fracture in adult life were also independent risk factors. Scores derived from these models showed good discrimination for fracture risk. Women with scores in the lowest quintile had a 5-year risk of hip fracture of 0.6% compared with about a 14-fold increase risk of 8.2% for those with scores at the highest quintile without BMD. From the relationship between AUC and gradient of risk, the gradient of risk can be computed at 2.5 per SD change in risk score assuming a normal distribution of risk score (our calculations from the data of Black et al. [57]). Good separation for those at high and low risk of vertebral fracture was also demonstrated. Gradients of risk were lower for non-vertebral fractures, there being about a threefold difference in risk between the highest and lowest quintile for risk score respectively (gradient of risk=1.4 and 1.5 without, or with BMD, respectively). As with our own models, the addition of BMD values to the models derived from clinical variables alone improved performance, although not markedly. These performance characteristics were independently assessed against the EPIDOS study. For hip fracture risk there was a 5.8-fold difference in risk between the lowest and highest quintile of risk score in the absence of BMD. When BMD was included in the model the risk ratio was 24.

In the present study, there was a 5.6-fold difference in hip fracture risk comparing the highest with the lowest quintile of risk with the use of clinical risk factors. When BMD was additionally added, the risk ratio was 20.9. The risk ratios in the two studies are not directly comparable, since age is used as a risk factor in the model of Black et al. [57], whereas the risk ratios we report are all age-specific. Since age is a very important determinant of fracture risk, and the risk ratios between the two studies are broadly comparable, this suggests that the performance characteristics of the present model represent some improvement in fracture risk prediction.

There are several aspects of validity that are of relevance to the present study. The first concerns the performance of the model in independent cohorts, and a second relates to the validity of the risk factors chosen. With respect to the first, there was some variation in the GRs and AUCs of the validation cohorts, but those that performed less adequately included those with missing risk factors. This would underestimate the gradient of risk of the affected cohorts. Significant heterogeneity was noted between cohorts in the gradients of risk, but there were marked differences in age between cohorts, and the gradient of risk varied by age. When the Poisson model included the interaction between age and risk score, heterogeneity was moderate. Overall, the performance characteristics of the test in the eleven independent cohorts were comparable to that of the original cohorts, as judged by the GR/SD. These validation cohorts, however, mainly comprised women and further studies in men are required. The performance characteristics of diagnostic tests are often expressed as the areas under the ROC curves (AUC). As shown in the Appendix, there is a mathematical relationship between AUC and GR. For example, a GR of 4.2/SD is equivalent to an AUC of 84% (i.e., hip fracture prediction with risk factors plus BMD at the age of 50 years). At the other extreme, a GR of 1.4 (other osteoporotic fractures at the same age) is equivalent to an area under the ROC curve of 60%. An area equivalent to 50% indicates a predictive value no better than chance.

The choice of risk factors for the present model was made on the basis of our previous meta-analyses. Aside from the availability of sufficient data, these risk factors were chosen for their ease of use in the setting of primary care. An important further consideration is whether the risk so identified by a risk factor is amenable to a therapeutic intervention. Liability to falls, for example, is a strong risk factor for fracture, but there is some uncertainty whether patients identified on the basis of such risk factors would respond to treatment with inhibitors of bone turnover [71]. The strongest level of evidence for the validity of the use of risk factors in this way would be provided by randomised controlled trials that recruit patients on the basis of these risk factors. Responsivity to pharmacological intervention has been shown for patients selected on the basis of low BMD, prior fracture or the use of oral corticosteroids [72–75]. In the case of the other risk factors, no trials have recruited on the basis of their presence. However, analyses of randomised controlled trials indicate that the beneficial effects of treatment are not adversely (or beneficially) affected by the presence or absence of the other risk factors [30, 76–79]. Moreover, since there is a significant correlation between the risk factors and BMD, case finding on the basis of the clinical risk factors used will capture a population with low BMD [28]. These considerations suggest that the risk factors chosen are appropriate, but would need to be validated in prospective studies of intervention.

We conclude that the combined use of clinical risk factors provides an assessment of fracture risk that can be used for the prediction of osteoporotic fractures. Moreover, clinical risk factors can be used to enhance the performance characteristics of BMD. The application of these models for the assessment of fracture probability will require the incorporation of hazard functions of death and calibration to the epidemiology of specific countries. This will permit the assessment of absolute fracture probabilities of hip fracture and other osteoporotic fractures to be determined on the basis of the clinical risk factors alone or in combination with BMD.

