RESULTS

Somatic *NLRP3* mosaicism in mutation-negative NOMID/CINCA syndrome patients. A heterozygous germline *NLRP3* mutation was detected in 1 of the 27 samples, and this was therefore excluded from the analyses. For each patient, 96 clones were selected at random for each amplicon. These were then sequenced. *NLRP3* mosaicism was detected in 18 of 26 patients (69.2%), and the level of allelic mosaicism ranged from 4.2% to 35.8% (mean ± SD 12.1 ± 7.9%; median 10.2%) (Table 1). Seven of the detected *NLRP3* mutations were novel (p.G307S, p.K355N, p.M406V, p.T433I, p.F566L, p.E567K, and p.K568N). The remaining mutations have been reported previously in NOMID/CINCA syndrome patients as disease-causing heterozygous germline mutations (p.L264F, p.D303H, p.G307V, p.A439P, p.Y570C, and p.G755R). Each of the 3 *NLRP3* mutations, p.F566L, p.E567K, and p.G755R, was detected in 2 unrelated patients. *NLRP3* mutation p.D303H was detected in 3 unrelated patients.

Analyses in family controls. To validate the clinical relevance of the *NLRP3* mosaicism identified in mutation-negative NOMID/CINCA syndrome patients, samples from 19 healthy relatives were investigated. No somatic mosaicism was detected in any of these samples. The *P* value from the comparison of cases and controls (18 of 26 versus 0 of 19) was statistically significant ($P < 0.0001$).

Functional effects of the identified somatic *NLRP3* mutations. Since disease-causing heterozygous germline mutations in *NLRP3* have been implicated in necrosis-like programmed cell death and ASC-dependent NF-κB activation (8), experiments were performed to determine whether the mutations identified in patients with somatic mosaicism showed the same effects. All of the identified mutations induced both THP-1 cell death (Figure 1A) and ASC-dependent NF-κB activation (Figure 1B). The in vitro effects of these novel mutations were similar to or even more pronounced than those of previously reported *NLRP3* mutations. This strongly suggests that all mutations showing somatic mosaicism have pathogenic effects, including the novel mutations identified in the present study.

Mutation frequency of *NLRP3* among various cell lineages and 1 tissue type. To explore the origin of the *NLRP3* mosaicism, mutational frequency was evaluated in various cell lineages and 1 tissue type from 4 Japanese patients with *NLRP3* somatic mosaicism. In each patient, the same mutations were found in all of the cell lineages investigated (neutrophils, monocytes, T cells, B cells) and in the buccal mucosa tissue, and no significant difference in mutation frequency was observed between these sources (Table 2).

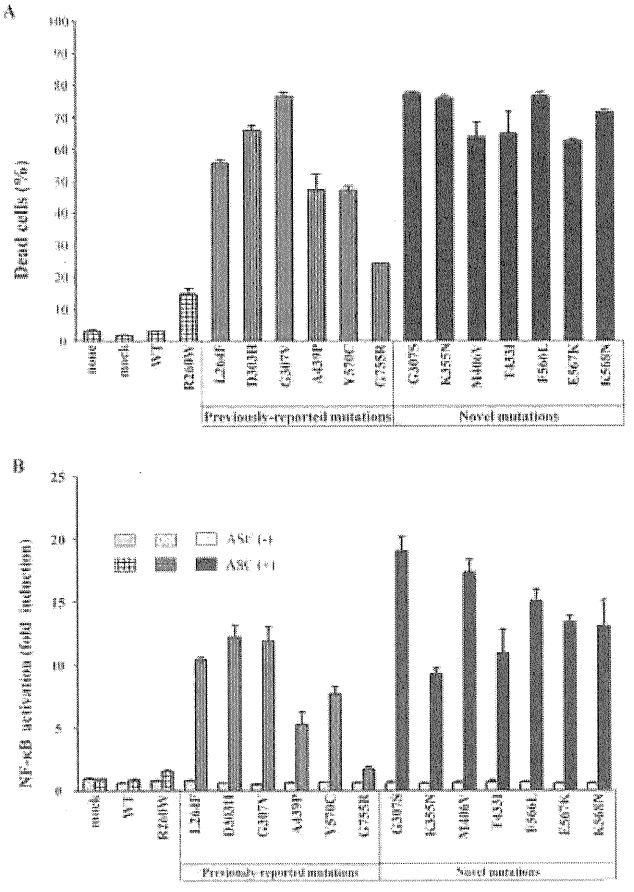

Figure 1. In vitro functional assessment of the identified *NLRP3* mosaicism mutations. **A,** Necrotic cell death of THP-1 cells induced by the identified somatic *NLRP3* mosaicism mutations. Green fluorescent protein (GFP)–fused mutant NLRP3 was transfected into THP-1 cells. The percentage of dead cells (7-aminoactinomycin D positive) among GFP-positive cells is shown. Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments. None = nothing transfected; mock = vector without NLRP3; WT = wild-type NLRP3; R260W = NLRP3 with p.R260W (frequent mutations in patients with cryopyrin-associated periodic syndromes). **B,** ASC-dependent NF-κB activation induced by the identified somatic *NLRP3* mosaicism mutations. HEK 293FT cells were cotransfected with WT or mutant NLRP3 in the presence or absence of ASC. The induction of NF-κB is shown as the fold change compared with cells that were transfected with a control vector without ASC (set at 1). Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments.

Table 2. Distribution and quantification of *NLRP3* mutations among sources of genomic DNA (4 cell lineages and 1 tissue type)*

Patient	Sequence variant	Protein variant	Mosaicism, %				
			Neutrophils	Monocytes	T cells	B cells	Buccal mucosa
J1	1709A>G	Y570C	12.6	9.8	8.0	9.5	8.3
J3	919G>A	G307S	9.1	10.8	6.9	10.6	9.0
J4	1699G>A	E567K	3.5	2.3	3.7	3.4	2.2
J5	907G>C	D303H	14.4	8.7	7.7	8.5	13.5

* No significant differences in the level of mosaicism were observed among the sources of genomic DNA.

Phenotype–genotype analysis. Given the previously reported genotype–phenotype association between the *NLRP3* gene and CAPS, the clinical characteristics of NOMID/CINCA syndrome patients with somatic *NLRP3* mutations were compared with those of patients from previous reports who had the same *NLRP3* mutations but with heterozygous germline status (1,4,10–13) (Figure 2) (further information is available at http://web16.kazusa.or.jp/download/). All of the patients in these 2 groups had an early onset of the disease, fever, and urticarial rash. The presence of arthritis, bony overgrowth, contractures, hearing loss, and seizure varied in each group of patients, and no significant difference was detected. However, whereas most patients with heterozygous germline *NLRP3* mutations presented with mental retardation, this was not the case for patients with somatic *NLRP3* mosaicism. A comparison was also made between the clinical data from patients with somatic *NLRP3* mosaicism and the data from patients with neither germline nor somatic *NLRP3* mutations. Again, a lower incidence of mental retardation was observed in patients with somatic *NLRP3* mosaicism

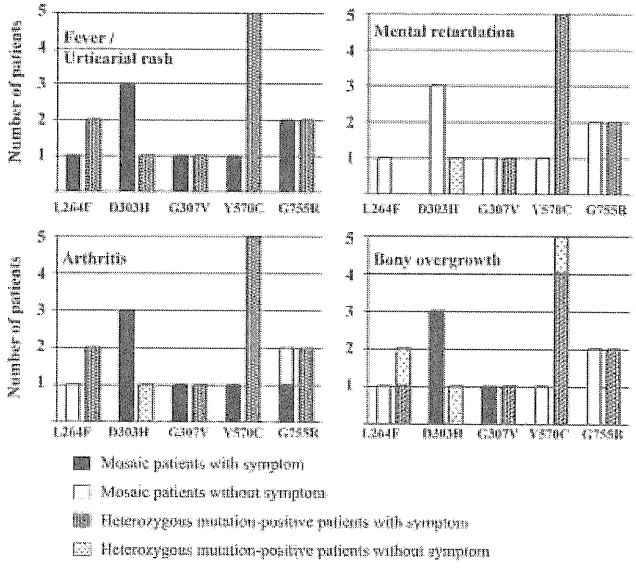

Figure 2. Comparison of the clinical profiles of patients carrying somatic *NLRP3* mutations and patients carrying the same mutation, but with germline status. Clinical profiles of patients with mosaicism and those of patients with heterozygous germline mutations are compared for each protein variant (L264F, D303H, G307V, Y570C, and G755R). The data on 4 typical clinical symptoms are shown. Total numbers of patients with mosaicism and total numbers of patients with heterozygous mutation examined are shown as a bar for each protein variant. Each bar is stratified according to the presence or absence of the symptom. For the protein variant L264F, no data on mental retardation were available for the patient with a heterozygous germline mutation.

Table 3. Clinical profiles of patients with somatic *NLRP3* mosaicism and patients with neither germline nor somatic *NLRP3* mutations*

	Patients with somatic *NLRP3* mosaicism (n = 18)	Patients with neither germline nor somatic *NLRP3* mutations (n = 8)
Age, median (IQR) years	12 (1–30)	10 (3–21)
No. of men/women	10/8	3/5
Age at onset, median (IQR) months	0 (0–24)	0.5 (0–33)
Fever	17/17	7/7
Urticarial rash	14/14	8/8
Mental retardation	4/17	6/8
Meningitis	13/17	5/8
Seizures	2/18	1/7
Hearing loss	10/18	2/7
Arthritis	14/17	7/8
Bony overgrowth	12/17	6/7
Contractures	7/17	4/7
Walking disability	8/18	3/7
Biologic therapy	10/15	3/8

* Except where indicated otherwise, values are the number with the feature/the total number of patients assessed. A lower incidence of mental retardation was observed in patients with somatic *NLRP3* mosaicism ($P = 0.03$). No other significant differences were detected between the groups. IQR = interquartile range.

