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H. 知的財産権の出願・登録状況
1. 特許取得
記載事項なし
2. 実用新案登録
記載事項なし
3.その他
記載事項なし

Ⅲ. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
林貴史、他.	膝軟骨損傷におけるアテロコラーゲン包埋自家軟骨移植後の評価T2mapとdGEMRICによる初期検討.	日本磁気共鳴医学会雑誌	28	95-100	2008
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Ⅳ. 研究成果の刊行物・別冊

膝軟骨損傷におけるアテロコラーゲン包埋自家軟骨移植後の評価：T2 map と dGEMRIC による初期検討［大会長賞記録］

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土江洋二[1]，吉廻　毅[1]，北垣　一[1]，蓼沼　拓[2]，
内尾祐司[2]，尾崎史郎[3]，原　真司[3]，Hasnine Haque[4]，
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緒　言

関節軟骨は，細胞増殖，血流に乏しい組織であり，修復能は低い．このため，自然治癒の可能性は低く，放置しておくと損傷は増悪する．そして，損傷の悪化に伴い，関節の機能障害を来し，患者の QOL 低下を招く．

このことから，様々な軟骨修復法が考案されているが，いまだにゴールドスタンダードはないのが現状である．その中で単層培養を利用した培養自家細胞軟骨移植（ACI）は良好な成績が報告され，世界各国で行われている．

単層培養による培養自家軟骨細胞移植は，骨膜でパッチした欠損部に培養軟骨細胞を浮遊液の状態で移植する方法である．

しかしながら，ACI の問題点として，①移植された軟骨細胞は三次元的空間である軟骨欠損部に均一に分布せず，偏在する可能性が高いこと，②移植された軟骨細胞は骨膜縫合部の隙間から漏出し得る可能性があること，③再生組織に最も必要な足場が存在しないことが挙げられる．

これらの問題点を克服する方法の一つとして，アテロコラーゲン包埋自家軟骨移植術がある．単層培養を用いた ACI の問題を克服するためには軟骨細胞移植ではなく，軟骨細胞と基質とで三次元的に構築された軟骨様組織を移植する方が有利である．

本法は，アテロコラーゲンゲルを軟骨細胞増殖の足場とし，軟骨細胞をゲルに包埋・培養し，軟骨細胞と基質からなる組織を欠損部に移植する．アテロコラーゲン包埋移植は組織学的に良好なヒアリン軟骨によって修復されることが証明されている．

単層培養自家軟骨移植において dGEMIC を用いた移植部の評価は既に報告があるが[1),2)]，本法についての報告はまだない．

アテロコラーゲンを担体とする本法は単層培養 ACI とは組織学的に異なる手法で，dGEMIC および T_2 により，移植部を評価することは臨床的に意義がある．

目　的

今回，我々はアテロコラーゲン包埋自家軟骨移植後患者について，膝軟骨移植部と健常部における造影後 T_1 緩和時間（T_{1post}）と T_2 緩和時間（T_2）を測定し，両者を比較検討した．

キーワード　cartilage, MR imaging, Gadolinium-enhanced, T2 map, autologous chondrocyte implantation

対象と方法

1. 対象

対象は，外傷，変形性膝関節症による関節軟骨欠損でアテロコラーゲン包埋自家軟骨移植を施行した患者4例（年齢：21～44歳．男性1例，女性3例．移植後2～8年経過）である．移植部位は大腿骨内顆および膝蓋骨である．

2. 方法

撮像機種は，1.5T MR装置(Gyroscan, Philips社製)，および3T MR装置（Signa HDx, GE社製）である．撮像条件は，1.5T MRIについては，プロトン密度強調像（2D-FSE）TR/TE＝2000/18 ms, slice/gap＝3.0/0.3 mm, FOV＝16×14 cm, matrix 512×512, NEX＝2 T_1 map（2D-FSEIR）: TR/TE＝760/11.9 ms, TI＝100, 300, 500, 1000, 1500 ms slice/gap＝3.0/1.0 mm, FOV＝16×16 cm, matrix＝256×160, NEX＝1.0 T_2 map（2D-FSE）: TR/TE＝760/11.9 ms, slice/gap＝5.0/0.0 mm, FOV＝23×23 cm, matrix＝256×256, NEX＝2. 3.0T MRIについては，プロトン密度強調像（2D-FSE）TR/TE＝3500/21 ms, slice/gap＝4.0/1.0 mm, FOV＝15×15 cm, matrix 384×256, NEX＝2 T_1 map（2D-SEIR）: TR/TE＝3000/11.9 ms, TI＝#1/#2, slice/gap＝3.0/1.0 mm, FOV＝16×16 cm, matrix＝256×160, NEX＝1.0（TI＝#1；50, 100, 300, 500, 1500 ms #2；100, 300, 500, 1000, 1500 ms）T_2 map（2D-FSE）: TR/TE＝1000/7.7～61.9 ms, slice/gap＝3.0/1.0 mm, FOV＝15×15 cm, matrix＝256×160, NEX＝2. 撮像断面は，大腿骨は矢状断像，膝蓋骨は横断像で撮像した．

3. 撮像手順

撮像手順は以下のとおりである．

① 形態画像（プロトン密度強調画像）撮像．

（② 造影前 T_1 mapを撮像）

③ Gd-DTPA^{2+} 造影剤（2倍量，0.2 mmol/kg）を静注（肘窩静脈）．

④ 歩行運動（ドレッドミルにて3 km/hourの設定で10分間施行）．

⑤ 静注2時間後 T_{1post} mapを撮像．

4. T_1, T_2値の測定

T_1, T_2の測定は，アテロコラーゲンゲルおよび健常軟骨，移植部について行った．

T_1 mapは，GEYMSより提供された DT_1 mapで作成した（Fig. 1a）．T_2 mapについては，Cartigram（Functool ver 4.4, GE社製）で作成した（Fig. 1b）．