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Appendix

Relationship between area under the ROC curve and gradient of risk for a normally distributed risk variable

Let X be a risk variable with the frequency function f_0 among the not diseased and f_1 among the diseased. Furthermore, assume that the probability of being diseased among the studied individuals is p. Then the conditional probability of belonging to the diseased group given the that X=x is

$$1/[1 + ((1-p)/p) \cdot (f_0(x)/f_1(x))]$$

We assume that f_0 and f_1 are frequency functions corresponding to normally distributed variables with the same standard deviation σ and the difference between means of diseased and not diseased equal to Δ. Then the conditional probability can be written as

$$1/[1 + \exp(-(\beta_0 + \beta_1 \cdot x))],$$

where $\beta_1 = \Delta/\sigma^2$. If individuals are followed for a short period so that the proportion of diseased are low, then the beta coefficients for a risk variable obtained by Cox regression, Poisson regression or logistic regression will be approximately the same and the standard deviation of the risk variable X in the population as a whole will be approximately as among the not diseased, σ. The gradient of risk per 1 standard deviation, GR, is $\exp(\beta_1 \cdot \sigma) = \exp(\Delta/\sigma)$, and thus

$$\ln(GR) = \Delta/\sigma \quad (1)$$

Let Y denote the value of the risk variable of a randomly chosen individual among the diseased individuals and let X be the corresponding quantity among not diseased individuals. We assume that Y tends to be larger than X. The area under the ROC curve is equal to the probability $P(Y>X) = 1-P(Y-X\leq 0)$. If Y and X have normal distributions with the same standard deviation σ and the difference Δ between the means, then the area under the curve is $1 - \Phi(-\Delta/(\sigma \cdot \sqrt{2})) = \Phi(\Delta/\sigma \cdot \sqrt{2})$, where Φ is the standardised normal distribution function. When we use the relationship (1), the area under the ROC curve can be represented as the following function of gradient of risk per 1 standard deviation, GR, *Area under the ROC curve* $= \Phi(\ln(GR)/\sqrt{2})$.

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Original article

Diet and lifestyle associated with increased bone mineral density: cross-sectional study of Japanese elderly women at an osteoporosis outpatient clinic

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Abstract

Background. Several studies have already demonstrated that lifestyle characteristics, such as physical activity, smoking, and alcohol intake, are associated with bone mineral density (BMD). Coffee intake was shown to be negatively associated with BMD, whereas tea drinking was reported to be associated with increased BMD. A review of the literature, however, revealed that few studies have described the association between BMD and lifestyle, including characteristic Japanese foods such as fish, *natto*, and Japanese green tea. The aim of this study was to identify lifestyle factors associated with BMD.

Methods. A total of 632 women age ≥60 years were enrolled in this study. Subjects were interviewed about their lifestyle by means of a questionnaire regarding the consumption pattern of dietary items. BMD was measured at the lumbar spine by dual energy X-ray absorptiometry.

Results. The BMD was higher in subjects with the habits of alcohol drinking, green tea drinking, and physical activity and lower in those with the habits of smoking and cheese consumption. Multiple regression analysis showed that factors associated with BMD were smoking, alcohol consumption, green tea drinking, and physical activity after adjusting for age and body mass index (BMI).

Conclusions. In this cross-sectional study at an osteoporosis outpatient clinic, patients with the habits of alcohol drinking, green tea drinking, and physical activity had significantly higher BMD, and those who smoked had significantly lower BMD than patients without each habit after adjusting for age, BMI, and other variables regarding lifestyle.

Introduction

As the population ages in Japan, osteoporosis has become a serious threat to society. Hip fracture is the most severe consequence of osteoporosis, leading to reduced activities of daily living, lowered quality of life, and increased mortality of patients.[1,2] Studies have demonstrated that lifestyle characteristics such as physical activity,[3] smoking,[4] and excessive alcohol intake[5] are associated with bone mineral density (BMD). Coffee drinking was shown to be negatively associated with BMD,[6,7] whereas tea drinking was reported to be associated with increased BMD.[8] However, few studies described the association between BMD and lifestyle regarding characteristic Japanese foods such as fish, *natto*, and Japanese green tea. The aim of this study was to identify lifestyle factors associated with BMD.

Subjects and methods

A total of 632 women age ≥60 years attending the Osteoporosis Outpatient Clinic at the Tokyo Metropolitan Geriatric Medical Center were enrolled in this study. Their mean age was 71.8 ± 7.5 years. The patients and/or families were informed that data from the case would be submitted for publication and gave their consent. Women who had complications associated with BMD, such as a history of hysterectomy or ovariectomy before menopause, gastrectomy or colonectomy, thyroid disease, parathyroid disease, severe diabetes mellitus, and/or steroid and bisphosphonate usage were excluded from the study.