($P = 0.03$). No other significant differences were detected (Table 3) (further information is available at http://web16.kazusa.or.jp/download/).

DISCUSSION

The present international multicenter study investigated 26 NOMID/CINCA syndrome patients who were mutation negative according to conventional sequencing along with 19 family controls to determine whether low-level mosaicism is a disease-causing genetic mechanism. Following our first report of low-level somatic mosaicism in a NOMID/CINCA syndrome patient (14), we reported a new method of detecting low-level *NLRP3* mosaicism, in which lipopolysaccharide (LPS) induced cell death specifically in *NLRP3* mutation–positive monocytes (8). However, this method requires fresh live monocytes, special equipment such as a cell sorter, and experience in its use due to the rapid time course of LPS-induced necrotic monocytic death. For these reasons, application of this method was not feasible in an international collaborative study. We therefore opted to use genomic DNA, since it is easier to handle and can be stored and shipped. Based on our previous study in Japanese patients showing that the frequency of mutant alleles could be <5%, we designed a subcloning and Sanger-sequencing strategy that could detect this very low allelic mutation frequency.

Presuming that the present cohort is representative of the 40% of NOMID/CINCA syndrome patients who are mutation negative according to conventional sequencing, the results suggest that ~28% of all NOMID/CINCA syndrome patients may carry somatic *NLRP3* mosaicism. CAPS patients present with a continuous spectrum of symptoms, and a degree of genotypic overlap is observed between disease subtypes. Although the present study focused on the most severe NOMID/CINCA syndrome phenotype, it is possible that somatic *NLRP3* mosaicism may also occur in milder forms of CAPS. The presence of somatic mosaicism should also be investigated in patients with other dominantly inherited autoinflammatory diseases caused by gain-of-function mutations and who are mutation negative according to conventional sequencing.

Among the 18 patients with somatic *NLRP3* mosaicism, we found 6 mutations that have previously been identified in NOMID/CINCA syndrome patients as heterozygous germline mutations. We also identified 7 novel mutations, which were confirmed as being functionally active and presumably pathogenic. Functional in vitro assays showed that these novel mutations had greater disease-causing capacity than the previously described mutations. This suggests that the novel mutations may be deleterious and unrecognized if inherited as heterozygous germline mutations.

The present study also addressed the important question of how somatic *NLRP3* mosaicism modifies clinical presentation. Although no statistically significant differences in age at disease onset, skin symptoms, joint involvement, or response to IL-1 blockade were detected, milder neurologic involvement was observed in patients with somatic mosaicism. Comparisons with NOMID/CINCA syndrome patients carrying the same *NLRP3* mutations but with heterozygous germline status made this tendency more prominent. Although the level of somatic mosaicism in blood leukocytes was relatively low, it remains unclear how these low-level mutations influence clinical presentation, including disease severity. One interesting hypothesis is that the difference in the severity of neurologic manifestations is a function of the level of mosaicism. For ethical and technical reasons, it was not possible to evaluate the level of mosaicism in central nervous system (CNS) cells or glial cells in the present study, and this therefore awaits investigation in future studies.

The mechanism through which *NLRP3* somatic mosaicism occurs also requires elucidation. The present study demonstrated that similar proportions of neutrophils, T cells, B cells, monocytes, and buccal cells carried the mutated allele. Therefore, the mutation leading to mosaicism must have arisen before the pluripotent stem cells committed to hematopoietic progenitor stem cells or ectoderm-derived nonhematopoietic cells. Several mechanisms for mosaicism have been proposed, including chimerism due to cell fusion with an aborted dizygotic twin and a mutational event during early embryogenesis (15). The latter mechanism is more likely in the present cohort, since mosaicism at similar frequency was detected in several cell types. To verify the hypothesis of a mutational event during embryogenesis, and to determine the point at which this occurred, it would be helpful to analyze other tissues. However, obtaining such tissues from patients may be ethically problematic.

Approximately 12% of the patients in the present cohort carried neither germline nor somatic *NLRP3* mutations and may therefore be considered to be genuinely mutation negative. However, it is possible that these patients have *NLRP3* mutations that have been overlooked. A recent report described a mutation in the 5′-untranslated region of *NLRP3* in a patient with FCAS

(16), although it remains unclear how this noncoding mutation causes disease. Another possibility is that an extremely low frequency of NLRP3 mosaicism may have been missed. The subcloning and Sanger-sequencing strategy used in this study set the detection limit of mosaicism at 5%. Considering the range of *NLRP3* mosaicism detected (4.2–35.8%), the median (10.2%), and the identification of 2 patients with <5% mosaicism, it is indeed likely that patients with an even lower level of *NLRP3* mosaicism may have been overlooked. Recent advances in next-generation DNA sequencing technology may resolve this technical problem, although the associated error rate could be problematic. Another possibility is that *NLRP3* mutations were present in uninvestigated cell lineages, such as those from CNS tissue, bone tissue, or skin. Future studies of NOMID/CINCA syndrome should investigate these tissues while searching for mutations in other genes.

In conclusion, the present study has clearly demonstrated that a significant proportion of NOMID/CINCA syndrome patients who were mutation negative according to conventional sequencing carried somatic *NLRP3* mutations with a variable degree of mosaicism. Clinicians should therefore consider somatic mosaicism as a possible cause of disease in mutation-negative NOMID/CINCA syndrome patients and implement appropriate therapy. The early diagnosis of NOMID/CINCA syndrome and prompt initiation of therapy would improve clinical outcome. Further goals in this research field are the refinement of genetic screening and the verification of the functional consequences of all detected somatic mutations. Systematic screening for somatic mosaicism will provide new insights into the etiology of human disease.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Ohara and Nishikomori had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Saito, Ohara, Nishikomori, Kambe.

Acquisition of data. Tanaka, Izawa, Saito, Oshima, Ohara, Nishikomori, Goldbach-Mansky, Aksentijevich, de Saint Basile, Neven, van Gijn, Frenkel, Aróstegui, Yagüe, Merino, Ibañez, Pontillo, Takada, Imagawa.

Analysis and interpretation of data. Sakuma, Morimoto, Kawai, Yasumi, Nakahata, Heike.

ROLE OF THE STUDY SPONSOR

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特集
小児医療における診断・治療の進歩　22
治療技術

Key words
induced pluripotent stem（iPS）細胞
臨床応用
疾患特異的 iPS 細胞
遺伝子治療
個別化医療

疾患特異的 iPS 細胞を用いた遺伝子治療・個別化医療

中畑 龍俊（なかはた たつとし）*

要旨

山中教授らにより成熟した細胞にたった 4 つの転写因子遺伝子を導入することにより作製されたヒト iPS 細胞は，未分化な状態のままほぼ無限に増やすことができ，われわれの身体を構成するすべての細胞への多分化能を有することから，幅広い医療への応用が期待されている．iPS 細胞のもつもっとも画期的な臨床的側面は，さまざまな疾患の患者から皮膚などの組織を用いて疾患特異的 iPS 細胞を樹立できることである．この細胞を用いて，診断，疾患の病態解析，新規治療法の開発，新規薬剤の有効性・毒性の検定などに応用されると考えられる．将来的には患者 iPS 細胞は異常遺伝子を修復した遺伝子治療と複合した再生医療や個別化医療への応用が期待されている．

はじめに

ヒト受精卵を滅失して樹立される ES 細胞は，社会的，倫理的に多くの問題を抱えているが，ほぼ無限に増殖でき，さまざまな細胞に *in vitro* で分化可能であることから，幅広い再生医療への応用が期待され，米国では昨年ヒト ES 細胞を用いた再生医療が開始された．人工多能性幹細胞（induced pluripotent stem cell：iPS 細胞）は受精卵を破壊することなく同様な能力を持った細胞を健常人や患者自身の皮膚や血液から作製可能なことから，再生医療への応用のみならず疾患解析や新規治療法の開発に応用されている．本稿では疾患特異的 iPS 細胞を中心にその遺伝子治療や個別化医療への応用について概説した．

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Ⅰ 人工多能性幹細胞（iPS 細胞）

2006 年，山中ら[1]はレトロウイルスベクターを用いてマウス胎児線維芽細胞に Oct3/4，Sox2，Klf4，および c-Myc というたった 4 つの転写因子遺伝子を導入することにより，ES 細胞と比べても遜色がない能力を持つ iPS 細胞の樹立に成功し，世界中に大きな衝撃を与えた．iPS 細胞は ES 細胞特異的なマーカーを発現するようになり，*in vitro*，*in vivo* で三胚葉系の細胞に分化することができ，さらにキメラマウスを作出することも可能であったことから，ES 細胞と同等の機能を獲得したものであると考えられた．翌年，彼らはヒト皮膚線維芽細胞にマウスと同様の 4 つの遺伝子を導入することによりヒト iPS 細胞の樹立に成功した（図 1）[2]．