また，健常軟骨は手術，関節鏡の所見を参考に健常と判断した非荷重部軟骨とし，移植部は，手術所見，プロトン密度強調画像を参考に部位を決定した．

これらにROI（円形，2～3 mm^2）を設定し，T_1, T_2値の測定を行った．

結　果

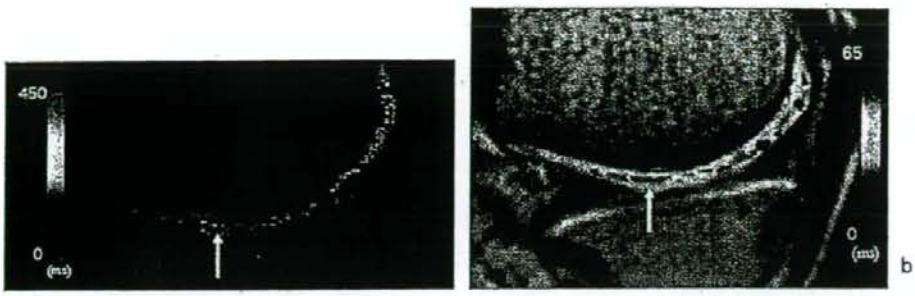
アテロコラーゲンゲルについては，T_1（1.5T/3T）＝2262/2437 ms, T_2（1.5T/3T）＝341/409 msであった．健常部および移植部について，T_2は，39±2.3（32～43）ms, 53±4.1 ms（41～58）であった．T_{1post}は，平均397±38 ms（324～487 ms），平均417±30 ms（366～498 ms）であった．

対応のあるt検定で，T_{1post}については両者間に有意な差は認められなかった（Fig. 2a）．T_2に関しては，健常部と移植部との間に有意差（$p=0.0006$）を認め，健常部に比べ移植部のT_2は延長傾向にあった（Fig. 2b）．

考　察

関節軟骨のT_2は，軟骨内の含水量やコラーゲン線維のネットワークを反映しているといわれている[3)～5)]．軟骨損傷で，膠原線維ネット

2007年12月6日受理



a b

Fig. 1. 44-years-old woman with ACI
a) T_{1post} map (dGEMRIC)
b) T_2 map
T_{1post} map (a) and T_2 (b) maps for a 44-year-old female with autologous chondrocyte implantation. According to the T_{1post} map, the reparative tissue (arrow) has a GAG level comparable to that of normal tissue, while T_2 makes it possible to distinguish reparative tissue (arrow) from adjacent tissue. An elevated T_2 level is therefore believed to relate to an incomplete collagen network in the graft.
ms : milliseconds
Arrow : the graft

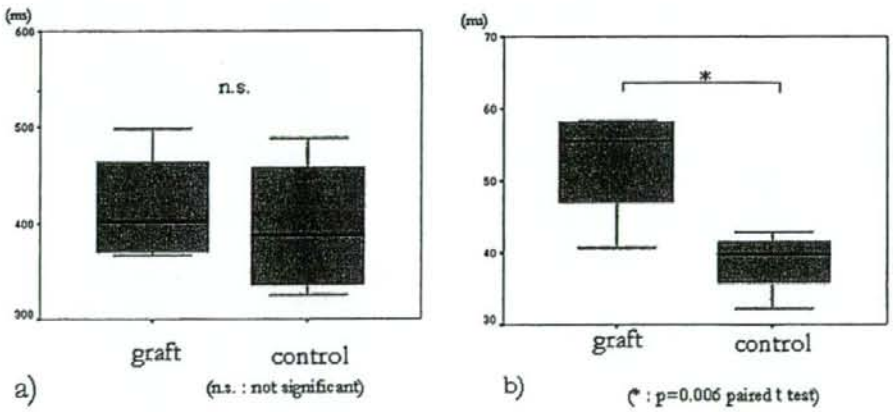


Fig. 2.
a) T_{1post} map (dGEMRIC)
b) T_2 map
The average T_{1post} values of graft and normal cartilage showed no significant difference (a). The average T_2 values showed a significant difference ($p=0.006$) (b).
ms : milliseconds

ワーク，三次元的層構造の消失や水分量の増加で，T_2は高値となる．

Glycosaminoglycan (GAG) は，プロテオグリカンの主成分で，軟骨の弾性力保持に重要な成分である．T_{1post}（造影後のT_1短縮）とこのGAG量とに一定の関係があると報告されている[6)~8)]．また，軟骨損傷で，T_{1post}は短縮するとされている．軟骨内のGAGは，負の電荷を

有するため，健常な状態では負の電荷を有し，Gd-DTPA^{2-}は相反する分布をとる．反対に損傷軟骨ではGAGは減少するため，Gdが分布するようになる．その結果，その領域のT_1短縮が生じる．

今回，T_{1post}については健常部と移植部の間に有意差がなく，両者GAG分布に差があるとはいえない．また，T_2に関しては，移植部の方が健常部よりもT_2は延長しており，軟骨の層構造，コラーゲン線維が健常部に比し乏しい，あるいは水成分が健常部に比し多いことが推定される．

以上の結果からは生検による組織所見（移植部は健常部に比べ，内部の三次元構造は異なるが，GAGの分布は近い）と類似したものとなっている．

今回の検討の問題点として，対象症例数が少ないこと，異なる磁場強度（3T, 1.5T）でのT_1値を一緒に評価していること，空間分解能等の撮像技術の問題，T_1の造影前後での変化については不詳であることが挙げられる．

今回の検討で1症例（39歳　女性；3T MRIで撮像）のみであるが，造影前T_1緩和時間（T_{1pre}）を撮像していた．その症例のT_{1post}は健常部と移植間（428 ms, 428 ms）には差がなかったが，T_{1pre}については両者間（健常部：422 ms，移植部：464 ms）で異なり，移植部のみで明らかなT_1短縮がみられた．

今回，軟骨細胞を包埋，増殖させた移植組織についてのT_1は未知であるが，アテロコラーゲンゲルのT_1の測定値から，移植組織の成熟度により健常軟骨との差は無視できないと思われた．

健常部と損傷軟骨のT_1にはほとんど差がなく，dGEMRICでは，時間の節約からT_{1pre}を省略し，T_{1post}のみを測定する[9)~11)]．一方で，Watanabeらは，造影MRによるACI後の軟骨評価では，ΔR_1（$R_{1post}-R_{1pre}$）のみがGAGと相関しており，R_{1pre}, R_{1post}は相関がなかったと報告し，ACI後の評価には，ΔR_1が重要と述べている[2)]．

以上のことから，今後は移植組織の成熟度を評価する観点から，アテロコラーゲン包埋自家軟骨移植でも，dGEMRIC（T_{1post}）に，T_{1pre}を追加する必要があると考えた．

結　　語

アテロコラーゲン包埋自家軟骨移植術後の軟骨評価について，dGEMRIC, T_2 mapを用いた初期検討を報告した．

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Delayed Gadolinium-enhanced MR to Evaluate Reparative Cartilage after Autologous Chondrocyte Implantation [Presidential Award Proceedings]

Takafumi HAYASHI[1], Kouji UCHIDA[1], Megumi NAKAMURA[1], Akihiko WADA[1], Youji TSUCHIE[1], Takeshi YOSHIZAKO[1], Hajime KITAGAKI[1], Taku TADENUMA[2], Yuji UCHIO[2], Shiro OZAKI[3], Shinji HARA[3], Haque HASNINE[4], Atsushi NOZAKI[4],

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Delayed gadolinium (Gd)-enhanced T_1 and T_2 values reflect the glycosaminoglycan (GAG) content and collagen network integrity in cartilage.