Upon entry into the study, body height and weight were measured, and the body mass index (BMI) was calculated. Subjects were interviewed about their lifestyle by means of a questionnaire regarding the consumption pattern of nine dietary items including milk, cheese, yogurt, fish, vegetable, tofu, *natto* (which contains a large amount of vitamin K), coffee, and green tea as well as their history of smoking and alcohol con-

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sumption and their level of physical activity. For each dietary item, subjects were divided into two groups: those consuming the food item ≥5 days per week and those consuming it <5 days per week. Subjects were categorized according to their history of smoking as nonsmokers or smokers, their history of alcohol consumption as nondrinkers or drinkers, and their level of physical activity as exercising ≥1 day a week or <1 day a week.

The BMD was measured at the lumbar spine by dual-energy X-ray absorptiometry with the Expert-5000 instrument (<1% CV; Lunar, Madison, WI, USA). Measurements were obtained from anteroposterior projections of the second to fourth lumbar vertebrae. Bone turnover markers and other serum levels such as serum osteocalcin, alkaline phosphatase, calcium (Ca), phosphorus (P), intact parathyroid hormone (iPTH), 1,25-vitamin D, and urine deoxypyridinoline were measured.

Table 1. Characteristics and the ratio of subjects with habits of each variable regarding lifestyles among 632 women aged ≥60 years

Age (years)	71.8 ± 7.5
Body height (cm)	148.5 ± 6.7
Body weight (kg)	48.7 ± 7.7
BMI (kg/m^2)	22.1 ± 3.2
BMD (g/cm^2)	0.802 ± 0.198
T score	−1.634 ± 1.633
Smoking (%)	20.6
Alcohol (%)	19.7
Milk (%)	30.9
Cheese (%)	16.8
Yogurt (%)	36.8
Fish (%)	31.0
Vegetables (%)	70.9
Tofu (%)	30.2
Natto (%)	24.2
Coffee (%)	28.3
Green tea (%)	91.8

BMI, bone mass index; BMD, bone mineral density

Table 2. Bone mineral density of lumbar spine according to lifestyle

	BMD at lumbar spine			
	Yes		No	
Lifestyle item	BMD (g/cm^2)	T score	BMD (g/cm^2)	T score
Smoking	0.772 ± 0.176**	−1.89 ± 1.45**	0.808 ± 0.194	−1.59 ± 1.60
Alcohol	0.842 ± 0.199*	−1.31 ± 1.65*	0.792 ± 0.198	−1.72 ± 1.64
Milk	0.802 ± 0.191	−1.64 ± 1.58	0.802 ± 0.207	−1.64 ± 1.71
Cheese	0.767 ± 0.209*	−1.93 ± 1.72*	0.812 ± 0.191	−1.56 ± 1.58
Yogurt	0.800 ± 0.205	−1.66 ± 1.70	0.805 ± 0.195	−1.61 ± 1.61
Fish	0.791 ± 0.192	−1.73 ± 1.59	0.809 ± 0.201	−1.58 ± 1.66
Vegetables	0.793 ± 0.191	−1.71 ± 1.58	0.818 ± 0.208	−1.50 ± 1.71
Tofu	0.799 ± 0.186	−1.66 ± 1.54	0.804 ± 0.204	−1.62 ± 1.69
Natto	0.797 ± 0.191	−1.68 ± 1.58	0.803 ± 0.201	−1.63 ± 1.66
Coffee	0.809 ± 0.199	−1.62 ± 1.64	0.805 ± 0.198	−1.58 ± 1.65
Green tea	0.807 ± 0.187*	−1.59 ± 2.70*	0.733 ± 0.182	−2.17 ± 2.08
Physical activity	0.856 ± 0.203*	−1.19 ± 1.68*	0.794 ± 0.198	−1.70 ± 1.64

Student's *t*-test was used to compare BMD between subjects with the habit and without the habit of each variable
* $P < 0.05$
** $P < 0.1$

Table 3. Lifestyles associated with BMD in women aged ≥60 years

	Coefficient of variation	SE	*P*
Age (year)	−0.002	0.001	<0.05
BMI (kg/cm^2)	0.017	0.003	<0.0001
Smoking (yes vs. no)	−0.058	0.034	<0.05
Alcohol (yes vs. no)	0.054	0.022	<0.05
Cheese (yes vs. no)	−0.032	0.024	NS
Green tea (yes vs. no)	0.064	0.033	<0.05
Physical activity (yes vs. no)	0.060	0.030	<0.05