ヒト iPS 細胞は，受精卵を滅失することなく作製できることから，ヒト ES 細胞の持つ社会的，倫理的な多くの問題を回避することができ

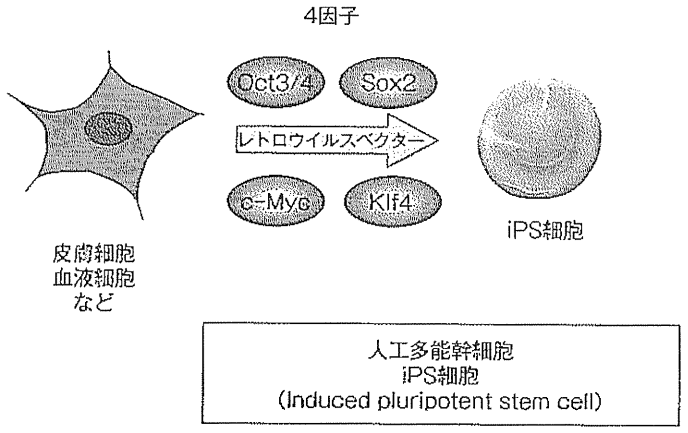

図1 ヒトiPS細胞の誘導

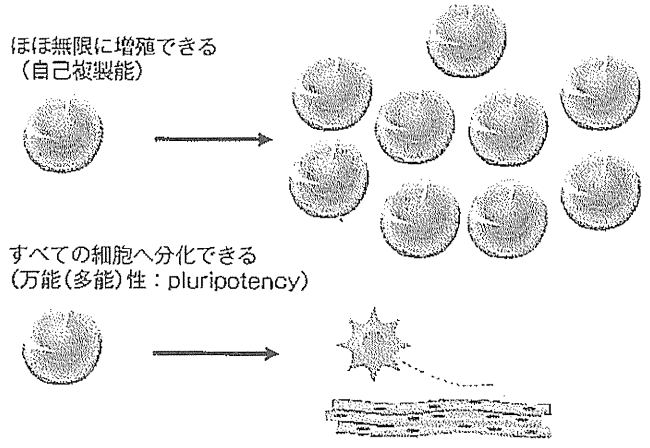

図2 iPS細胞の特性

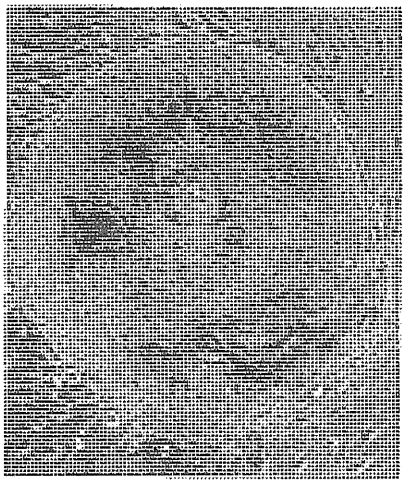

図3 ある患者から樹立されたiPS細胞
細胞が密に接触しながら増殖している.

る．ヒトiPS細胞はES細胞と同様に未分化な状態のままほぼ無限に増やせること，培養条件を変化させると，神経細胞，心筋，骨格筋，血管内皮細胞，軟骨や骨の細胞，膵島細胞，肝細胞，各種血液細胞などさまざまな細胞に*in vitro*で分化可能であることから，幅広い再生医療や遺伝子治療への応用が期待されている（図2）．臨床応用可能な，より安全性の高いiPS細胞を樹立するためさまざまな検討が行われている．

当初，皮膚線維芽細胞から樹立されていたiPS細胞はその後，骨髄細胞，臍帯血，末梢血，毛根細胞，歯髄細胞，脂肪組織などさまざまな組織から樹立可能となり，どの組織を材料にして作製したiPS細胞がもっとも優れているか検討が行われている．

当初Oct3/4, Sox2, Klf4, c-Mycの4つの転写因子遺伝子を導入してiPS細胞の樹立が行われていたが，最近ではがん遺伝子でもあるc-

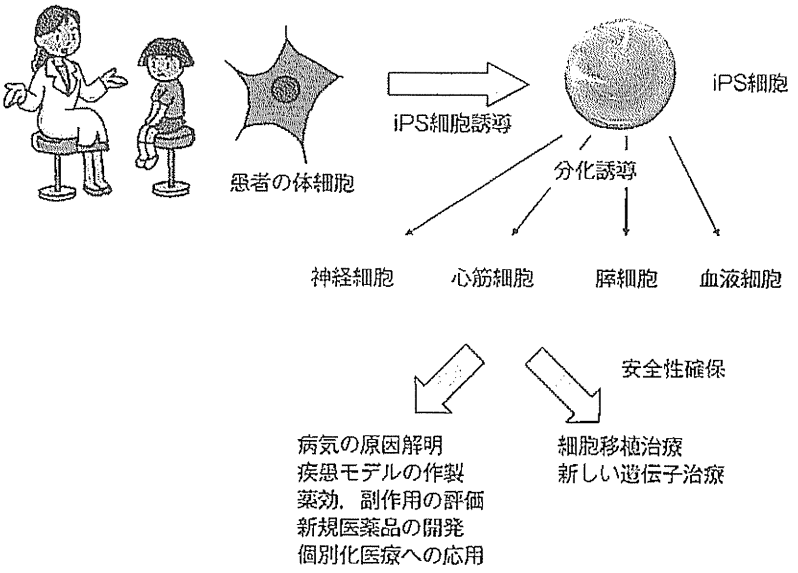

図4　疾患特異的 iPS 細胞を用いた新しい医療の開発

Myc の代わりに L-Myc が用いられるようになった[3]．また，遺伝子導入にはレトロウイルスベクターが用いられていたが，染色体に組み込まれないアデノウイルスベクター，センダイウイルスベクターや episomal plasmid vectors などが用いられるようになってきている[4]．

2010 年 11 月 1 日に改正された「ヒト幹細胞を用いる臨床研究に関する指針」ではヒト iPS 細胞を用いた再生医療も指針の中に含まれている．iPS 細胞を用いた再生医療はそう遠くない将来わが国でも始まるであろう．iPS 細胞を用いた遺伝子治療についても現在見直しが始まろうとしている．

Ⅱ 疾患特異的 iPS 細胞

iPS 細胞の持つ意義として，基礎研究への貢献，再生医療や創薬への応用などさまざまな面がいわれているが，わが国では再生医療への応用を目指した研究を中心に研究費が投下されてきた．一方，われわれは当初から，患者の細胞から疾患特異的 iPS 細胞を樹立し（図 3），それを用いた疾患の病因，病態解析，遺伝子治療への応用を中心に研究してきた．

iPS 細胞の持つもっとも画期的な臨床的側面は，さまざまな疾患の患者から皮膚などの組織を用いて疾患特異的 iPS 細胞を樹立できることである．この細胞を用いて，疾患の病態解析，新規治療法の開発，新規薬剤の有効性・毒性の検定などに応用されると考えられる．将来的には患者 iPS 細胞は異常遺伝子を修復した遺伝子治療と複合した再生医療につながるものと期待される（図 4）．

1．疾患特異的 iPS 細胞の診断・病態解析への応用

疾患特異的 iPS 細胞を用いることによりさまざまな研究が進展すると予想される．その中でも，iPS 細胞を用いた診断，病態解析，疾患モデルの作製など患者の治療に直結する成果が期待されている．

a．診断への応用

iPS 細胞から生検が困難な組織の細胞を作り出し，それを用いた疾患の診断が期待される．患者皮膚などから樹立した iPS 細胞を生検困難

な大脳，小脳，脊髄などの中枢神経組織に分化させ，それを使った診断や病態解析が考えられる．心筋，軟骨，肺，膵臓などの組織は生検可能だが，大量の組織を繰り返し採取することはできないので，そのような場合も対象となりえるだろう．

b．病気の発症機序の解明

患者からつくったiPS細胞を患部と同じ組織に分化させることができれば，病気発症機序を詳細に解析できる可能性がある．健常人と患者のiPS細胞を同じように分化させて，各分化段階の細胞を回収し比較することにより，いままでとはまったくちがう手法で病気の本態に迫ることができるかもしれない．

また，Kostmann症候群の一部では，好中球減少と中枢神経の異常を，Shwachman症候群では膵外分泌の異常と造血障害を合併することが知られているが，どうみても関係のない2つの異常がなぜ合併するのかまったくわかっていない．これらの患者から疾患特異的iPS細胞を樹立し，その細胞から血液（好中球），神経，膵臓に分化させ，その過程を正常と比較することにより新たな知見が得られるのではないかと考えている．

c．疾患モデルの構築

iPS細胞技術は，今までモデル動物のなかった疾患に対して新しいモデルを提供すると考えられる．たとえば脊髄性筋萎縮症（spinal muscular atrophy：SMA）と筋萎縮性側索硬化症（amyotrophic lateral sclerosis：ALS）は筋組織を支配している運動ニューロンが選択的に変性，死滅し，その結果筋力低下，筋萎縮が生じ，不幸な転機をとる疾患である．しかし，なぜ運動ニューロンが選択的に変性，死滅してしまうのかまったくわかっていない．ともによい動物モデルがないことから病態解明が進まず，治療法の開発のためには新しいモデルの開発が待たれている．iPS細胞技術を使うことで，SMA，ALS患者の皮膚細胞から疾患特異的iPS細胞を樹立し，この細胞からSMAやALS患者と同じ遺伝子セットをもった運動ニューロン細胞を大量に作り出すことができるようになった．この細胞を使うことで，なぜ患者の運動ニューロンは正常iPS細胞由来運動ニューロンに比べ壊れやすいのかの解明が可能となり，これら疾患の病態解明や薬剤探索のスタートラインに立つことができるようになった．

d．時空を超えて病気の本態に迫る

Duchenne型筋ジストロフィー患者は一般に10歳頃に歩けなくなり，20歳ぐらいで人工呼吸器が必要となる．たとえば10歳の患者から筋生検をして診断する際，皮膚または筋肉の細胞からiPS細胞を樹立し，その細胞から分化させた骨格筋組織は，いわば生まれたての筋肉の細胞と同じ状態であることが予想される．つまり10年間という時間を過去に遡って，そのときの筋肉の状態と現在の筋組織を比較することが可能となると考えられる．このような手法は，今までには考えられなかったことであり，iPS細胞が持つ強力なインパクトではないかと思っている．