We evaluated graft integrity after autologous chondrocyte implantation (ACI) with atelocollagen by measuring T_1 and T_2 relaxation time after intravenous injection of contrast medium.

We examined 4 patients (aged 21 to 44 years) with ACI, using 1.5- and 3.0-tesla magnetic resonance (MR) scanners. Contrast medium containing Gd-DTPA was administered intravenously at double doses, subjects were made to exercise their knee joint for 10 min, and MR images were taken 2 hours after contrast injection. T_1- and T_2-calculated images were produced, and the regions of interest (ROI) were set in the reparative tissue and normal cartilage in each knee.

The average postcontrast T_1 values (T_{1post}) of graft and normal cartilage were 417 ± 30 ms and 397 ± 38 ms, which were not significantly different. The average T_2 values were 53 ± 4.1 ms and 39 ± 2.3 ms, which were significantly different ($P = 0.006$).

The results suggest that the grafts had an almost normal GAG content, but not a normal collagen network in the cartilage.

However, we think that the maturation or integrity of the graft should be interpreted based on both the T_{1post} value and the precontrast T_1 (T_{1pre}) value of the reparative tissue because the T_{1pre} value of reparative tissue may vary with the degree of maturation or integrity.

Delayed gadolinium-enhanced MR imaging using T_1 and T_2 measurements is therefore considered a useful, noninvasive method to evaluate the graft after ACI with atelocollagen.

Spontaneous Recurrent Hemarthrosis of the Knee in the Elderly: Arthroscopic Treatment and Etiology

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Purpose: To elucidate the etiology of and find a preferable surgical treatment for spontaneous recurrent hemarthrosis in osteoarthritic knee joints arthroscopically. **Methods:** Nineteen patients referred to our institution from affiliate hospitals between April 1998 and October 2006 were involved in this study. Their demographics, preoperative radiographic findings, preoperative magnetic resonance imaging (MRI) findings, arthroscopic findings and procedures performed, the patient's medical history, and the postoperative clinical course were retrospectively reviewed. **Results:** There were 9 male and 10 female patients with average age of 61.9 years (range, 41 to 83 yrs). The average number of joint aspirations before surgery was 5.4. The average time from onset to arthroscopy was 10 months. Radiographs showed 2 knees with isolated lateral compartment osteoarthritis (OA), one with isolated patellofemoral (PF) OA, 14 with medial and lateral compartment OA, and 2 with tricompartmental OA. Classifying them according to the dominant compartment, 6 knees were medial-dominant OA, 11 lateral-dominant OA, and 2 PF-dominant OA. The MRI scans revealed 18 grade III lateral menisci and 1 grade II lateral menisci. Even with 6 medial-dominant OAs, lateral meniscal involvement was more obvious than medial meniscal involvement on MRI. Subtotal lateral meniscectomy accompanied with coagulation of the bleeding points was performed on 17 cases. For 2 PF OA cases, synovectomy and a histologic examination of synovium were performed. Remission was obtained for 18 cases. The unsuccessful case had cirrhosis of the liver. **Conclusions:** A majority of the patients (17 of 19) had degenerative torn lateral menisci confirmed with MRI and at arthroscopy. Successful outcomes were achieved by meniscectomy and coagulation. Most so-called spontaneous recurrent hemarthroses in OA knee joints appear to be attributable to torn lateral menisci. **Level of Evidence:** Level IV, therapeutic case series. **Key Words:** Arthroscopy—Elderly—Knee—Lateral meniscus—Osteoarthritis—Spontaneous recurrent hemarthrosis.

In 1959, Wilson[1] reported 5 cases of spontaneous recurrent hemarthrosis in the elderly as spontaneous hemarthrosis in knee joints with osteoarthritis (OA). He described the common features of hemarthrosis as being of rapid onset and having tense effusion without any initiating injury.[1] In his report, patellofemoral (PF) joint OA was implicated as the cause of this condition. Since then, several reports have described this disease, including mostly successful treatment outcomes.[2-5] As for surgical treatment, in 1964 Burman et al.[2] reported that synovectomy accompanied with medial and lateral meniscectomy was successful. Morii et al.[3] also reported successful surgical outcomes for 16 patients, and stressed the importance of synovectomy through unicompartmental

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The authors report no conflict of interest.

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knee arthroplasty for 2 cases, lateral meniscectomy for 4 cases, transfer of tibial tuberosity for 2 cases, and the resection of osteopytes for 5 cases; these were performed as accompanying procedures. In 1994, Kawamura et al.[4] first reported a specific pathology of the knee joint as the cause of hemarthrosis (i.e., degeneratively torn lateral meniscus). They reported that lateral meniscectomy alone gave successful results for 5 surgically treated cases. Pellacci et al.[5] and Ogawa et al.[6] supported this etiology. A few questions, however, remain unanswered: is it proper to put all patients into the same category? When conservative treatment has failed, what is the proper treatment choice? To address these questions, we reviewed our experience with 19 cases of spontaneous hemarthrosis of the knee joint that were treated arthroscopically. We hypothesized that the cause of spontaneous recurrent hemarthrosis in the elderly could be attributed to a degeneratively torn lateral meniscus concomitant with lateral compartmental OA, and that lateral meniscectomy would be an adequate procedure when surgical treatment was necessary.

