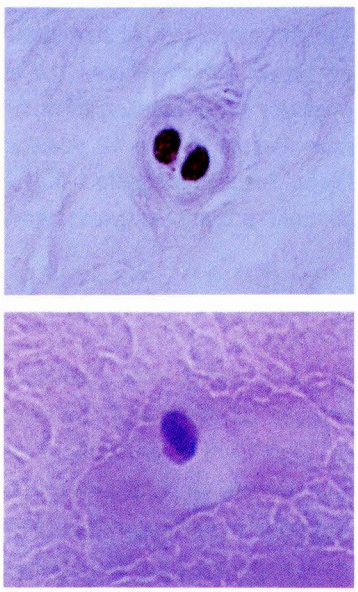
When disc derived cells were stimulated by IL-1α in combination with 10 μg/ml lipopolysaccaride (LPS) simultaneously, the concentration of NO in the culture media increased in a dose dependent fashion of IL-1α. Significant enhancement of NO production was observed by the treatment of 10 U/ml IL-1α with 10 μg/ml LPS. However, IL-1α stimulation without LPS failed to induce NO generation of disc derived cells in vitro (Figure 4). These results suggest that disc derived cells might be capable of generating NO by stimulation of proinflammatory cytokines in inflammatory conditions.

4. Effect of NO on DNA synthesis and DNA fragmentation of disc derived cells

In order to investigate whether NO suppresses disc derived cell proliferation and induces DNA fragmentation, cells were treated with NOC-18 at doses ranging from 10^{-6}-10^{-4} M. Incubation with a varying concentration of NOC-18 for 72 hours demonstrated that ^{3}H-TdR incorporation into the disc derived cells was decreased by addtion of only 100 μM NOC-18. Statistical significance of the results was observed at 100 μM of NOC-18 (Figure 5).

Intracellular DNA fragmentation, which is a hallmark of apoptosis, was induced by NOC-18 treatment in disc derived cells and the extent of DNA fragmentation increased by treatment of NOC-18 in the dose dependent fashion.

Statistical analysis showed a significant increase of apoptotic rate at doses over 25 μM NOC-18 (Figure 6). The assay for DNA fragmentation applied in the present study does not define the number of apoptotic cells and the data do not always provide information on the percentage of cells that underwent apoptosis in given culture. The extent of apoptosis detected in culture condition with 25 to 50 μM of NOC-18 seems not to be large amount comparing with the results obtained from ^{3}H-TdR incorporation assay. This may be the reason why lack of dose dependency by NOC-18 on disc derived cell proliferation was observed.



(a)Fragmented DNA was visualized as a brown color in the nucleus of apoptotic cells.

(b)Non-apoptotic cell in annulus fibrosus tissue shows a violet color stained by hematoxyline which indicates that digoxygenin-labeled dUTP was not incorporated at ends of DNA breaks.

Figure 1. Histological findings of tissue sample stained by TUNEL method. Tissue samples were processed by using TUNEL procedure stated in Materials and Methods.

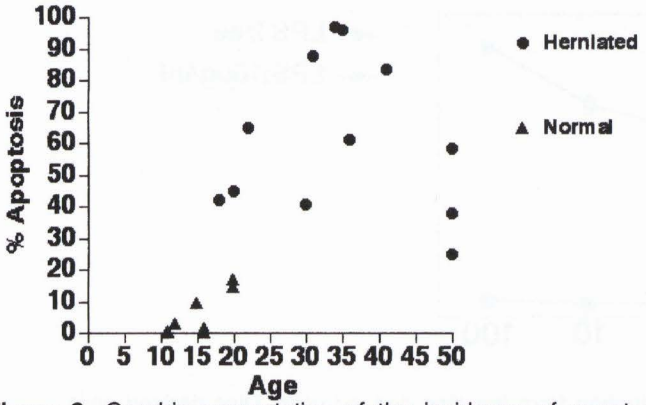
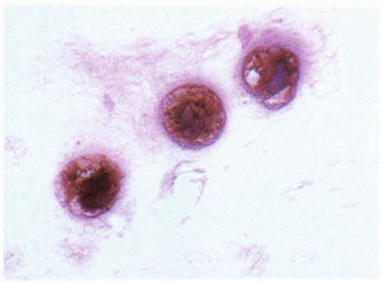
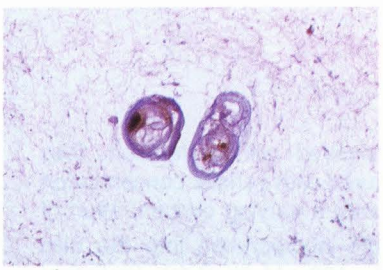


Figure 2. Graphic presentation of the incidence of apoptosis in specimens from the normal control subjects and herniated subjects. The number of cells positively stained by TUNEL method was compared between degenerative disc samples and non-degenerative control samples. The ACI % was calculated by formula stated in materials and methods. The incidence of apoptotic cell death expressed by ACI % was 61.3 ± 24.5 % in the herniated disc group and 5.6 ± 6.8 % in control group.