Ⅲ 疾患特異的iPS細胞の遺伝子治療・個別化医療への応用

1．遺伝子治療への応用

遺伝子治療は疾病の治療を目的として遺伝子または遺伝子を導入した細胞をヒトに投与することにより疾病の治療を直接の目的とするものを指すが，遺伝子マーキングのような新しい治療法の開発や従来の治療法の検証に使われるものも広義の遺伝子治療に含める場合もある．しかし，アンチセンスを用いた治療は一般には遺伝子治療には含めない．各種疾患の遺伝子異常を修復し，正常の遺伝子に戻すことが究極の目標であるが，現在そのための相同組み換えの技術はヒトの遺伝子治療では確立されたとはいえない．そのため，子孫に影響が残る可能性がない体細胞を標的として，欠陥遺伝子の補充

(gene complimentation)，あるいは正常の遺伝子の付加（gene addition）の形で遺伝子治療が行われている．

遺伝子の投与法は *in vivo* 法と *ex vivo* 法に分けられる．前者は治療用の遺伝子を直接患者に投与する方法で簡便ではあるが，目的とする標的細胞へ選択的に遺伝子を導入することが難しく，安全性のチェックも容易でない．後者は治療の標的となる細胞を一旦体外に取り出し，遺伝子導入を行った後患者に戻す方法である．この方法は特定の細胞だけに遺伝子を導入できより安全な方法といえるが，対象が体外に取り出せる造血幹細胞などの細胞に限られていた．また，この造血幹細胞にしても確実に遺伝子が付加あるいは補充できている細胞だけを取り出してそれを治療に用いることはできなかった．さらに造血幹細胞への遺伝子導入は容易ではなく，加えて造血幹細胞の持つ自己複製能は限定的なことから遺伝子治療後導入細胞が時間とともに消滅していくこともしばしばみられた．

このような従来の遺伝子治療の抱える問題点を解決してくれそうなのがiPS細胞を用いた遺伝子治療であり，大きな期待が寄せられている．その主な理由は患者皮膚や血液からiPS細胞を作製すれば，この細胞は患者が侵されている臓器を含むすべての細胞に分化できることから，従来の方法では細胞を体外に取り出すことができないために対象とならなかった疾患にも応用できること，iPS細胞に遺伝子を導入し確実に遺伝子が付加あるいは補充できているiPS細胞クローンを選別し，これを増やすことができるようになることなどである．さらに従来は不可能と考えられていたヒトにおける相同組み換え技術を用いた究極的な遺伝子修復も，iPS細胞を用いれば可能になるのではないかと期待されている．

すでにマウスでは，鎌状赤血球のモデルマウスの尻尾から採取した細胞に山中4因子を導入して作製したiPS細胞の異常遺伝子を修復し，修復されたiPS細胞から作られた造血前駆細胞を用いた移植実験で，鎌状赤血球マウスの貧血が良くなったモデルが報告されている（図5）[5]．また，さまざまな致死的代謝異常症に対する新しい治療として，羊水検査で得られた細胞からあらかじめiPS細胞を作製し，この細胞の遺伝子を修復したiPS細胞を用意しておき，出生直後にこの細胞を用いて治療するモデルも提唱されている（図6）[6]．将来の遺伝子治療は，iPS細胞を使って行う治療法が主となっていくのではないかと筆者は考えている．

②．個別化医療への応用

a．患者自身の細胞を使った薬物試験

新薬の開発に当たっては，一般的に前臨床試験として動物実験を行うが，動物実験の結果が必ずしもヒトに当てはまるわけではない．次に健常人で薬物試験を行い，ある程度安全性を確認してから患者での臨床試験に移ることが多い．しかし，健常人には異常がなくても，患者では重篤な副作用が出現することがありしばしば問題となる．実際薬を投与されるのは患者なのだから，患者の細胞そのものを使って毒性試験や有効性試験をするほうがよいはずであり，疾患特異的iPS細胞から目的とする細胞に分化させ，その細胞を用いて毒性試験や有効試験をする時代が始まろうとしている．

b．個別化医療への応用

最近米国では，QT延長症候群のiPS細胞樹立が盛んに行われている．QT延長症候群は8つ以上のタイプに分かれ，タイプによって使用する薬が違うため，タイプを決めるのに薬物負荷試験をする必要がある．ところがこの薬物負荷試験が非常に危険な検査で，致死的な不整脈が出現することがあり，除細動器を横に置き，循環器専門医が慎重に薬物負担を行い診断している．そこでQT延長症候群の患者の皮膚から作製したiPS細胞を *in vitro* で心筋に分化させ，この細胞を用いて薬物負荷試験が行われている．従来患者で行っていた負荷試験を，iPS

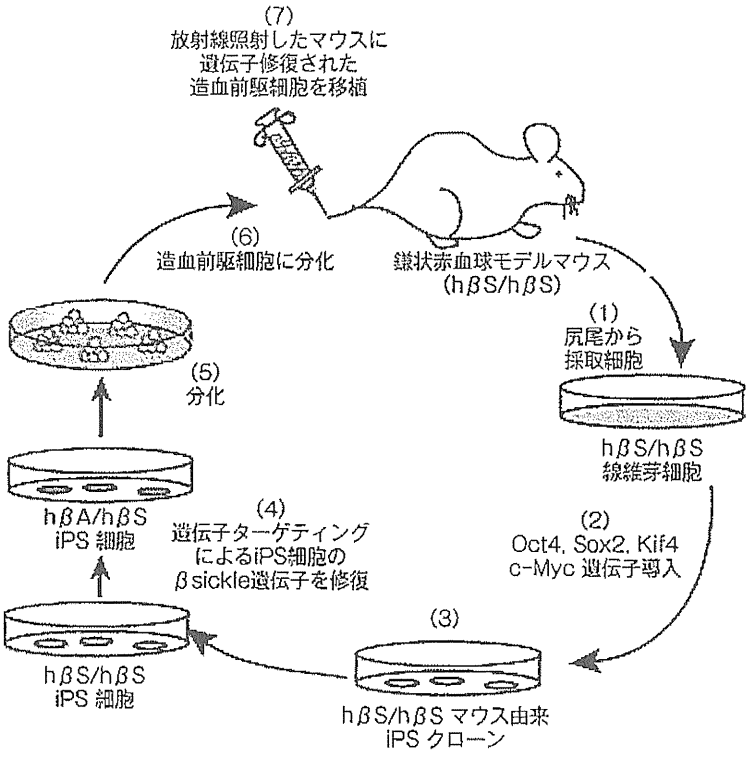

図5　iPS 細胞を用いた遺伝子治療の可能性：鎌状赤血球症マウスの例（Hanna J et al, 2007[9]より改変）

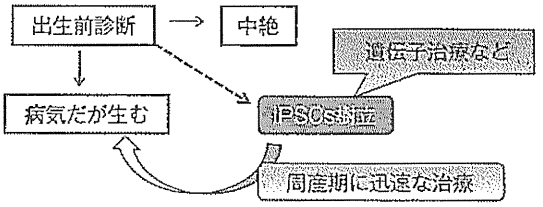

図6　出生前診断を遺伝病治療に応用できるよう，羊水検査や絨毛検査で得られた細胞から iPS 細胞を樹立（Ye L et al, 2009[6]より改変）

細胞で代用できるようになるかもしれない．このような iPS 細胞から分化させた細胞を用いてさまざまな負荷試験を行う時代がくることも考えられる．

おわりに

遺伝的疾患では iPS 化（初期化：リセット）した細胞を再び分化させることで，疾患特異的な現象を再現できる可能性が高いと考えられる．いままでとは違ったアプローチにより病気の本体に迫っていけるかもしれない．疾患特異的 iPS 細胞は，今まで考えられなかった新しい研究手段を提供しており，患者に還元される多くの知見が得られることが期待される．疾患特異的 iPS 細胞は疾患の病因，病態の解明に有用であるとともに，遺伝子治療や個別化医療などの新規治療法の開発へ結び付くことを考えると，iPS 細胞技術は小児科（子ども）のために開発してくれたのではないかと思ってしまう．この分野の研究の発展に期待したい．

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arthritis research&therapy

RESEARCH ARTICLE Open Access

Prostaglandin E2 receptor type 2-selective agonist prevents the degeneration of articular cartilage in rabbit knees with traumatic instability

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Abstract

Introduction: Osteoarthritis (OA) is a common cause of disability in older adults. We have previously reported that an agonist for subtypes EP2 of the prostaglandin E2 receptor (an EP2 agonist) promotes the regeneration of chondral and osteochondral defects. The purpose of the current study is to analyze the effect of this agonist on articular cartilage in a model of traumatic degeneration.