METHODS

Patients

Patients referred to our institution from affiliate hospitals because of recurrent hemarthrosis of the knee between April 1998 and October 2006 were involved in this retrospective study. Inclusion criteria were being over 40 years of age, the injury having no association with trauma, having no other findings other than OA changes on radiographic examination, and having no previous surgical treatment on the corresponding knee. Knees with obvious causes of hemarthrosis, such as pigmented villonodular synovitis, hemangioma, or other intra-articular abnormalities other than degenerative changes validated on magnetic resonance imaging (MRI) scans or at arthroscopy were excluded. Several items were recorded for each patient, including: (1) demographic information; (2) preoperative radiographic findings; (3) preoperative MRI findings; (4) arthroscopic findings and procedures performed; (5) the patient's medical history; and (6) the postoperative clinical course.

Radiographic Grading and Magnetic Resonance Imaging

All patients underwent radiographic imaging and MRI scans (Signa 1.5T; GE Yokokawa Medical Systems, Tokyo, Japan) of the affected knees. Radiographic grading with the Kellgren and Lawrence (KL) scale[7] was recorded for each compartment as follows: grade 0, no radiographic findings of osteoarthritis; grade 1, doubtful osteophyte formation; grade 2, definite osteophytes with unimpaired joint space; grade 3, definite osteophytes with moderate joint space narrowing; and grade 4, definite osteophytes with severe joint space narrowing and subchondral sclerosis. Among the 3 compartments, the OA compartment receiving the highest grade on the KL scale was considered the dominant osteoarthritic compartment. When the KL grade was the same for 2 or 3 compartments, a single author (K.N.), who had 19 years' experience in musculoskeletal radiology and was blinded to the clinical and arthroscopic information, determined the dominant osteoarthritic compartment according to degree of sclerosis and subchondral cysts formation. MRI was performed on fast spin echo, proton-weighted sagittal and coronal signals (TR 2000 ms, TE 16 ms, field of view 14 to 16 cm, matrix 512 × 256, number of excitations 2, and 3-mm slice thickness, without an interslice gap). Menisci were graded according to the scheme used by Crues et al.,[8] as follows: grade 0, normal; grade 1, intrameniscal focus of signal; grade 2, intrameniscal linear or wedge-shaped signal; and grade 3, linear or globular signal extending to an articular surface.

Operative Findings and Procedures

Arthroscopic findings and operative procedures were determined from operative records. A laser apparatus (VersaPulse; Coherent Japan, Tokyo, Japan) was used to coagulate bleeding points until the year 2003, and after that, a radiofrequency system (VAPR; DePuy Mitek, Raynham, MA) was used.

RESULTS

Patient Demographics

A total of 19 patients were enrolled in this study. Nine patients were male and 10 were female, with ages ranging from 41 to 83 years (mean, 61.9 yrs). On their first visit, all patients save for 1 had experienced aspiration of bloody joint fluid more than twice at affiliate hospitals. The average number of joint aspirations before surgery was 5.4 (range, 1 to 20 aspirations). The average time from onset to arthroscopy was 10 months (35 days to 8 yrs, 7 mos). Nine cases received arthroscopy within 3 months; 8 cases within 4 to 12 months; 1 case at 1 year and 2 months; and 1 case at 8 years and 7 months.

Preoperative Radiographic Findings and Magnetic Resonance Imaging

OA was diagnosed when the KL grade was found to be higher than 2. Two knees had isolated lateral compartment OA, 1 knee had isolated PF OA, 14 knees had medial and lateral compartment OA, and 2 knees had 3-compartment OA. Classifying knees according to the dominant compartment, 6 knees were medial-dominant OA, 11 were lateral-dominant OA, and 2 were PF-dominant OA.

Based on MRI scans, 18 lateral menisci were classified as grade III and the other was grade II. Eight medial menisci were grade II, 7 were grade I, and 4 were grade 0. Even with 6 medial-dominant OA, lateral meniscal involvement was more obvious than medial meniscal involvement on MRI.

Surgical Findings and Procedures Performed

Brown-colored diffuse synovitis in the knee was observed in all cases. Seventeen knees (89.5%) had degenerative torn lateral menisci (89.5%). Bucket handle–type tears were predominant (12 of 17), 3 were flap tears, and 2 were longitudinal tears. Among them, 6 were considered discoid lateral menisci because of their thicker rims and free margins. Two cases with PF-dominant OA (10.5%) did not have lateral meniscal injury. Two medial-dominant OA and a lateral dominant knee had degenerative torn medial menisci at the posterior segment. All 3 tears were horizontal and did not exceed half width of the meniscal body; trimming of the free margin was performed for these lesions.

In 17 torn lateral meniscus cases, hypertrophy of synovium around the rim of the lateral meniscus was observed. Subtotal meniscectomy was performed on these 17 cases, and bleeding from the lateral meniscus around the popliteus tendon was observed in the process of debridement in 16 cases. For 2 PF OA cases, synovectomy using VAPR and a histologic examination of synovium were performed.

Medical History

Eleven patients did not have any history of other diseases. Hypertension was found in 7 patients. Among them, 1 patient was taking an anticoagulant because of a transient ischemic attack. One patient had an elongation of bleeding time caused by late-stage liver cirrhosis.

Postoperative Course

Recurrence of hemarthrosis after arthroscopic surgery was found in the single case with liver cirrhosis (5.3%). The other 18 patients have not had a recurrence of hemarthrosis for an average of 5 years and 2 months (1 yr, 10 mos to 9 yrs, 7 mos).

Case 1

A 65-year-old woman had been suffering from left knee pain for about 10 years. She felt severe knee pain without any traumatic episode, went to a hospital, and received arthrocentesis that produced 40 mL of bloody joint flood. Her knee pain was relieved by this arthrocentesis. After that, she experienced similar episodes 7 times in 3 months and was referred to our hospital. Because of the sudden onset of hemarthrosis and severe pain, her chief complaint was fear and apprehension about knee pain and swelling. Her radiographic findings revealed KL grade 3 lateral type OA with KL grade 2 medial compartment (Fig 1), and MRI scans showed a degenerative torn lateral meniscus (Fig 2). Arthroscopy was performed and revealed a degenerative torn lateral meniscus. Removal of a small meniscal segment induced pulsatile bleeding from the residual rim of the lateral meniscus. We

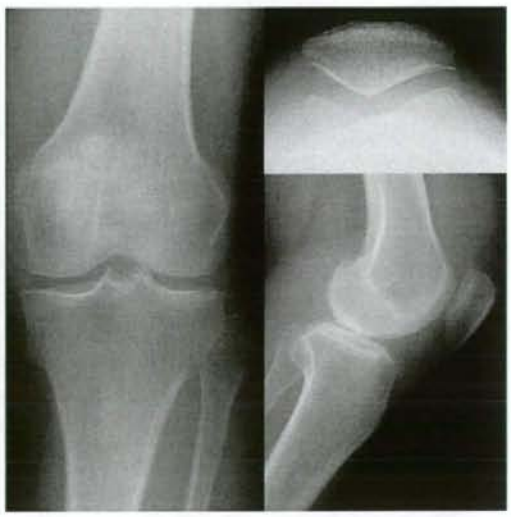

FIGURE 1. Radiographic findings of case 1 (a 65-year-old woman). An anteroposterior radiograph reveals lateral-dominant osteoarthritis along with involvement of the medial compartment (left) and minimum involvement of osteoarthritic changes of the patellofemoral joint (upper right and lower right).