(a) Strongly expressed iNOS visualized as a wide brown color area in the cyotoplasm of herniated intervertebral disc cells.



(b) Weakly expressed iNOS visualized as a limited brown color area in the cytoplasm of intervertebral disc cells in nucleus pulposus.

Figure 3. Detection of iNOS protein by immunohistochemistory analysis. Tissue samples were processed stated in Materials and Methods. Brown color area in the cytoplasm showed the expression of iNOS.

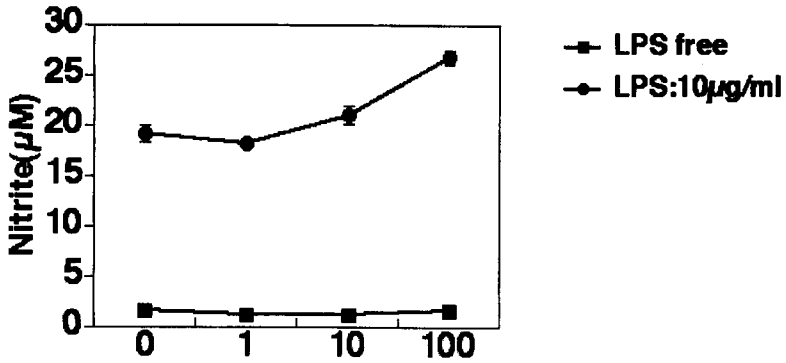


Figure 4. Nitric oxide production from the disc derived cells. Disc derived cells plated into 24-well plates were stimulated with inflammatory cytokines such as IL-1α. Generation of NO was detected by nitrite accumulation using the Griess reaction. When disc derived cells were stimulated by IL-1α in combination with 10 μg/ml LPS simultaneously, the concentration of NO in the culture media increased in a dose dependent fashion of IL-1α. Significant enhancement of NO production was observed by the treatment of 10 U/ml IL-1α with 10 μg/ml LPS.

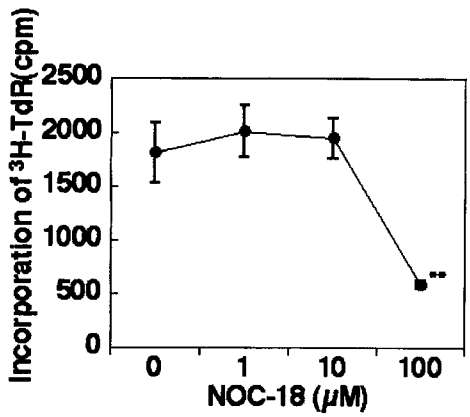


Figure 5. Effect of NO on intervertebral disc-derived cell proliferation. Disc derived cells plated into 96-well plates were cultured with various concentration of NO donor, NOC-18 for 72 hours. Incubation with a varying concentration of NOC-18 for 72 hours demonstrated that ^{3}H-TdR incorporation into the disc derived cells was decreased by addtion of only 100 μM NOC-18. Statistical significance of the results was observed at 100 μM of NOC-18 (**: $P<0.01$ vs. unstimulated cells).

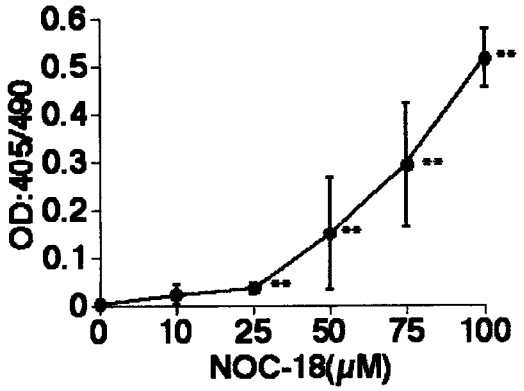


Figure 6. Effect of NO on intervertebral disc-derived cell DNA fragmentation. The amount of fragmented DNA was measured by quantitation of cytosolic oligonucleosome-bound DNA with cell death detection ELISA. Intracellular DNA fragmentation was induced by NOC-18 treatment in disc derived cells and the extent of DNA fragmentation increased by treatment of NOC-18 in the dose dependent fashion. Statistical analysis showed a significant increase of apoptotic rate at doses over 25 μM NOC-18 (**: $P<0.01$ vs. unstimulated cells).