Methods: The model of traumatic degeneration was established through transection of the anterior cruciate ligament and partial resection of the medial meniscus of the rabbits. Rabbits were divided into 5 groups; G-S (sham operation), G-C (no further treatment), G-0, G-80, and G-400 (single intra-articular administration of gelatin hydrogel containing 0, 80, and 400 μg of the specific EP2 agonist, ONO-8815Ly, respectively). Degeneration of the articular cartilage was evaluated at 2 or 12 weeks after the operation.

Results: ONO-8815Ly prevented cartilage degeneration at 2 weeks, which was associated with the inhibition of matrix metalloproteinase-13 (MMP-13) expression. The effect of ONO-8815Ly failed to last, and no effects were observed at 12 weeks after the operation.

Conclusions: Stimulation of prostaglandin E2 (PGE2) via EP2 prevents degeneration of the articular cartilage during the early stages. With a system to deliver it long term, the EP2 agonist could be a new therapeutic tool for OA.

Keywords: prostaglandin E2, EP_2, ONO-8815Ly, osteoarthritis, ACLMT

Introduction

Osteoarthritis (OA) is the single most common cause of disability in older adults [1]. It is a complex process involving a combination of cartilage degradation, repair, and inflammation. However, its pathogenesis is not yet fully understood [2]. Articular cartilage is composed of chondrocytes, and an extensive extracellular matrix (ECM). The major ECM components are type II collagen and aggrecan. In normal cartilage, catabolic and anabolic activities are in dynamic equilibrium. Chondrocytes can produce several catabolic cytokines such as IL-1 and TNF-α, which in turn induce the production of proteinases including matrix metalloproteinases (MMPs) and disintegrin-like and metalloproteinase with thrombospondin, that lead to the destruction of the matrix network [3,4]. Among the MMPs, MMP-13 (collagenase 3) plays a particularly important role in causing OA [5]. Indeed, transgenic mice carrying an inducible human *MMP-13* gene develop pathological changes similar to those observed in human OA patients, when the transgene is expressed in articular cartilages of postnatal mice [6]. Moreover, inhibitors of MMP-13 prevent the degradation of articular cartilage [5,7]. Chondrocytes also produce anabolic cytokines such as the bone morphogenetic protein family members and insulin-like growth factor-1 (IGF-1), which induce the synthesis of collagen and initiate the proliferation of chondrocytes [3]. A disruption of

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the equilibrium between the catabolic and anabolic activities results in catastrophic damage to the articular cartilage, ultimately inducing the pathological condition known as OA.

Prostanoids, including prostaglandin (PG) D2, PGE1, PGE2, PGF2α, prostacyclin (PGI2), and thromboxane A2, are lipid mediators produced in a sequence of cyclooxygenase (COX) -1, -2-catalyzed reactions [8]. The role of PGE2 in the development of OA is controversial. Some reports point to an important role in inflammation [9]. Pro-inflammatory signaling mediators such as IL-1 and TNF-α induce the synthesis of PGE2 by promoting the expression or activities of COX-2 and microsomal PGE synthase-1 [10]. PGE2 then promotes IL-1 expression as part of a positive feedback mechanism, degrades the cartilage ECM [4,10-13], and finally induces apoptosis of chondrocytes [3]. Other reports insist that PGE2 opposes the effect of IL-1 [14] and stimulates the gene expression of type II collagen [3,15]. In addition, PGE2 stimulates the synthesis of proteoglycan and collagen through the expression of an IGF-1-binding protein [16,17]. PGE2 works through four isoforms of the EP receptor, EP1 to EP4. Previously, we considered that the controversy could result from differences in the mode of action and tissue distribution of each receptor [18]. Using an EP2 selective agonist, we showed that EP2 receptor-mediated PGE2 signaling enhances the growth of chondrocytes [18,19] and promotes the regeneration of articular cartilage in rabbits with cartilage defects [19].

In the current study, we investigate the effect of an EP2 agonist on articular cartilage in a rabbit model of traumatic degeneration.

Materials and methods

Materials

Microspheres loaded with a selective EP2 agonist, ONO-8815Ly (lysine salt) [20], were prepared by the emulsion-solvent evaporation method [19,21]. Briefly, ONO-8815Ly and polylactic-co-glycolic acid (PLGA) were mixed to form a water/oil emulsion, and added to the outer water phase containing polyvinyl alcohol under stirring with a turbine-shaped mixer at 5000 rpm to obtain a water/oil/water emulsion. PLGA microspheres that did not contain ONO-8815Ly in its free form were recovered by centrifugation and lyophilized to remove residual organic solvent and water. Then, a gelatin aqueous solution (20%, w/w) was poured into the microsphere suspension to form a gel. For the crosslink reaction, a glutaraldehyde aqueous solution (12.5 mg/ml) was poured into the microsphere suspension. Small cylinder-shaped gelatin hydrogels (4 mm in diameter and 2 mm in thickness) containing ONO-8815Ly (0, 80, or 400 μg of ONO-8815/gel) were obtained by hollowing out the gelatin hydrogel sheet. Diffusion kinetics analyses showed that ONO-8815Ly is gradually released from the microsphere over a period of seven days *in vitro* (Figure 1).

Animal model for traumatic degeneration

Four-month-old female Japanese white rabbits (weighing approximately 3 kg) were used. Traumatic degeneration was induced as described for the anterior cruciate ligament and menisectomy transection (ACLMT) model [22]. Operations were performed under general anesthesia, and a skin incision was made on the medial side of the patella. Soft tissues and articular capsules were cut to expose the knee joints. The anterior cruciate ligament was transected at the attachment to the tibia in the knee-flexed position, and the anterior horn of the medial meniscus was resected. The articular capsule and skin were sutured in layers with 4-0 nylon sutures. After the operation, rabbits were allowed to move freely. Preliminary experiments revealed that osteoarthritic changes were observed in this model at as early as two weeks after operation (data not shown).

Treatments with the EP2-agonist

A total of 64 animals were randomly assigned to five groups: G-S (sham operation), G-C (no further treatment), G-0, G-80, and G-400 (single intra-articular administration of gelatin hydrogel containing 0, 80, and 400 μg of ONO-8815Ly, respectively). Sham-operated rabbits (G-S; n = 4) received no further treatment, and were sacrificed either 2 (n = 2) or 12 weeks (n = 2) after the operation.

The ACLMT surgery was performed on both the knees of each of the remaining 60 rabbits to avoid any unequal bearing of weight due to pain on one side. No further treatment was performed in animals of the

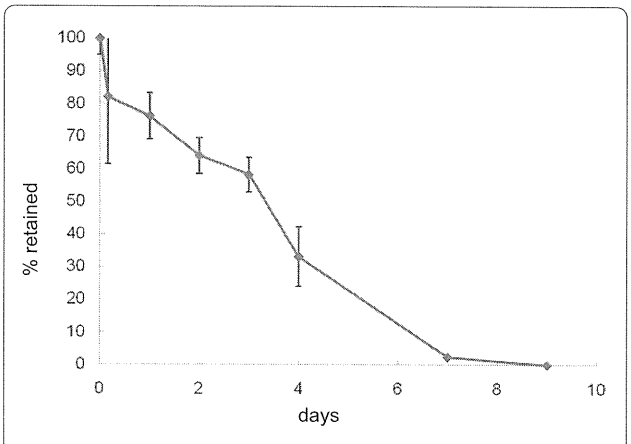

Figure 1 The diffusion kinetics of ONO-8815Ly from microspheres *in vitro*. Microspheres loading ONO-8815Ly were soaked in PBS, and the amount of retained ONO-8815Ly at each time point was measured by high-performance liquid chromatography and calculated as the ratio to the initial amount (n = 5).

control group (G-C; n = 12). In the treatment groups, no further treatment was performed on the right knee, but a gelatin hydrogel cylinder containing ONO-8815Ly (G-0, G-80, and G-400; n = 16 per group) was placed on the fatty pad of the left knee at the time of operation. Rabbits were sacrificed two weeks (G-C, n = 6; G-0, G-80, and G-400, n = 10 per group) or 12 weeks (n = 6 per group) after the operation. All the experiments with animals were approved by the institutional animal research committee, and performed according to the Guidelines for Animal Experiments of Kyoto University.

Histological examination

Rabbits were sacrificed 2 or 12 weeks after surgery, and the distal femur and proximal tibia of the left side of each animal were resected, fixed at 4°C overnight in a 10% formalin solution, and decalcified in formic acid for three days. After neutralization by 10% sodium sulfate for 24 hours, the samples were embedded in paraffin. Serial sections were prepared in the coronal plane through the middle of the femoral and tibia condyles, and one section from each sample was used for each of the histological analyses. In every section, the entire cartilage portion in full depth was evaluated. The specimens were stained with safranin O/Fast Green or H&E using standard procedures. The histological grade of cartilage degeneration was evaluated using the modified Mankin's scoring system [23], which was adopted as the original system [24] for the evaluation of the rabbit model. All the results shown herein represent the combined scoring data of two researchers.