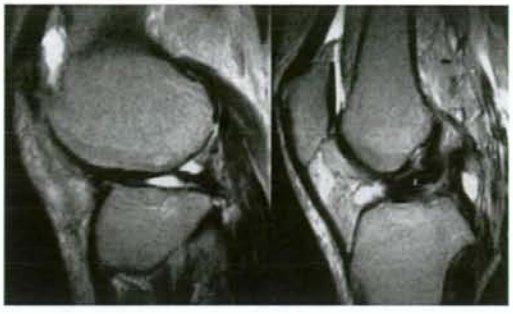

FIGURE 2. Magnetic resonance imaging findings of case 1. The irregularly shaped triangle of the posterior horn indicates torn lateral meniscus. The lack of cartilage is obvious on both the femoral and tibial surfaces in the lateral compartment (left). Dislocated lateral meniscal fragment can be seen adjacent to the anterior cruciate ligament (right, asterisk).

performed a subtotal meniscectomy with air tourniquet followed by coagulation using VAPR without air tourniquet (Fig 3). No recurrence has been observed for more than 2 years.

Case 2

An 83-year-old woman received arthrocentesis 6 times in 9 months, which produced bloody joint fluid each time, before referral to our hospital. Her chief complaint was fear and apprehension about the recurrence of hemarthrosis. Her radiographic findings exhibited KL grade 2 OA involvement of the PF joint (Fig 4). Arthroscopy was performed. A diffuse proliferation of brown synovium was observed, and a tumorous lesion was found floating in the suprapatellar pouch with no connection to the joint capsule (Fig 5). A pathologic examination found that this lesion was a hematoma in its process of organization (Fig 6). Arthroscopic synovectomy was performed.

DISCUSSION

Spontaneous hemarthrosis of the knee is a relatively rare condition observed in older OA patients. In 1959, Wilson[1] reported 5 cases of spontaneous hemarthrosis in OA knees. He suggested a vascular fringe of synovial membrane around the deformed patella as a cause of hemarthrosis because of the high prevalence of retropatellar arthritis in patient radiographs. A successful outcome of conservative treatment was also reported. In 1964, Burman et al.[2] reported that synovectomy accompanied with medial and lateral meniscectomy was successful. Morii et al.[3] also reported successful outcomes for 16 patients who received synovectomy, although only 7 knees received synnovectomy alone, and the other 9 knees were treated with several other accompanying procedures.

Until 1991, synovium had been considered to be the origin of bleeding; therefore, synovectomy appeared to be the most reasonable treatment. But in 1994, Kawamura et al.[4] suggested that the cause of this hemarthrosis was most likely the peripheral arteries of the posterior horn of the lateral meniscus. They underwent arthroscopic resection of the posterior portion of the remaining degenerative lateral meniscus and achieved satisfactory results. Pellacci et al.[5] also achieved successful outcomes with total lateral meniscectomy. Ogawa et al.[6] reported a case of recurrent

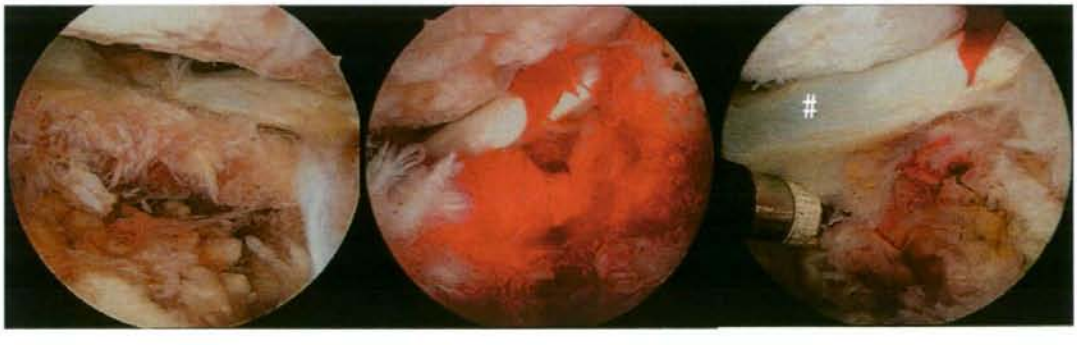

FIGURE 3. Arthroscopic treatment of case 1. The degenerative torn lateral meniscal rim is present between the femoral and tibial surfaces that are covered with patchy fibrous cartilage (left). The removal of a small meniscal segment induces pulsatile bleeding from the residual rim of the lateral meniscus (middle). Subtotal meniscectomy followed by coagulation using VAPR was performed (right). Note that nothing but the popliteus tendon can be seen in the lateral compartment (right, number sign).

