a notochordal phenotype. In fact several studies have used rat cells to explore the notochordal molecular phenotype in the disc[30,31]. Therefore, results from the rat, as well as mouse or rabbit have to be extrapolated with care to the human situation. Apart from the different developmental pathways of the NP cell populations the large deviations in IVD size and thus nutrition and mechanical conditions are also likely to influence the molecular characteristics of NP cells. Nevertheless, the expression profile of KRT19 demonstrates that selected genes may be valuable as markers even in different species.

When comparing the present study with previous studies on beagles, it also has to be noted that the human samples in this study are very heterogeneous with respect to age, while the animals all had the same age. This may explain the less pronounced differences between NP, AF, and cartilage cells in human individuals compared to the dog species, while general trends were identical in both species. Consequently, the age of the animal always has to be taken into consideration for the interpretation of results from an animal model. Moreover, although a study in the rat did not reveal major differences in gene expression pattern between RNA extracted from isolated cells and RNA extracted directly from the tissues, enzymatic cell isolation might have contributed to the reduced tissue-related differences observed in human specimens[7]. Besides, it is sometimes difficult to clearly distinguish human NP from AF tissue, especially in aged discs, which can also result in lowered gene expression differences between NP and AF cells. In view of these difficulties and the observed age-related alterations, young individuals are clearly preferred for the study of the phenotype of the healthy human NP cell.

Age-related changes have been detected regarding matrix composition, expression of matrix degrading enzymes, and other processes[3,6,32–34]. Although differences between aging and (early) degeneration were recently described in rabbits, a strong correlation exists between age and degeneration grade in human patients[3,35,36], which is confirmed in this study. Thus, it is not possible to clearly separate the influence of aging from that of degeneration mechanisms. Accordingly, Adams and Roughly defined a degenerate disc as one with structural failure combined with accelerated or advanced signs of aging[37]. This has recently been demonstrated also for the cervical spine, where in a longitudinal study no other factor except for age was related to the progression of degeneration[38]. Although cases of early disc degeneration have been described and are of particular value for specific investigation of degenerative processes, the present study evaluated individuals with "natural" disc development, where aging is accompanied with a certain degree of degeneration. This is particularly reflected in the mRNA expression of MGP in the NP, which strongly correlated with both aging and degree of degeneration. MGP is a Bone morphogenetic protein-2 (BMP-2) regulatory protein that is known as a calcification inhibitor in cartilage and in arteries[39,40]. Interestingly, MGP serum levels of community-based cohorts were also elevated with increasing age and were associated with individual risk factors for coronary heart disease[41]. It was suggested that induction of MGP expression may be a feedback mechanism to prevent mineralization of calcium deposits in the arteries[41]. Similarly, one could speculate that induction of MGP in the NP may be an attempt to prevent calcification processes that have been observed in the aging disc[42,43]. The presence of MGP in areas of mineralized cartilage in the endplate and in cells adjacent to sites of degeneration would support this hypothesis. The same is true for the expression at the fibrocartilaginous attachment of the outer AF where ectopic ossification (i.e., exophyte growth) would be prevented. Interestingly MGP expression in non-calcified AC is restricted to the superficial regions in monkeys and is barely detectable in senile human cartilage tissue[44]. In chondrocytic cells, both over-expression of MGP in maturing chondrocytes and under-expression of MGP in proliferative and hypertrophic chondrocytes may induce apoptosis[45]. As it has been reported that some cells of the IVD may differentiate towards the hypertrophic chondrocyte phenotype with age, MGP might function to prevent apoptosis in these cells[46]. Further studies are required to clarify the role of MGP expressed in the disc.

The observed rise in PTN gene expression in the AF with aging may result from a cellular attempt to restore a slowly degrading tissue. PTN functions as a growth and differentiation factor in many cell types and has been shown to induce the synthesis of matrix molecules in articular chondrocytes[47]. In cartilage, elevated PTN levels have been related to both osteoarthritis and rheumatoid arthritis[48,49]. Interestingly, an increased amount of PTN-immunopositive cells was observed in degenerated and in prolapsed disc samples and was associated with vascularization of diseased or damaged tissue[50]. Since blood vessels are mostly localized in the outer AF and rarely penetrate into deeper zones of the IVD, an increasing expression of PTN in the AF with aging would support the suggestion that PTN may function as an angiogenic factor in the degenerating IVD[50]. Age- or degeneration-associated changes might become clinically useful markers to determine the "juvenileness" or the regenerative capacity of IVD tissues sampled from discectomies or nucleotomies. More extended studies will be required to validate the potential of such markers to individually evaluate the most appropriate treatment of an IVD disorder.