Immunohistochemical analyses

Immunohistochemical examination was performed as follows. In brief, after deparaffinization, sections were incubated with 0.3% hydrogen peroxide for 30 minutes. Then, sections were treated with proteinase K for two minutes (proliferating cell nuclear antigen [PCNA] staining) or with hyaluronidase for 60 minutes (MMP staining), after which they were incubated with the following primary antibodies: mouse anti-human PCNA monoclonal antibody (1:100; Dako, Glostrup, Denmark), mouse anti-human MMP-13 monoclonal antibody (1:20; AnaSpec Inc., San Jose, CA, USA), or mouse anti-rabbit MMP-3 monoclonal antibody (1:50; Daiichi Fine Chemical Co. Toyama, Japan). All antibody dilutions were made in PBS. After an overnight reaction with the primary antibody at 4°C, sections were incubated with horseradish peroxidase-conjugated anti- mouse IgG (Vector Laboratories, Southfield, MI, USA) at room temperature for 30 minutes. Signals were visualized with 3, 3'-diaminobenzidine tetrahydrochloride, and nuclei were counterstained with hematoxylin. The percentage of PCNA-, MMP-13-, and MMP-3-positive cells in the cartilage was calculated by methods similar to those described above. Results of histological and immunohistochemical analyses were evaluated by two observers who were blinded to the identity of each sample.

Primary chondrocyte cultures

Primary culture of chondrocytes was performed using articular cartilage tissues harvested from non-treated rabbits (NRC cells) or ACLMT-operated rabbits (ORC cells). Briefly, thinly sliced cartilage tissues were incubated with collagenase (4 mg/ml; Sigma Aldrich, St. Louis, MO, USA) in DMEM for 12 hours. Cells were then collected by centrifugation, seeded into type I collagen-coated dish (Corning International K.K.,Tokyo, Japan), and cultured with DMEM containing 10% FBS supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. Chondrocytes were grown in monolayer cultures, and were passaged when reaching confluence. Cells at the second passage were used for the assay. ONO-AE1-259-01, a selective agonist of EP2, was used to stimulate EP2 signaling in the presence or absence of IL-1β (Sigma Aldrich, St. Louis, MO, USA).

Real-time PCR

Total RNA was extracted from cultured cells using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. All reverse transcription reactions were performed with an RT-PCR kit using 1 μg of total RNA with a Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for conversion into cDNA. The mRNA expression levels of *MMP-13* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were quantified by real-time PCR using SYBR Green (Applied Biosystems, Foster City, CA, USA) and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were run in triplicate, and the amount of PCR product of each gene was calculated using the standard curve method and normalized to *GAPDH* levels, which were used as an internal control. Using the ratio obtained for the untreated sample as a standard (1.0), the relative ratio of the treated samples was presented as the relative expression levels of the *MMP-13* gene. Sequences of primers used in this experiment were as follows: 5'-aggagcatggcgacttctac-3' and 5'-taaaacagctccgcatcaa-3' (*MMP-13*) and 5'-gctctccagaacatcactcctgcc-3' and 5'-cgttgtcataccaggaaatgagct-3' (*GAPDH*).

Statistical analysis

The statistical analyses were performed using the Statcel2 software (The publisher OMS Ltd., Saitama, Japan). The results are shown as the mean ± standard deviation (SD). The Kruskal-Wallis test was performed for screening

purposes, and the Steel-Dwass method for multiple comparisons was used if there was a significant difference between samples. A P value less than 0.05 was considered to be significant.

Results

Therapeutic effect of ONO-8815Ly in the early stages of degeneration

At two weeks after the operation, articular cartilages in medial condyles of G-C (Figure 2a, b) and G-0 (Figure 2a, c) showed severe degenerative findings such as surface irregularity including clefts and reactive changes such as clonal proliferation of chondrocytes. The intensity of safranin O staining was reduced in G-C (Figure 2a, g) and G-0 (Figure 2a, h). The grade of degenerative findings was less prominent in sections of G-S (Figure 2a, a), G-80 (Figure 2a, d) and G-400 (Figure 2a, e) than in those of G-C or G-0. Safranin O staining was stronger in sections of G-80 (Figure 2a, i) and G-400 (Figure 2a, j). Similar findings were observed in sections prepared from lateral femoral condyles. The degenerative changes were less prominent and the safranin O staining was stronger in sections of G-S (Figure 2b a and 2f), G-80 (Figure 2b, d and 2i) and G-400 (Figure 2b, e and 2j) than in those of G-C (Figure 2b, b and 2g) or G-0 (Figure 2b, c and 2h).

Histological grade was evaluated using a modified Mankin's scoring system [23,24]. The grades of medial condyle in each sample were scored and mean values were compared (Figure 2c). Scores were significantly better for G-80 than for G-0. The effect of ONO-8815Ly was more prominent in lateral condyles, and both G-80 and G-400 showed much better scores than G-C or G-0 (Figure 2d).

Similar findings were observed in medial (Figure 3a) and lateral (Figure 3b) condyles of tibiae. The degenerative changes were less prominent and the safranin O staining was stronger in sections of ONO-8815Ly-treated groups (G-80 and G-400) than in those of non-treated groups (G-C and G-0). The effect of ONO-8815Ly was similar between G-0 and G-80 in medial condyles (Figure 3c), whereas G-80 and G-400 showed better values than G-C or G-0 in lateral condyles (Figure 3d). These results suggested that ONO-8815Ly prevents degenerative change in articular cartilages during the early stages.

Therapeutic effect of ONO-8815Ly in the late stages of degeneration

Similar analyses were performed using sections prepared at 12 weeks after surgery. In the case of femoral condyles, no improvements of cartilage degeneration were observed in sections of ONO-8815Ly-treated groups (G-80 or G-400) (Figure 4a, d and 4e) and the staining of safranin O also showed no difference (Figure 4a, i and 4j). Similar results were obtained in lateral condyles of femora (Figure 4b). In agreement, there was no significant difference in Mankin's score in the analyses of medial (Figure 4c) or lateral (Figure 4d) condyles of femora.

Similar results were obtained in the tibiae. Neither medial nor lateral condyles showed better histological features by the treatment with ONO-8815Ly, and the Mankin's score showed no improvements (data not shown).

These results suggested that the effect of ONO-8815Ly failed to last, at least when using this drug delivery system.

Growth promoting effect of ONO-8815Ly

The proliferating activity of chondrocytes was evaluated by PCNA staining (Figure 5). The proportion of PCNA-positive cells in femoral (Figures 5a and 5b) and in tibial (Figures 5c and 5d) condyles at two weeks after operation were similar among all groups, suggesting that the improvement of cartilage degeneration by the EP2 agonist was not due to the acceleration of cell proliferation.

EP2- selective agonist inhibits the expression of MMP-13 in ACLMT

MMP-3 and MMP-13 are major proteases degrading the ECM. The expression of these enzymes was analyzed by immunohistochemistry using samples prepared at two weeks after the operation. For MMP-3, there were no significant differences in staining intensity or number of positive cells between any of the groups (Figure 6). For MMP-13, however, significant differences were observed (Figure 7). The staining of MMP-13 was much stronger in G-C and G-0 (Figure 7a, b and 7c) than in G-S, G-80, or G-400 (Figure 7a, a, d, and 7e). The proportion of MMP-13-positive cells was significantly lower in sections of G-80 and G-400 than in sections of G-C or G-0 (Figure 7b). Similar results were obtained for the intensity (Figure 7a, f, i, and 7j) and the ratio of MMP-13-positive cells (Figure 7c) in the analyses of lateral condyles.

EP2-selective agonist inhibits IL-1β-induced *MMP-13* mRNA expression

To confirm the effect of EP2 agonist on MMP-13 expression, the expression of the *MMP-13* gene by primary cultured chondrocytes was evaluated by quantitative real-time PCR (Figure 8). The expression levels of *MMP-13* were similar in NRC and ORC cells under basal culture conditions. Similarly, EP2 agonist treatment showed no significant effects on *MMP-13* levels on either cells. When NRC and ORC cells were treated with IL-1β (50 pg/ml), the expression levels of *MMP-13* mRNA were significantly increased in both cells. IL-1β-induced expression of MMP-13 mRNA in ORC cells was reduced by