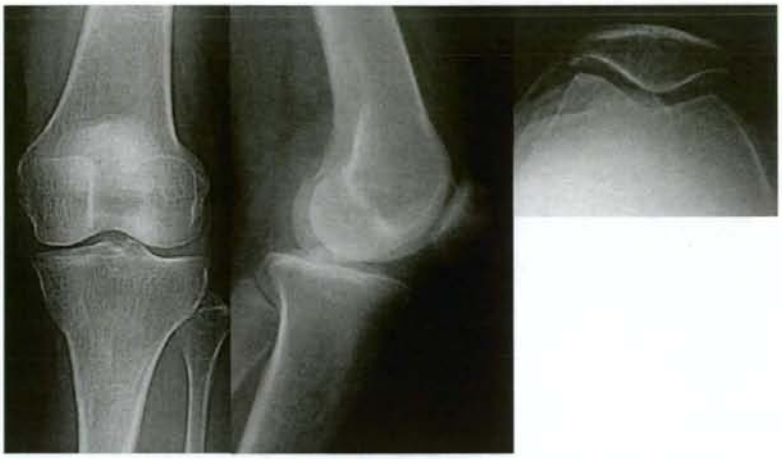

FIGURE 4. Radiographic findings of case 2 (an 83-year-old woman) showing no arthritic changes in the tibiofemoral joint (left) and slight osteoarthritic involvement of the patellofemoral joint (middle and right).

hemarthrosis associated with meniscal ganglion and treated with resection of the lateral meniscus and meniscal ganglion, speculating that the torn lateral meniscus caused the bleeding. From our experience, a majority of the patients (17 of 19) had degenerative torn lateral menisci as determined with MRI and at arthroscopy. The mostly successful treatment was meniscectomy and coagulation using a laser or radiofrequency system. A majority of so-called spontaneous recurrent hemarthroses of the knee in the elderly appear to be attributable to torn lateral menisci. Recurrent hemarthrosis of the knee caused by degenerative torn lateral meniscus or meniscogenic recurrent hemarthrosis (MRH) could be legitimate names for this condition. A small portion of patients might be suffering from bleeding from synovium, possibly around the PF joint, as Wilson speculated,[1] and only this condition should be called spontaneous recurrent hemarthrosis (SRH) of the knee in the elderly.

Considering that recurrent hemarthrosis occurs only in older patients, a combination of other factors, such as age-related mechanical and structural changes of vessels,[9-11] may also be necessary for this condition. The relatively high percentage of patients with hypertension in this case series might enhance these changes.[12,13]

As a treatment option, we recommend subtotal meniscectomy and coagulation using a radiofrequency system for MRH cases. For SRH cases, we are not positive that arthroscopic synovectomy is an effective treatment, because we could not detect a bleeding point arthroscopically. Two out of two SRH patients have not experienced hemarthrosis for more than 6 years after arthroscopy.

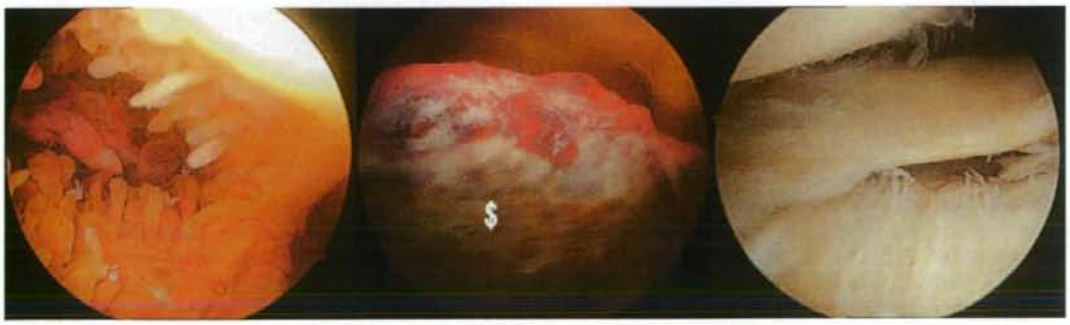

FIGURE 5. Arthroscopic treatment for case 2. Diffuse proliferation of brown synovium can be seen (left). A tumorous lesion can be seen in the suprapatellar pouch that is floating in the pouch with no connection to the joint capsule (middle, dollar sign). The lateral meniscus has surface fibrillation without tears (right).

FIGURE 6. H&E staining of the tumorous lesion in case 2 reveals a hematoma in the process of organization. The paucity of cellularity and abundant collagenous tissue are the main constituents of the lesion. Areas of hemosiderin deposition can also be seen. (Left: ×100; right: ×320.)

Considering that they had received arthrocentesis on about a once-a-month basis before surgery, arthroscopic synovectomy could be called a curative treatment. Therefore, arthroscopic synovectomy is a promising treatment, but it might be possible that the lesions are self-limiting conditions where conservative treatment might have given satisfactory results. Another possibility is that with arthroscopy or MRI, we could not detect intra-articular lesions that may lead to hemarthrosis, such as hemangioma or localized type pigmented villonodular synovitis, and these were removed coincidently upon synovectomy.

It is known that medial-dominant OA is more predominant in the population than lateral-dominant OA,[14] but in the present case series, most of the patients had lateral-dominant OA. Even with 6 cases of medial-dominant OA, they had OA changes in their lateral compartment, and torn lateral menisci were the most apparent arthroscopic findings. Therefore, the existence of OA changes in the lateral compartment is considered a typical radiographic finding of MRH. Pre-existing discoid lateral menisci might be a risk factor for MRH, because 6 of 17 cases had discoid lateral menisci. We have not yet identified why degenerative torn lateral meniscus but not medial meniscus exclusively causes hemarthrosis. Possible explanations for this disparity are the size difference of vascularity and different positional relationship between the genicular artery and the menisci. The lateral inferior and middle genicular arteries that supply the lateral meniscus are much larger than the medial genicular artery.[15] The proximity of the genicular artery to the meniscal rim is observed only on the lateral meniscus.[16] In the present study, throbbing bleeding indicting arterial bleeding was observed in the process of debridement of lateral menisci in 5 cases, which suggested direct bleeding from the lateral genicular artery. Negative pressure during knee motion, especially at the mid-flexion angle, might enhance bleeding and prevent coagulation.[17]

A couple of limitations should be noted. The small number of cases might not have been enough to describe the detailed etiology of the disease. But even with only 19 cases, this study appears to be the biggest surgically treated case series in the literature. Involvement of only arthroscopically treated cases might also be another limitation. The inclusion of conservatively treated cases might aid in the comprehension of a complete picture of the disease.

CONCLUSIONS

A majority of the patients (17 of 19) had degenerative torn lateral menisci confirmed with MRI and at arthroscopy. Successful outcomes were achieved by meniscectomy and coagulation. Most so-called spontaneous recurrent hemarthroses in OA knee joints appear to be attributable to torn lateral menisci.

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Original article

RGD peptide-induced cell death of chondrocytes and synovial cells

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Abstract

Background. Small peptides including the Arg-Gly-Asp (RGD) motif have been used in studies on cell-extracellular matrix (ECM) attachment due to their ability to disturb integrin-mediated attachment on the cell surface. As another biological action of RGD peptides, several reports have shown that RGD peptides are incorporated into cytoplasm and induce apoptosis by direct activation of caspase-3. This study evaluated the effect of RGD peptides on chondrocytes and synovial cells and studied the involvement of caspases.