In conclusion, from a selection of NP phenotype markers identified in animal studies, KRT19 and NCAM1 expression were found to be more pronounced in NP than AF and AC cells in human individuals. Whereas NCAM1 levels are relatively low even in NP cells, KRT19 may be regarded as a marker for human NP cells, being highly expressed in NP and at significantly lower levels in AF and AC cells. This observation on the subject of gene expression is at least partially reflected at the protein level, where KRT19 positive cells are almost exclusively identified in the NP of juvenile and young adult discs. This suggests that KRT19 transcripts are translated into detectable amounts of protein primarily in notochordal-like cells of juvenile NP and occasionally in young chondrocyte-like NP cells. MGP is found in a variety of human IVD tissues and thus cannot serve as a characteristic NP marker.

Conflict of interest

The authors confirm that they have no financial and personal relationships with other people or organisations that could inappropriately influence their work.

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特集●腰痛疾患に対する interventional therapy—現在から未来へ—

細胞レベルからの椎間板再生
—細胞移植療法のその先に—

酒井 大輔　　持田 譲治

Key words ■ 椎間板(intervertebral disc), 再生医療(regenerative medicine), 髄核細胞(nucleus pulposus cell)

要旨：椎間板障害は腰痛のみならず，椎間板ヘルニア，すべり症などを誘引しうる重大な問題である．われわれは髄核細胞の質を評価するアッセイ系を確立，髄核細胞の"heterogeneity"とその特色を明らかにした．また元来，椎間板を構成する細胞数は少ないが，ヒト検体を当研究室で用いた臨床研究の検討の結果，その細胞数は年齢とともに減少，その活性度(細胞増殖能,基質産生能)，構成細胞分画についても年齢と相関関係にあることを実証した．椎間板細胞の質を迅速に評価できるアッセイ系は再生医療を行う上でセルプロセッシングセンターでの細胞品質評価に有用なツールになると考える．

Summary
Intervertebral disc disease are one of the most frequent reason to see a doctor. In order to establish a simple and effective method to evaluate the quality of disc cells taken from surgery, we performed a colony assay and found that several different colonized cells can be seen. With the use of the colony assay, we were able to detect the biological potential of intervertebral disc cells for clinical application.

はじめに

本邦における腰痛の有訴者率は男性1位，女性2位であり，社会の高齢化に伴い近年ますます増加している[1]．その主因の一つである椎間板障害の診療にかかる医療費は年間約1,700億円超とされ医療経済に与える影響も大きい[2]．さらに椎間板障害の好発年齢は青壮年期の男性に多く，労働力への影響も大きいため社会的医療問題であるが，病態の詳細なメカニズムは未知である．椎間板障害は腰痛のみならず" motion segment"における" imbalance"をきたし，椎間板ヘルニア，変形性脊椎症，脊柱管狭窄症，すべり症などを誘引しうる重大な問題である．現行の脊椎固定手術などは脊椎の可動性を犠牲にするため，