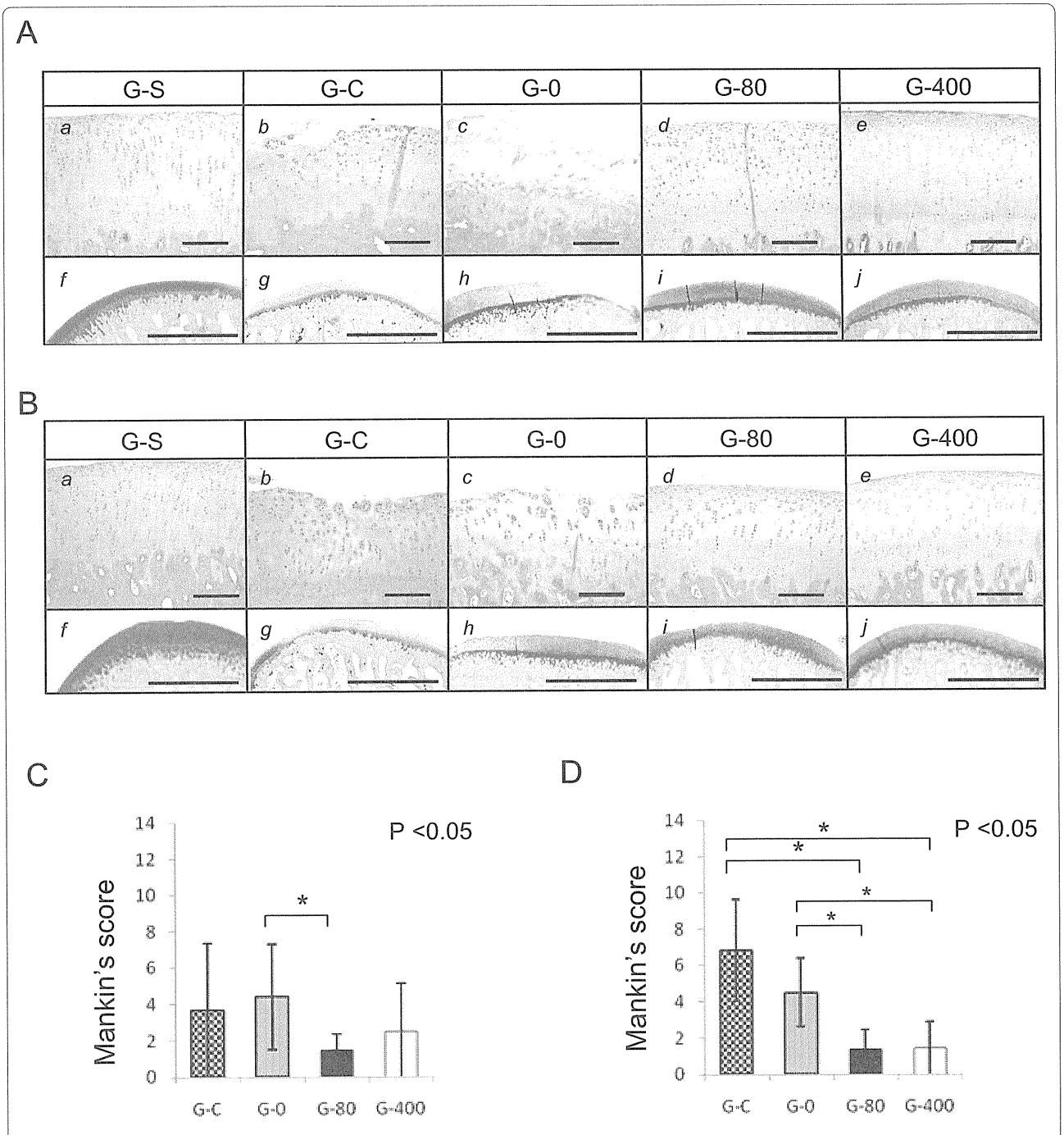

Figure 2 Effect of ONO-8815Ly in the early stages of OA (femur). Histological findings and grade of degeneration, scored using Mankin's system, in (**a**) medial and (**b**) lateral femoral condyles at two weeks after the operation. Sections were prepared from samples of (*a* and *f*) G-S, (*b* and *g*) G-C, (*c* and *h*) G-0, (*d* and *i*) G-80, and (*e* and *j*) G-400, and stained with (*a* to *e*) H&E or (*f* to *j*) safranin-O. Magnification, 200× (H&E) and 40× (safranin-O). Scale bar, 200 μm (HE) and 2.0 mm (safranin-O). *$P < 0.05$. Values are the mean ± standard deviation. OA, osteoarthritis.

co-treatment with the EP2 agonist in a dose-dependent manner, and the maximum reduction was 37% at 1 μM of EP2 agonist. In the case of NRC cells, the maximum reduction (27%) was observed at the concentration of 0.1 μM.

Discussion

The effect of PGE2 on the progression of OA is still a matter of debate. In some reports, PGE2 was shown to destroy articular cartilage by degrading cartilage ECM [12,13]. It has also been reported to down-regulate the

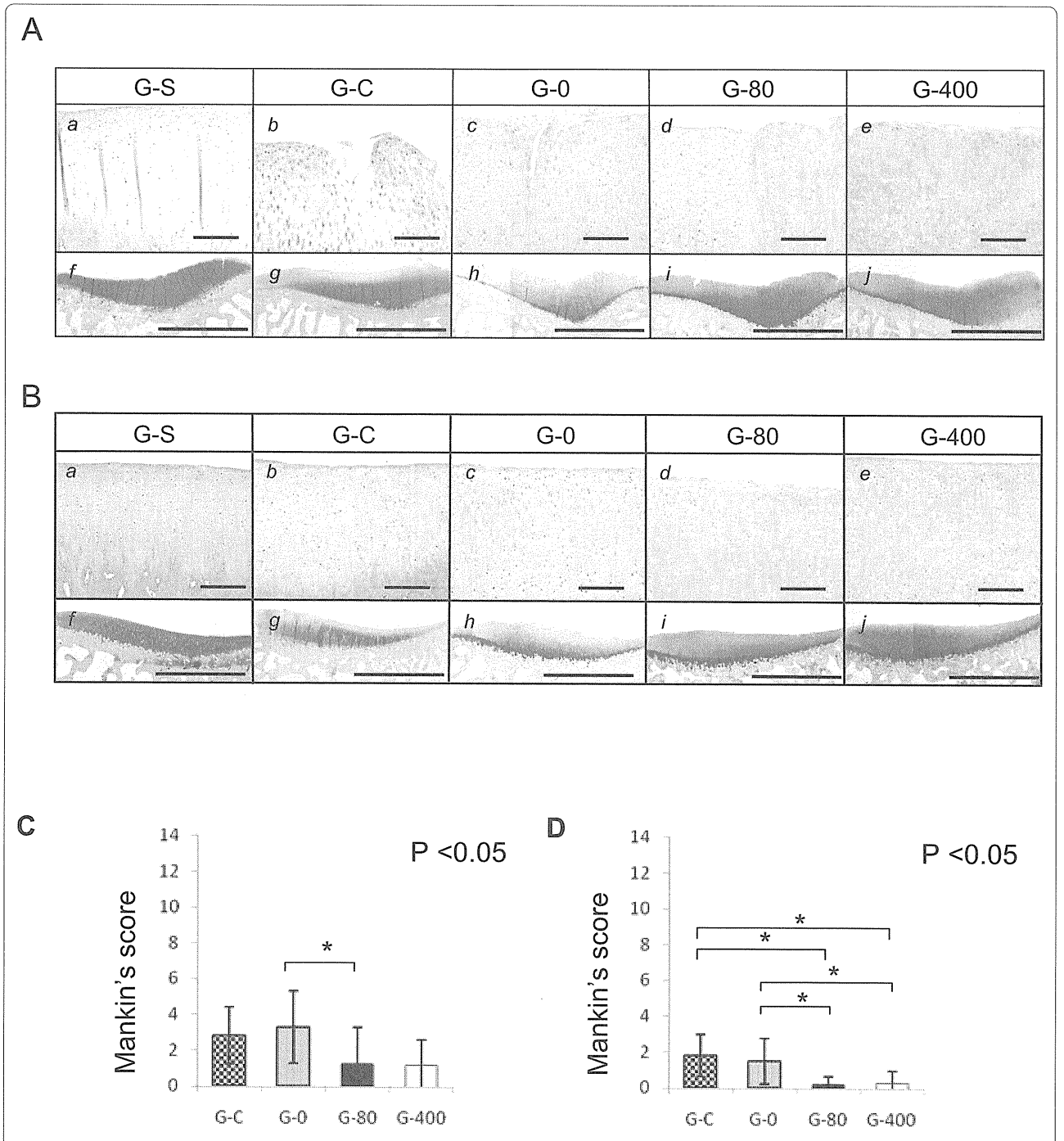

Figure 3 Effect of ONO-8815Ly in the early stages of OA (tibia). Histological findings and grade of degeneration, scored using Mankin's system, in (**a**) medial and (**b**) lateral tibial condyles at two weeks after the operation. Sections were prepared from samples of (*a* and *f*) G-S, (*b* and *g*) G-C, (*c* and *h*) G-0, (*d* and *i*) G-80, and (*e* and *j*) G-400, and stained with (*a* to *e*) H&E or (*f* to *j*) safranin-O. Magnification, 200× (H&E) and 40× (safranin-O). Scale bar, 200 μm (H&E) and 2.0 mm (safranin-O). *$P < 0.05$. Values are the mean ± standard deviation. OA, osteoarthritis.

production of IL-6 by IL-1α and IL-1β via EP2/EP4 receptors [25,26]. PGE2 at very low concentrations inhibits the production of IL-1β, TNF-α, and MMP-13 in the articular cartilages of OA patients [27]. In the current study, the production of MMP-13 was decreased by an EP2 agonist (Figures 7 and 8), which is consistent with the *in vitro* data described in a recent report [28]. Continuous administration of non-steroidal anti-inflammatory drugs to patients with OA exacerbates OA [29,30]. These contradictory results