Methods. Chondrocytes and synovial cells were isolated and cultured from the knee joints of New Zealand White rabbits. Cells were incubated in serum-free medium with peptides (RGD, RGDS, GRGDSP, GRGDNP, RGES), and the survival rates were evaluated. The rate of apoptotic cells was measured by flow cytometry in cells treated with RGDS, GRGDSP, and RGES. Caspase-3, -8 and -9 activity was measured in cells treated with RGDS and GRGDSP. Osteochondral explants harvested from rabbits were also incubated with RGD peptides (RGDS, GRGDSP, and GRGDNP), and the survival rate of chondrocytes was evaluated.

Results. The survival rate of cultured chondrocytes was significantly decreased in the GRGDSP- and GRGDNP-treated groups. The survival rate of synovial cells was significantly decreased with four of the RGD peptides (RGD, RGDS, GRGDSP, and GRGDNP) at 5 mM, and in the RGDS- and GRGDSP-treated groups at 1 mM. Flow cytometric assay revealed increases of apoptotic chondrocytes with GRGDSP and increases of apoptotic synovial cells with RGDS and GRGDSP. Caspase-3 was activated in chondrocytes treated with GRGDSP and it was also activated in synovial cells treated with RGDS and GRGDSP. Caspases-8 and -9 were not activated in chondrocytes or in synovial cells. The survival rate of chondrocytes in explants decreased in the superficial layer with all three RGD peptides (RGDS, GRGDSP, and GRGDNP) and in the middle layer with GRGDSP.

Conclusions. RGD peptides induced apoptosis in cultured chondrocytes as well as in cells in cartilage explants and synovial cells, presumably through direct activation of caspase-3.

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Introduction

Small peptides including the arginine-glycine-aspartate (RGD) motif are present in the extracellular matrix (ECM) and work as ligands for integrins.[1] Thus, RGD peptides have been used in studies on cell-ECM attachment due to their ability to disturb integrin-mediated attachment.[2,3] Cell attachment is crucial for cell survival in a variety of cells; therefore, adhesion-blocking agents, including RGD peptides, cause caspase-dependent apoptotic cell death, known as anoikis.[4] However, Buckley et al.[5] reported that RGD peptides were incorporated into cytoplasm and induced apoptotic cell death in lymphocytes through direct activation of caspase-3.

Caspases are a family of cysteine proteases that cleave their substrates at aspartate residues and play key roles in apoptosis.[6] Caspases exist in cells as catalytically inactive zymogens, referred to as procaspases.[7] Once activated, most caspases can process and activate themselves and other inactive procaspases. This characteristic suggests that caspases may execute the apoptotic program through a cascade of sequential activation of initiators, such as caspases-8 and -9, and executioners, such as caspases-3 and -7.[8]

The purpose of this study was to evaluate the effect of RGD peptides on cells in two major components of joint disease, cartilage and synovium. Specifically, the effect of RGD peptides to induce cell death was examined. Apoptotic cell death is one of the major factors in the progression or initiation of osteoarthritis (OA). Synovial cell proliferation is a typical feature of arthritic diseases. Knowledge of differences in the susceptibility to RGD peptides between chondrocytes and synovial

cells might be clinically useful in treating synovial proliferating diseases, or in creating a new osteoarthritic animal model. Thus, we first examined induction of cell death in cultured chondrocytes and synovial cells by RGD peptides, followed by examination of caspase involvement and annexin V induction in the pathway leading to cell death. Last, we examined the induction of cell death in chondrocytes in osteochondral explants by RGD peptides.

Materials and methods

Experiments were conducted in accordance with the guidelines for animal experimentation of the Ethics Review Committee of Chiba University.

RGD peptides

Four kinds of RGD peptides: i.e., RGD (Arg-Gly-Asp), RGDS (RGD-Ser), GRGDSP (Gly-RGDS-Pro), and GRGDNP (GRGD-Asn-P), and non-RGD peptide RGES (RG-Glu-S), were purchased (Funakoshi, Tokyo, Japan). Stock solutions (50 mM) were prepared by dissolving the peptides in phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA) and stored at −20°C. RGDS peptide labeled with fluorescein isothiocyanate (FITC; Funakoshi) was also prepared as a 50-mM stock solution.

Cell culture

Cartilage and synovium were harvested from the knee joints of 1-month-old New Zealand White rabbits. Chondrocytes were isolated by an enzymatic preparation. Briefly, cartilage specimens were minced and washed with PBS. The specimens were then treated with 0.05% hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 10 min and 0.2% trypsin (Wako, Osaka, Japan) for 15 min, followed by digestion with 0.2% collagenase (Asahi Techno Glass, Tokyo, Japan) for 1 h. Isolated chondrocytes were collected by centrifugation (1500 rpm, 10 min) and washed with PBS. Then they were suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM of L-glutamine (Gibco) and antibiotics consisting of penicillin G sodium and streptomycin sulfate (Gibco), and incubated at 37°C.

Synovial cells were isolated by enzymatic digestion as described previously.[9] Briefly, synovial tissues were minced and digested with Dispase Grade II (Roche Diagnostics, Indianapolis, IN, USA) for 2 h at 37°C. Isolated synovial cells were collected and washed with PBS. The cells were then seeded in RPMI1640 (Gibco) containing 10% FBS and antibiotics and incubated at 37°C.

Cells were cultured to first or second passage until cells increased sufficiently for analysis.

Viability of cultured cells

Chondrocytes and synovial cells were collected and resuspended at a density of 2×10^5 cells/ml in a 12-well plate with serum-free medium and incubated for 48 h at 37°C with 1 or 5 mM of peptides, i.e., RGD, RGDS, GRGDSP, GRGDNP, and RGES. Concentrations of peptides were determined according to previous reports.[5,10] Control cells were incubated in medium that did not contain any peptides. For detection of cell death, the Live/Dead Double Staining Kit (MBL, Nagoya, Japan) was used, following the manufacturer's instructions. Briefly, cells were collected and resuspended in staining solution containing 1 μM of CYTO-Dye (MBL, Nagoya, Japan), a cell-permeable green fluorescent dye, and 2.5 μg/ml of propidium iodide (PI), a cell nonpermeable red fluorescent dye, and incubated for 15 min at 37°C. Live cells were stained only with the CYTO-Dye and dead cells were stained with both CYTO-Dye and PI. The numbers of live and dead cells from each specimen were counted under a fluorescence microscope (Nikon, Tokyo, Japan) until the total cell number reached 100 cells in randomly selected fields of view. Survival rate was expressed as the percentage of live cells.

