Original Article

Discoid Meniscus: High Levels of Apoptotic and Autophagic Genes

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Abstract

Background: How the meniscus adapts to the morphological changes in the lateral tibiofemoral compartment, in terms of gene expression, was the reason to establish this present study. **Objective:** This study aimed to determine the changes in the mRNA levels of the apoptotic and autophagic genes in the discoid meniscus. **Subjects and Methods:** We have investigated the apoptotic and autophagic gene levels in discoid and normal lateral menisci of 21 patients (11 discoid and 10 control). The RNAs were isolated from the fresh discoid and healthy meniscal tissue. Gene expression was defined based on the threshold cycle (Ct), and Actin beta was used as a reference gene that acts as an internal reference to normalize RNA expression, which was calculated as 2−ΔΔCT. **Results:** Apoptotic and autophagic gene levels were significantly higher in the discoid meniscus group. In discoid meniscus samples, the Bcl-2 mRNA, BclXL, BAK mRNA, ATG 12, ATG 7, ATG 5, ATG 3, and Beclin1 mRNA levels were higher by 4.2, 5.9, 9.1, 8.3, 23.2, 6.1, 12.4, and 18.1 times, respectively, with statistically significant differences ($p < 0.001$). **Conclusion:** The discoid meniscus etiology should be considered both in morphological and genetic modulation manners: apoptotic and autophagic genes play roles with tibiofemoral morphological differences.

Keywords: *Apoptosis, autophagy, discoid, etiology, gene, meniscus, upregulation*

Received: 26-Apr-2020;
Revision: 25-May-2020;
Accepted: 04-Aug-2020;
Published: 20-May-2021

Introduction

The term "discoid" represents the non-C shape of the meniscus with an increased rate of snapping and/or locking of the knee joint in the younger population, while the word "meniscus" comes from the Greek word meniskos meaning "crescent."[1] The incidence of discoid meniscus may vary because the criteria for judging the morphology of meniscus is inconsistent; however, current reports indicate the rate from 2 to 40%.[2] The commonly known types of Watanabe are complete, incomplete, and Wrisberg ligament type,[3] with a higher prevalence among Asians and Caucasians.[4] Young first published an article in 1889 about an external semilunar cartilage in the knee joint, possibly related to a meniscus,[5] and Jeannopoulus first mentioned about the discoid meniscus in 1950.[6] Since that time, some debates have taken place about discoid meniscus etiology, and the exact mechanism of the formation of discoid meniscus morphology has remained uncertain.

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Quick Response Code:

Website: www.njcponline.com

DOI: 10.4103/njcp.njcp_212_20

Apoptosis is "programmed cell death" which plays critical roles in physiological functions such as elimination of unwanted tissues and reshaping of tissues during embryological development, balancing of cell number during immune system development in continuously renewed tissues, and elimination of cells that are damaged during aging or at the end of the lives of healthy tissues.[7] The Bcl2 gene family consists of a group of genes that contribute to apoptosis. The first discovered member of this gene family was named Bcl-2. While Bcl-XL is antiapoptotic, the Bax and Bak genes are proapoptotic. Apoptosis is regulated by the balance between these molecules.[8]

Autophagy is a biological process that means the "cell to digest itself". Autophagy is rapidly activated

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How to cite this article: Atik A, Avcikurt AS, Sargin S. Discoid meniscus: High levels of apoptotic and autophagic genes. Niger J Clin Pract 2021;24:647-50.

in conditions leading to cellular stress, such as lack of nutrients and growth factors, to remove damaged organelles, long-lived proteins, and protein aggregates. It may be triggered by pathogen infections, hypoxia, nutrient depletion, or cellular stress, such as reactive oxygen species (ROS). This feature allows the cell to adapt to stressful conditions, and it may also cause death by consuming vital organs. When its control is impaired, it may cause unwanted conditions like cancer, early dementia, some hereditary diseases, Alzheimer's disease, or infections.[9] There are casual relationships between apoptosis and autophagy in cell death. While some proapoptotic signals may induce autophagy, antiapoptotic signals could suppress it. This connection is made possible via mitochondria.[10]

Upregulation is the increase of the product of a gene, namely mRNA, and then the protein product. This means an increase in the function of that protein. Bcl-2 plays a role in the regulation of autophagy by engaging directly with key autophagic proteins such as Beclin-1 (Bcl-2 inhibits autophagy by direct interaction with Beclin-1.). However, the autophagy-regulating functions of Bcl-2 proteins in mitochondria have not been fully elucidated. The overexpression of antideath members such as Bcl-2 and Bcl-XL protects the cells from autophagic cell death, while Bax, a proapoptotic member, causes autophagic cell death. Bax leads to cell death by causing the accumulation of ROS, lipid peroxidation, and plasma membrane changes.[11]