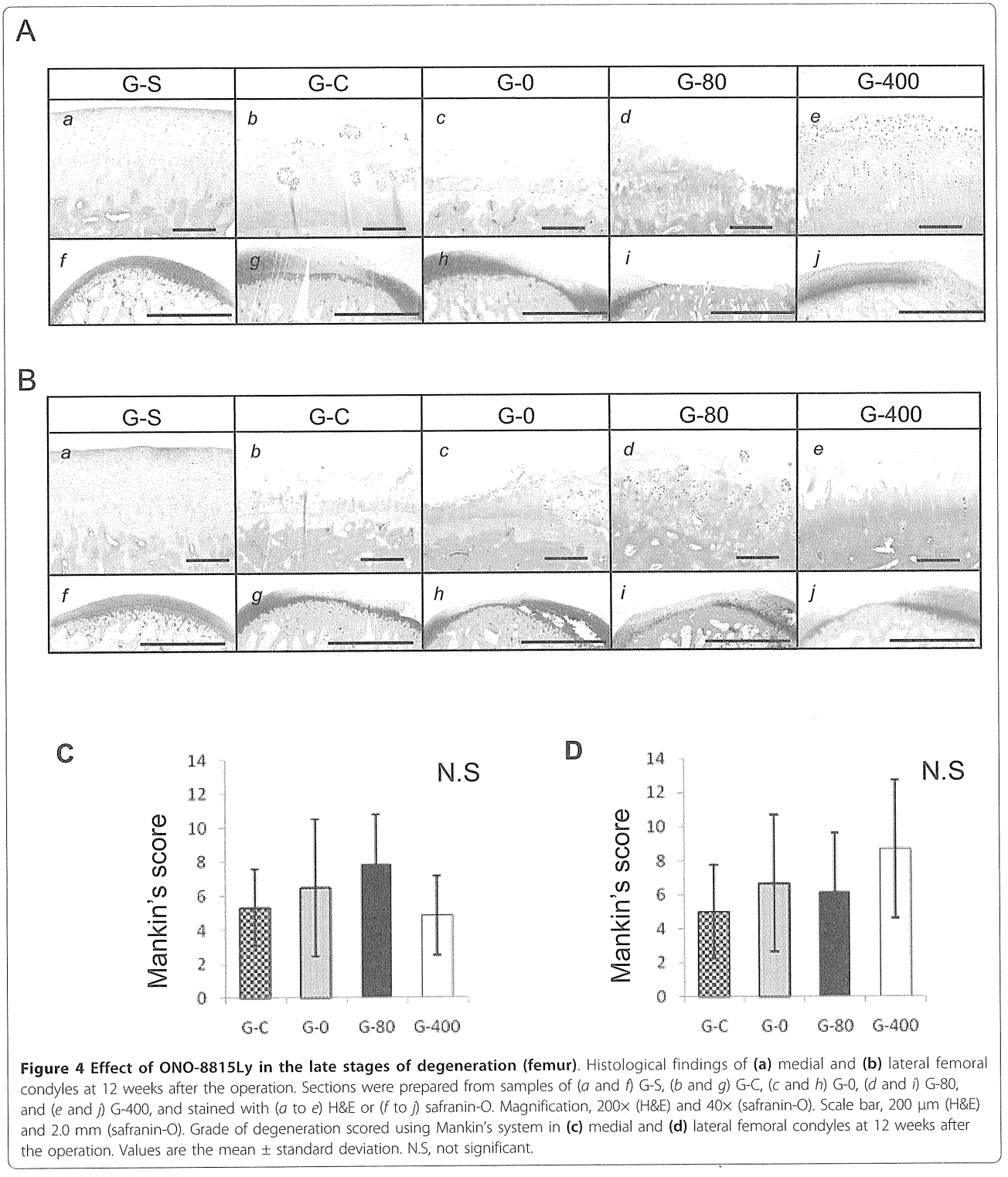

Figure 4 Effect of ONO-8815Ly in the late stages of degeneration (femur). Histological findings of **(a)** medial and **(b)** lateral femoral condyles at 12 weeks after the operation. Sections were prepared from samples of (*a* and *f*) G-S, (*b* and *g*) G-C, (*c* and *h*) G-0, (*d* and *i*) G-80, and (*e* and *j*) G-400, and stained with (*a* to *e*) H&E or (*f* to *j*) safranin-O. Magnification, 200× (H&E) and 40× (safranin-O). Scale bar, 200 μm (H&E) and 2.0 mm (safranin-O). Grade of degeneration scored using Mankin's system in **(c)** medial and **(d)** lateral femoral condyles at 12 weeks after the operation. Values are the mean ± standard deviation. N.S, not significant.

may be due to the differences in the experimental dose of PGE2 agonist used, or due to the pleiotropic effects of PGE2 through different types of receptors (EP1 to EP4). Therefore, analyses should be conducted with agonists specific for each type of receptor. IL-1β-induced expression of *MMP-13* mRNA was reduced by EP2 signaling both in NRC and ORC cells *in vitro* (Figure 8). Moreover, IL-1β-induced expression of *MMP-13* mRNA was reduced in ORC cells, but not in NRC cells, in a dose-dependent manner, that is, *MMP-*

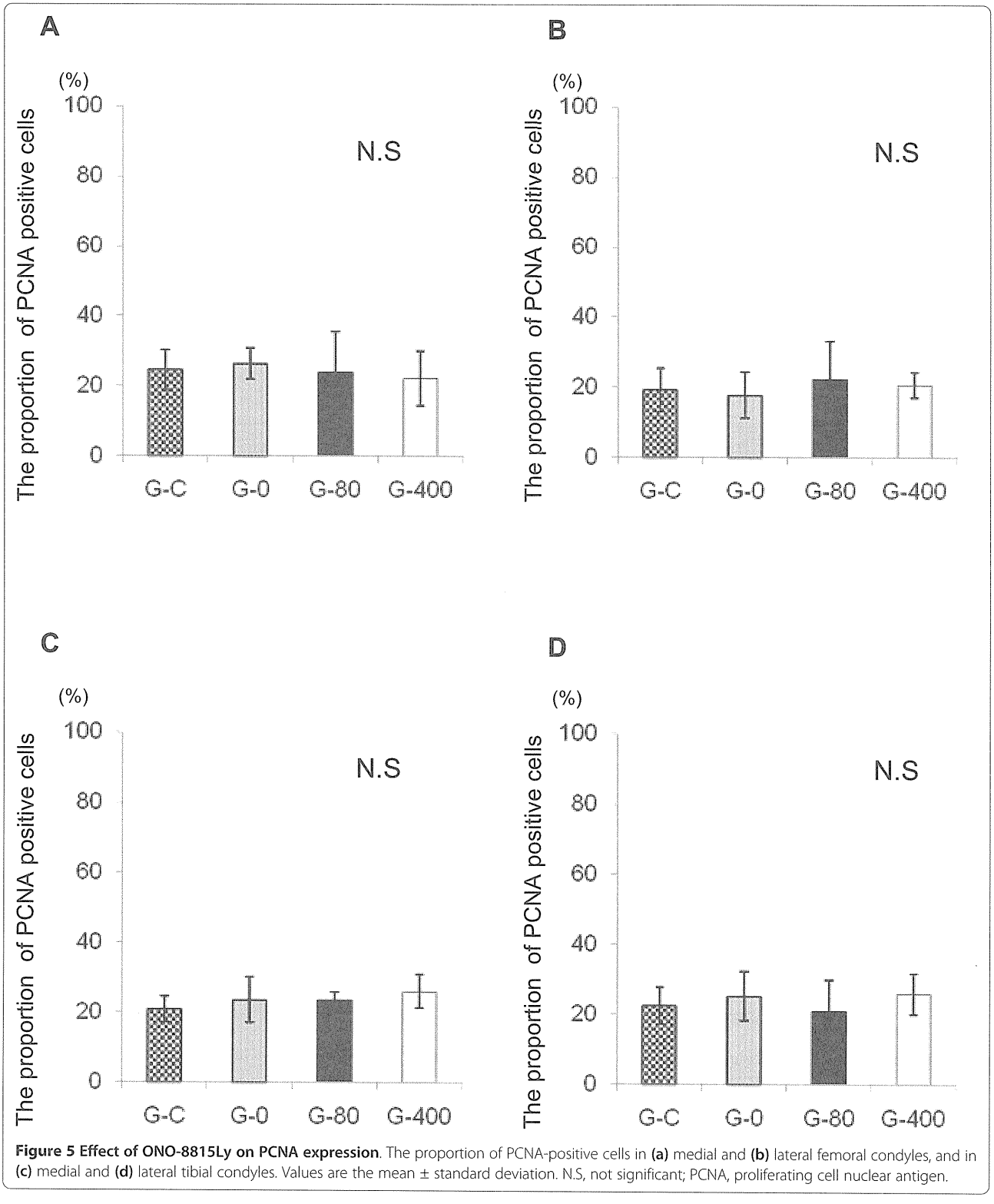

Figure 5 Effect of ONO-8815Ly on PCNA expression. The proportion of PCNA-positive cells in **(a)** medial and **(b)** lateral femoral condyles, and in **(c)** medial and **(d)** lateral tibial condyles. Values are the mean ± standard deviation. N.S, not significant; PCNA, proliferating cell nuclear antigen.

13 expression was higher in the presence of 1 μM of ONO-AE-259-01 than in the presence of 0.1 μM of ONO-AE-259-01 (Figure 8). An EP2 agonist acts as an anti-inflammatory drug at low doses, but if the concentration exceeds 1 μM, the anti-inflammatory effect may become weak (Figure 8). In fact, some authors have reported that excess EP2 agonists may act rather as inflammatory-inductive drugs.