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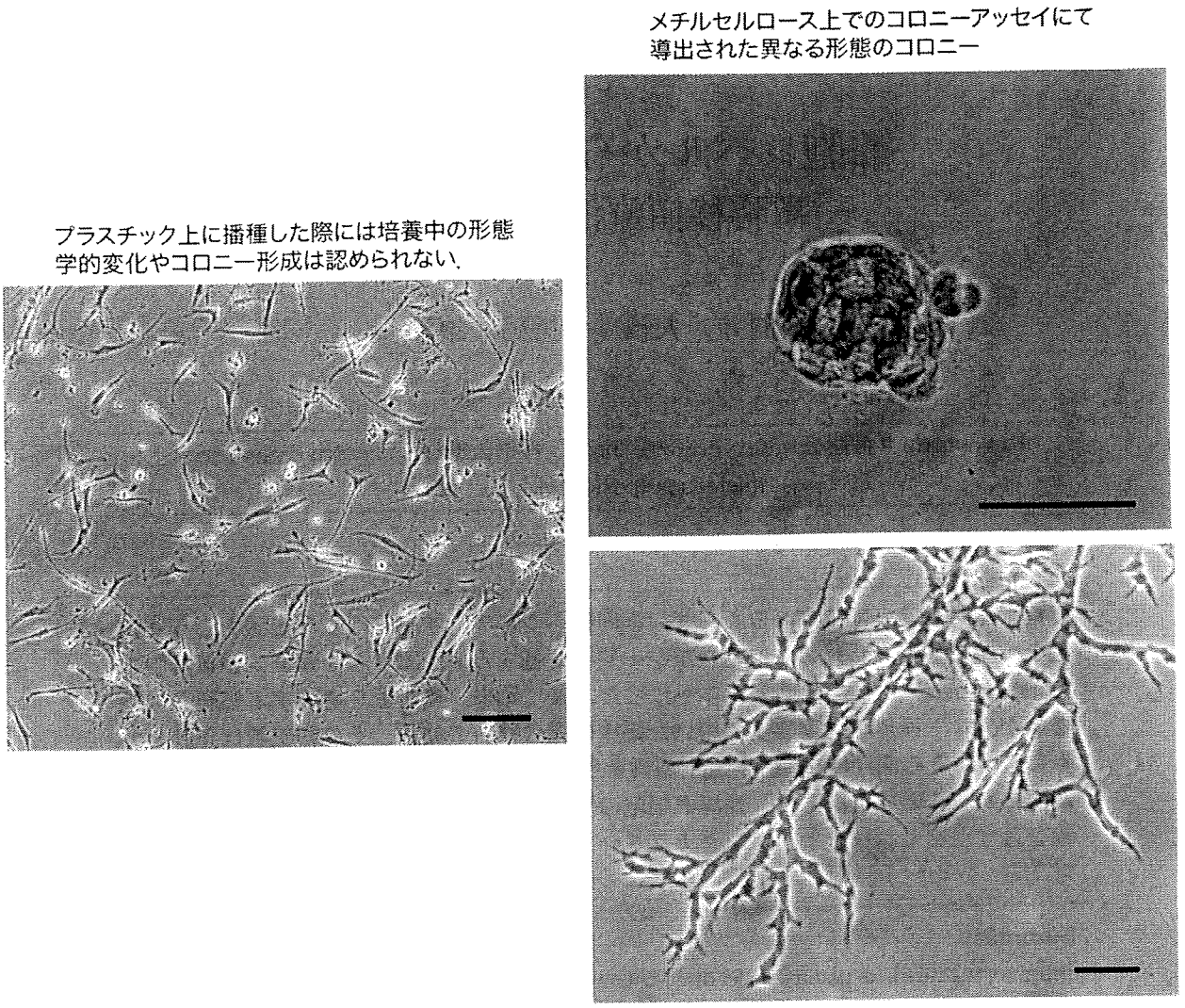

図1

隣接椎間障害をきたし，人工椎間板も脊椎の持つ生体力学特性から問題も多い．以上のような現状から可動性を温存した生物学的治療による椎間板障害治療を通じた脊柱全体の老化予防が望まれている．東海大学医学部外科学系整形外科学領域ではこれまで細胞移植治療を主軸に椎間板の変性抑制，再生研究を内外に多数報告しており，特に幹細胞との共培養により活性化された髄核細胞移植は厚生労働省ヒト幹細胞臨床応用研究の承認の下，目下試験中である[3~5]．さらに筆者らは大動物レベルにて世界で初めて椎間板に自己間葉系幹細胞移植を行い，その移植後の分化評価や体外での分化誘導法などを報告している．その後われわれのみならず，海外でも複数の研究が追試され細胞移植療法の将来性につき注目されている．しかしながら，椎間板障害の病態は多岐にわたり，実験動物モデルの限界や構成細胞の発生，分化，運命，脊索性髄核細胞の意義などが未知なこと，椎間板再生と腰痛改善についてなど，真の椎間板再生を目指すためには椎間板内の微小環境における細