DISUCUSSION

The goal of this study is to clarify the role of nitric oxide and significance of apoptosis in the pathogenesis of intervertebral disc degeneration. Intervertebral disc cell density is reported to decrease with aging.[4] Probably, it may be the result of disc cell apoptosis. However, there are fewer reports of apoptosis in intervertebral disc. Thus, it is still elusive what induces disc cell apoptosis and how apoptosis contributes to the progression of disc degeneration.

In mouse model, Juliao et al.[11] has shown that the percent apoptotic cell ratio of the nucleus proposus was increased with increasing age. Percentage of cells undergoing apoptosis in the intervertebral disc is also reported to be dependent on the magnitude and duration of spinal loading.[14, 15]

In human model, Gruber et al.[8] has shown that the incidence of apoptotic cell death was significantly greater in the control group (73.1%) than among surgical specimens (53.5%). He suggested that this was probably a result of the significantly greater average age in the control population (57.2 years) compared with that in the patient population (44.3years).

In this study, the tissue samples from degenerative group had larger number of apoptotic cells than those from control group. The incidence of apoptosis in the herniated

disc was average 61.3 %, while control specimens showed only average 5.6 % in ACI %. Indeed, we did not compare the histological findings stained by TUNEL method in the age-matched groups between degenerative and control and further examination should be required. However, it is speculated that increase of the apoptotic cell number in disc tissue might result from aging or result in the disease such as low back pain due to disc degeneration.

In related to the degenerative joint disease, researchers are gaining a better understanding of the role of apoptosis. In the pathomechanism of osteoarthritis, chondrocyte apoptosis induced by aging or other biological factors were recently focused.[1-3, 9] Nitric oxide is reported to induce the apoptosis of various kinds of cells including articular chondrocytes.[3, 5, 16] It is demonstrated that NO production and chondrocyte apoptosis occur during the development of osteoarthritis[2, 3] and are correlated with the severity of cartilage degeneration.[9]

Nitric oxide is also reported to induce the apoptosis of both osteoblasts and osteoclasts. Osteoblast apoptosis induced by NO was suggested to be one of the pathological mechanisms in osteoporosis.[5] Therefore, it is possible that inhibitor of NO synthase is utilized for the treatment of osteoporosis clinically. On the other hand, it is reported that NO might prevent the progress of osteoporosis due to inhibiting the bone resorption by osteoclast.[16]

Thus, NO is strongly related to the development of degenerative disease in orthopaedic field. Apoptosis in the intervertebral disc is likely to be induced by both aging and mechanical load. NO may also induce the apoptosis of cells constituting intervertebral disc, which may disrupt the synthesis of extracellular matrix to maintain the mechanical property of the disc. Apoptosis seems to cause the intervertebral disc degeneration same as that apoptosis of articular chondrocytes yields osteoarthritis.

At present, Kang et al.[12, 13] reported that intervertebral disc specimens in culture increased their production of NO when they were stimulated by interleukin-1β. Hashizume et al.[10] reported that nitric oxide in a lumbar disc herniation was mainly produced by cells in granulation tissue around the intervertebral disc. These indicate that intervertebral disc specimen is capable of producing NO in vitro. However, there is no reports whether cells constituting intervertebral disc can release NO directly in vitro. We found that the production of NO from disc derived cells was enhanced by the stimulation of proinflammatory cytokine, IL-1 in combination of LPS. According to the histological examination, iNOS proteins were localized more or less in the disc cells form both degenerated and healthy disc samples and these findings may suggest that NO is generated from L-arginine by iNOS in situ. To the best of our knowledge, this is the first report concerning the release of nitric oxide by intervertebral disc cell. Localization of iNOS in intervertebral disc cells has not been well explored either and we detected it

immunohistologically.

It is possible that mechanical load induces intervertebral disc cell apoptosis via its production of nitric oxide same as the mechanical load induces nitric oxide production of articular chondrocytes.[6] Whether mechanical load induces nitric oxide production of intervertebral disc cell is unknown. NO production was enhanced by the stimulation of proinflammatory cytokine, IL-1 in combination of LPS, which suggests mechanical load might stimulate NO release from the disc directly or indirectly through the inflammatory cytokine production by infiltrated inflammatory cells or disc cells by themselves.

It is also found that cell proliferation was suppressed and DNA fragmentation in cultured disc cells was promoted by adding nitric oxide donor, NOC-18. These results suggest that nitric oxide is generated in inflammatory conditions and nitric oxide may affect the cell viability through inducting the apoptosis of disc cells. Therefore, We conclude that excessive increase in NO concentration in situ by biological or mechanical inflammatory stimulation may accelerate disc degeneration because of the excessive decrease of disc cell density by apoptosis. Although this mechanism of cell death needs further investigation, regulation of NO concentration could be targeted for new treatment strategies in future.

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