Autophagy occurs in at least five steps: 1) induction, 2) expansion, 3) completion, 4) adhesion and fusion, and 5) degradation. There are about 30 known autophagy-related genes (ATG). The genes functioning at the beginning of autophagy (ATG-1), vesicle nucleation (bec-1), protein conjugation system (ATG-7, ATG-12), uptake, and vesicle transformation (ATG-18) have been identified. Inhibition of mTOR releases ULK1/ATG-1 which in turn stimulates the redistribution of mATG-9 from golgi to endosome. Activation of type III PI3K hVPS34/Beclin 1 (ATG-6) initiates the formation of a bilayer membrane. Then activation of ATG-7 activates ATG-12 and makes a covalent bond with ATG-5. ATG-16 is interfered with this complex to form the ATG5-ATG12-ATG16 triple complex. With this complex, vesicle enlargement occurs.[12]

When they work precisely, all these procedures help to shape normal morphology. Because discoid meniscus is a morphological pathology, we aimed to determine the changes in the mRNA levels of the apoptotic genes which are responsible for the clearance of embryological residues and to determine the changes in the mRNA levels of autophagic genes that are suppressed or enhanced by apoptotic genes in the embryological residual discoid meniscus, the mechanism of which is not yet fully elucidated.

Materials and Methods

This study was conducted in our institute between 2016 and 2018. The local ethical committee approved the study. All patients signed informed consent forms. Symptomatic discoid meniscus patients, who needed surgical intervention, had arthroscopic surgery by the same surgeon (AA), and the diagnosis of "discoid meniscus" were confirmed under arthroscopic vision. An arthroscopic partial central meniscectomy (saucerization) and stabilization of the capsule (if unstable) and repair of any tear (if present) were performed. The samples were collected with an arthroscopic punch during saucerization and kept in the TRIzol™ reagent in the operating theater, immediately within an icebox and sent to the medical genetics department for investigation. For the control group, patients who had undergone total knee arthroplasty surgery with intact and morphologically normal lateral menisci were selected, and specimens were collected in the same manner. All samples were stored in the refrigerator at -80°C. Any patients with a history of genetic disorders were excluded from the study. No age or gender discrimination was made. 11 discoid meniscus patients and 10 control group patients formed the study. Of the total patients, 9 were male and 12 were female.

Gene expression

The RNAs were isolated from the fresh discoid and healthy meniscal tissue. The total RNA was extracted using the High Pure RNA Isolation Kit (ROSHE) per the manufacturer's protocol. Complementary DNA isolation from RNA (Gene All, Hyperscript first-strand synthesis kit, Cat no: 601-005, Lot no: FS015B04002) was performed in two steps. Applied Biosystem Step One Plus equipment was used for the real-time PCR (GeneAllSybr Green Master Mix, Cat No: 801-520, Lot No: QP116G25001) analyses. Samples were analyzed three times, and the reactions were established by using both Actin beta (ACTB) and their own genes. Gene expression levels were quantified using 7500 Fast Real-Time Sequence detection system Software (Applied Biosystems, Foster City, CA). Gene expression was defined based on the threshold cycle (Ct) and ACTB was used as a reference gene that acts as an internal reference to normalize the RNA expression, which was calculated as $2^{-\Delta\Delta CT}$. The sequences of the primers and Tm temperatures are summarized in Table 1.

Statistical analyses

In the molecular studies, for statistical evaluation, one-way ANOVA and Student-*T* test were used. Data was presented as mean percent ± SD. A p-level of <0.05 was accepted as statistically significant.

Results

In discoid meniscus samples, the Bcl-2 mRNA levels were higher 4.2 times, and BclXL mRNA levels were 5.9 times higher when compared to the control group. This increase is highly statistically significant (p = 0.000). The BAX levels were 1.3 times higher than the control group, but this increase was not statistically significant (p = 0,134). BAK mRNA levels were 9.1 times higher with a highly statistically significant difference (p = 0.000). ATG 12, ATG 7, ATG 5, ATG 3, and Beclin1 mRNA levels were higher by 8.3, 23.2, 6.1, 12.4, 18.1 times, respectively, with statistically significant differences (p = 0,00). LC3 mRNA levels were decreased by 0.5 times, which was not statistically significant (p = 0192) [Figure 1].

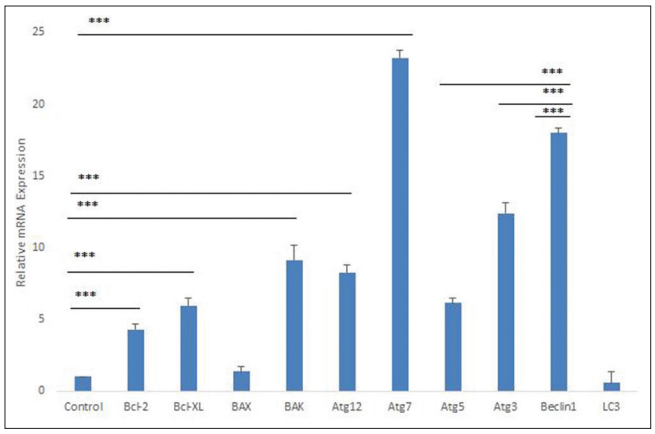

Figure 1: Quantitative gene expression analyses by real-time PCR studies revealing the expression levels. The expression levels of Bcl-2, BclXL, BAK, ATG 12, ATG 7, ATG 5, ATG 3, and Beclin1 mRNA were higher in discoid menisci. ***P = 0,000