Flow cytometric analysis with Annexin V-FITC

Chondrocytes and synovial cells were harvested from five knee joints of New Zealand White rabbits. The cells were collected and resuspended at a density of 2×10^5 cells/ml in a 12-well plate with serum-free medium supplemented with 5 mM of peptides (RGDS, GRGDSP, and RGES). Cells were then incubated for 4 h at 37°C. Control cells were incubated in medium that did not contain any RGD peptides. A MEBCYTO apoptosis kit (MBL, Nagoya, Japan) was used for detection of apoptosis, according to the manufacturer's instructions. Briefly, the collected cells were washed and resuspended in 85 μl of binding buffer. Then, 10 μl of Annexin V-fluorescein isothiocyanate (FITC) and 5 μl of PI were added. After 15-min incubation at room temperature, the cells were counted with a FACScan cytometer (Beckton-Dickinson, San Diego, CA, USA) up to 5000 cells. The percentages of apoptotic cells in the total number of cells were calculated and were used for analysis.

Treatment of cells with FITC-labeled RGD peptide

To examine the localization of RGD peptides, cultured chondrocytes and synovial cells were collected and resuspended in serum-free medium supplemented with

5 mM FITC-labeled RGDS and incubated at 37°C. After 2 h, the cells were evaluated with a fluorescence microscope.

Caspase activity assay

Cultured cells were collected and resuspended at a density of 1×10^6 cells/ml in a 6-well plate with serum-free medium supplemented with 1 or 5 mM of RGD peptides (RGDS and GRGDSP). Cells were then incubated for 4 or 24 h at 37°C. Control cells were incubated in medium that did not contain any RGD peptides. After 4 or 24 h, intracellular caspase-3, -8, and -9 activity was measured with the APOPCYTO colorimetric assay kit (MBL) according to the manufacturer's instructions. Briefly, the cells were collected and resuspended in cell lysis buffer for 10 min. The cell lysates were centrifuged at 10000 *g* for 5 min at 4°C and the supernatants were obtained. Then, 50 µl of reaction buffer containing 10 mM dithiothreitol (DTT), 50 µl of cell lysates, and 5 µl of caspase substrates were added to each well of a 96-well microplate and incubated overnight at 37°C. These substrates were labeled with p-nitroanilide (pNA); i.e., DEVD-pNA for caspase-3, IETD-pNA for caspase-8, and LEHD-pNA for caspase-9. When substrates were recognized and cleaved by the corresponding caspases, pNA was released. Caspase activity was calculated by measuring the amount of free pNA at a wavelength of 405 nm with a microplate reader (Asahi Techno Glass, Tokyo, Japan).

Viability of chondrocytes in osteochondral explants

The bilateral proximal epiphyses of the tibiae (osteochondral explants) were harvested from four 15-month-old New Zealand White rabbits at a thickness of 5 mm horizontally. Special care was taken not to touch the cartilage surface. The explants were cultured in serum-free DMEM supplemented with 5 mM of RGD peptides (RGDS, GRGDSP, or GRGDNP). Control specimens were cultured without any peptides. After 48 h, two 1-mm-wide pieces of cartilage that were vertical to the joint surface were cut out from each condyle and stained with the Live/Dead Double Staining Kit (MBL). Cell viability of the superficial and middle layer was assessed separately with a confocal microscope (Carl Zeiss, Heidelberg, Germany). The deep layer was excluded in this study, because cells in the deep layer might have been affected at the time of cartilage preparation, i.e., necrotic cell death might have occurred at the bottom of cartilage when the cells were separated from the underlying subchondral plate. The survival rate of chondrocytes was determined by manual counting.

Statistical analysis

Values for results were expressed as the mean plus or minus standard deviation (SD). Statistical comparisons were done with the paired *t*-test. A significant difference was set at a level of $P < 0.05$.

Results

Viability of cultured cells

Representative images of RGD peptide-treated chondrocytes stained using the Live/Dead Double staining Kit are shown in Fig. 1A, where dead cells are stained yellow and live cells are stained green. The survival rate of chondrocytes was significantly decreased with both 1 mM and 5 mM GRGDSP and GRGDNP, compared to control (Fig. 1B). These two peptides induced cell death in more than 30% of chondrocytes. By contrast, dead cells accounted for less than 20% of RGES-treated cells and control cells. With 5 mM RGDS the survival rate (70.8 ± 6.1%) tended to be low, but this difference was not significant ($P = 0.052$).

The survival rate of synovial cells was significantly decreased with four of the RGD peptides at 5 mM (Fig. 1C, RGD, 65.0 ± 12.1%; RGDS, 65.3 ± 12.9%; GRGDSP, 60.9 ± 13.1%; and GRGDNP, 66.4 ± 6.9%), compared to RGES-treated cells and control cells. However, the survival rate at 1 mM was significantly decreased in only the RGDS and GRGDSP groups (58.8 ± 10.8% and 60.0 ± 15.3%, respectively). The difference in survival rates between 1 and 5 mM concentrations of these two peptides was not significant.

Flow cytometric analysis by Annexin V-FITC

The representative result of FACScan is shown in Fig. 2A. The percentage of apoptotic cells in chondrocytes treated with GRGDSP was significantly higher than that in RGES-treated cells and control cells (Fig. 2B; 7.00 ± 4.27%, 3.06 ± 1.75%, and 3.68 ± 2.05%, respectively). However, significant apoptotic cell death was not observed in the cells treated with RGDS (5.39 ± 2.03%).

The percentages of apoptotic cells in synovial cells treated with RGDS and GRGDSP were significantly higher than those in RGES-treated cells and control cells (Fig. 2C; 14.8 ± 5.46%, 10.8 ± 2.15%, 3.27 ± 1.93%, and 3.73 ± 0.87%, respectively).

Localization of RGD peptide

FITC-labeled RGDS peptide was used to determine the location of RGD peptide in chondrocytes and synovial cells. The labeled peptides were found in the cytoplasm and not on the cell surface in both chondrocytes and