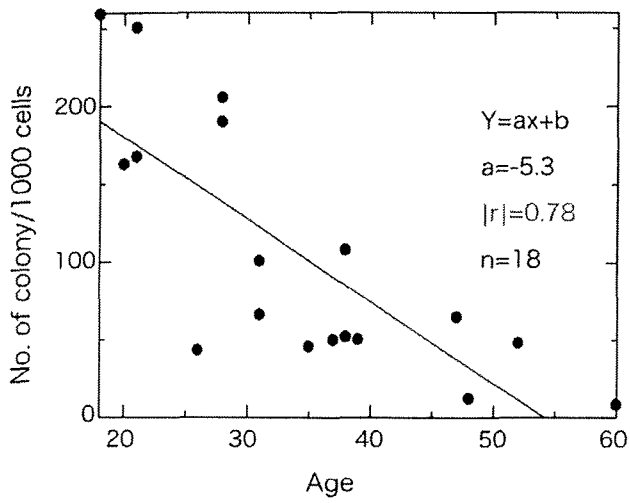

図2　球状コロニー形成数は年齢と相関

胞レベルでのさまざまな変化，恒常性維持機構を解明する必要がある．一方，椎間板障害は致死的疾患ではないがゆえに細胞移植治療を検討する上でさらなる妥当性，安全性の確保が重要と考える．特に椎間板構成細胞の恒常性維持に必要な細胞集団の動態把握と細胞移植治療に用いる有効な細胞集団同定は不可欠な検討項目である．そこで現在は真の椎間板再生に向け，細胞移植療法の本研究では椎間板内構成細胞と内在性未分化幹細胞の同定，幹細胞ニッチと老化制御システムの解析を行っている．本稿ではいくつかのテーマのうち，培養髄核細胞のheterogeneityないしは有効性を簡便に評価できるassay系の開発につき報告する．

Ⅰ．方　法

Institutional Review Board承認の下，C57BL6マウス(12週齢，n=30)，SD系ラット(16週齢，n=3)，ビーグル成犬(24カ月齢，n=3)の腰椎あるいは尾椎よりおのおの髄核と線維輪細胞をtrypsin，collagenaseにて酵素処理し分離した．初代細胞を400 cells/dishでメチルセルロース上にて28日間培養，経時的に細胞増殖率，コロニー形成能，形態を評価した．次にヒト髄核細胞(18～52歳，平均33.8歳，n=9)を同様の手法にて評価した．また出現したコロニーにつき免疫染色を行い基質合成能につき評価した．

Ⅱ．結　果

マウス，ラット，イヌすべての髄核および線維輪細胞で接着型と球状型の2種類のコロニー形成細胞および細胞のheterogeneityを確認し，少なくとも椎間板の構成には3種類以上の形質の違う細胞が関与していることが示唆された(図1)．最もヒトに近い軟骨異栄養犬種であるビーグルでの結果，細胞増殖率は腰尾椎間で差は認めなかったが(培養4，16，28日目で腰椎平均4.0倍，53.9倍，83.597.5倍，尾椎で平均3.7倍，86.3倍，71,593.7倍，$p>0.05$)，より器質合成能力が高い細胞を多く含むとされる尾椎髄核から多くのコロニーが出現した(コロニー数：腰椎では培養4，28日目で接着型が平均66個，155個，球状型79個，57個，尾椎で接着型94個，179個，球状型99個，191個，$p<0.05$)．ヒト髄核細胞でもこの2種類のコロニーを認め，提供者の年齢が若い方が球状コロニーの出現数が顕著に多く(図2)，免疫染色の結果，球状コロニーにより多くプロテオグリカン，Ⅱ型コラーゲンを産生する細胞が存在することが判明した．

Ⅲ．考　察

椎間板変性の治療を科学的根拠に基づき開発するためには椎間板の細胞・分子レベルでの理解が必須であるが，椎間板はいまだその発生，構成細胞の分化，恒常性維持，そして

変性機序に至るまで骨や関節軟骨に比し未知である．これまで脊索性髄核と軟骨性髄核という細胞集団が報告されてきたが，その根拠は*in vivo*での発生学的知見と単層培養下での形態学的知見によるものであり，厳密に細胞形質を分別できているかは定かでなかった．メチルセルロースを用いた本アッセイ法はこれまで椎間板細胞で用いられてきた手法に比べてより多彩な培養形質を検出できた．また培養形質ごとに細胞集団を回収，解析できるため，椎間板細胞のheterogeneityを検討する上で有利な手法といえる．本研究の結果，生物学的活性度(基質産生能)の高い髄核細胞集団は球状型コロニーを多く作り，その傾向は培養4日目から評価できることが証明された．今後球状型コロニー形成細胞集団を中心にそのマーカーや分別法を解析することで，より再生治療に効果的な細胞組成や変性機序を解明できる研究に応用できることが示唆された．椎間板細胞の質を迅速に評価できる本アッセイは再生医療を行う上でセルプロセッシングセンターでの細胞品質評価に有用なツールになると考える．

以上のように，椎間板障害に対する細胞移植療法を検証する目的で始まった一連の研究の結果，椎間板における細胞レベルでの微小環境をより理解し，さらに質の高い治療法の探索へと椎間板再生研究は進んでいる．今後，多くのことが明らかになるにつれて椎間板障害の予防，治療に応用できることと考える．

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