Table 1: The primers and Tm temperatures

			TM (°C)
BCL2	F	CTGCACCTGACGCCCTTCACC	67
	R	CACATGACCCCACCGAACTCAAAGA	
BCL-XL	F	GATCCCCATGGCAGCAGTAAAGCAAG	69
	R	CCCCATCCCGGAAGAGTTCATTCACT	
BAX	F	TTTGCTTCAGGGTTTCATC	58
	R	TCCTCTGCAGCTCCATGTTA	
BAK	F	ACCAGCCTGTTTGAGAGTGG	60
	R	AGTGATGCAGCATGAAGTCG	
BAD	F	CCAGATCCCAGAGTTTGAGC	60
	R	CCAGATCCCAGAGTTTGAGC	
ATG12	F	TCTATGAGTGTTTTGGCAGTG	57
	R	ATCACATCTGTTAAGTCTCTTGC	
ATG7	F	AGGAGATTCAACCAGAGACC	58
	R	GCACAAGCCCAAGAGAGG	
ATG5	F	GGGAAGCAGAACCATACTATTTG	61
	R	AAATGTACTGTGATGTTCCAAGG	
ATG3	F	TCACAACACAGGTATTACAGG	53
	R	TCACCGCCAGCATCAG	
BECLİN1	F	TGTCACCATCCAGGAACTCA	58
	R	CTGTTGGCACTTTCTGTGGA	
LC3-II	F	GAGAAGCAGCTTCCTGTTCTGG	64
	R	GTGTCCGTTCACCAACAGGAAG	
ACTB	F	CCTGACTGACTACCTCATGAAGATCCTC	59
	R	CGTAGCACAGCTTCTCCTTAATGTCAC	

Discussion

Because the lateral femoral condyle deflects more laterally than the medial condyle in the longitudinal axis of the femur, and the lateral tibial plateau is smaller than the medial tibial plateau on the anteroposterior axis, many authors consider this asymmetric morphology and asymmetric motion as a reason for the discoid meniscus with a higher incidence rate laterally.[13] More variations in the shape of lateral meniscus than the medial meniscus[14] supported the theory for morphology/movement based adaptation in terms of different moments of load–transmission onto the menisci.[13]

In 1948, Smilie reported that the residual prenatal morphology of the menisci might result in morphological variations.[15] Nevertheless, Kaplan revealed an embryological study stating no discoid formation at any time of the development of the human fetus in 1957.[16] Then Ross *et al.* suggested that discoid meniscus is a congenital anomaly in 1958.[17] However, studies regarding human fetuses were limited and quantitative analyses of the development of menisci were lacking.

One recent milestone study from Fukazawa *et al.* examined fetus and adult menisci comparatively and reported that the histogenesis occurs earlier in the lateral than in the medial menisci, which means a higher incidence of accidents and more frequent overloads in the lateral menisci soon after birth.[13] They found that the lateral menisci were oval, more rounded, and covered most part of the lateral tibial plateau in all fetuses, while the medial menisci were longer and more slender in fetuses than in adults.[13] Tibial plateau morphology was the same in lateral and medial sides in the early gestational phase. However, the medial plateau increased in size more rapidly than the lateral plateau in correlation with the menisci sizes, and also, the circularity index of lateral meniscus was significantly higher in fetuses than in adults.[13]

These embryological findings caused us to think about the abnormal regulation of meniscal genes regarding

tibiofemoral morphological changes. That is for sure, the gestational morphological changes were different in lateral and medial tibiofemoral compartments. Stimulation of tibiofemoral contact on the meniscus probably regulates the formation of the crescent shape of the meniscus; therefore, when this stimulation does not occur adequately anatomically, the apoptotic and autophagic processes are not regulated and the meniscus forms a discoid shape. So, to question a deficit in meniscal adaptation to this morphological complexity in terms of gene expression was the reason to establish this present study.

All discoid menisci in our study presented statistically significantly higher levels of autophagic and apoptotic genes without exception. Although apoptotic and antiapoptotic gene expressions were higher in the discoid tissue, apoptosis did not occur and discoid formations were seen. In other words, apoptosis did not occur which led to discoid shape and this may be the cause of the overexpression of apoptotic and antiapoptotic genes.

Apoptosis and autophagy are fundamental mechanisms in cellular events that are interrelated through mitochondria. The interrelationship between tibiofemoral morphology, hypoinduction of lateral compartment due to delayed development of tibial plateau, and broader lateral meniscal coverage over plateau might need a more autophagic and apoptotic activity to shape the meniscus normally. Thus, when one or more of these inductors decrease, the upregulation of these genes may begin. This may lead to a new pathway definition in discoid meniscus etiology.

Several limitations of the present study should be noted. First, the protein levels of the investigated genes have to be measured to conclude a more exact relationship. Secondly, a correlation between tibiofemoral radiological morphology and gene levels could be analyzed. Of course, if the number of samples would be higher, then the validity of the results would be higher.

However, this present study suggests that the discoid meniscus etiology should be considered both morphological and genetic modulation manners and that the autophagic/apoptotic genes play roles in this etiology.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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