SE, standard error
Variables were chosen according to the results of Student's *t*-test (Table 2, $P < 0.01$)
Multiple regression analysis was used to determine the lifestyles associated with BMD after adjusting for age and BMI

Table 4. Bone turnover markers according to the lifestyles associated with BMD

Marker	Smoking		Alcohol		Green tea		Physical activity	
	Yes	No	Yes	No	Yes	No	Yes	No
Osteocalcin	8.65 ± 3.71	9.08 ± 5.31	8.38 ± 3.82	9.00 ± 5.12	8.95 ± 5.09	8.86 ± 3.60	7.51 ± 2.93	9.13 ± 5.24
Alkaline phosphatase	308.0 ± 153.2*	264.5 ± 93.8	271.8 ± 90.3	268.2 ± 103.1	268.8 ± 106.6	275.1 ± 98.9	243.1 ± 79.9	273.2 ± 107.5
Deoxypyridinoline	7.11 ± 3.22	6.91 ± 3.32	6.81 ± 2.66	6.95 ± 3.36	6.89 ± 3.28	7.28 ± 2.34	5.76 ± 1.81	7.07 ± 3.38
Intact PTH	43.1 ± 35.7*	31.7 ± 22.2	32.3 ± 18.7	32.4 ± 24.7	32.0 ± 23.0	31.0 ± 16.8	26.9 ± 8.7	32.8 ± 25.1
Ca	9.65 ± 0.47	9.56 ± 0.72	9.50 ± 0.69	9.59 ± 0.71	9.58 ± 0.69	9.55 ± 0.59	9.55 ± 0.38	9.59 ± 0.69
P	3.59 ± 0.55	3.59 ± 0.49	3.56 ± 0.51	3.59 ± 0.50	3.59 ± 0.49	3.57 ± 0.45	3.63 ± 0.45	3.58 ± 0.50
1,25-Vitamin D	51.5 ± 19.2	50.7 ± 16.4	52.2 ± 18.3	50.2 ± 15.9	50.6 ± 16.3	50.4 ± 15.2	49.3 ± 15.3	50.5 ± 15.8

PTH, parathyroid hormone
Student's *t*-test was used to compare bone turnover markers between subjects with the habit and without the habit for each variable
*$P < 0.05$

Statistical analyses were performed using the statistical software package Statview 5.0 (Abacus Concepts, Berkeley, CA, USA). Student's *t*-test was used to compare the BMD and bone turnover markers between subjects with habits of each variable and those without the habit. The effects of the lifestyles associated with BMD by using Student's *t*-test ($P < 0.1$) were subsequently analyzed using multiple regression analysis after adjusting for age and BMI. Statistical significance was defined as $P < 0.05$.

Results

Table 1 shows the characteristics and lifestyles of the subjects. The BMD was higher in subjects with the habits of alcohol drinking, green tea drinking, and physical activity; it was lower in those with the habits of smoking and cheese consumption (Table 2). Table 3 shows the result of multiple regression analysis of BMD with age, BMI, and the aforementioned variables regarding lifestyle. Factors associated with BMD were age, BMI, smoking, alcohol consumption, green tea drinking, and physical activity. No significant associations were found between any serum or urinary levels and the aforementioned factors regarding lifestyle, except alkaline phosphatase and iPTH, between smokers and nonsmokers (Table 4).

Discussion

In this study, lifestyle habits such as smoking, alcohol consumption, green tea drinking, and physical activity were associated with increased BMD. According to multiple regression analysis, smoking was associated with low BMD, which agreed with findings in previous studies.[4] In contrast, alcohol consumption was associated with an increased BMD. Excessive alcohol consumption was reported to lower BMD,[5] although moderate alcohol consumption has been shown to be associated with increased BMD.[9] The amount of alcohol consumption was not examined, although it may be moderate in most alcohol drinkers. Japanese green tea was also associated with increased BMD.

To the best of the authors' knowledge, this study is the first to investigate the relation between consumption of Japanese green tea and BMD. An epidemiological case-control study suggested that Japanese green tea drinking was a factor in protecting against hip fracture.[10] The reason was unclear, although flavonoids that are contained in Japanese green tea have been shown to have a weak estrogenic effect,[11] which may increase BMD. Recent evidence suggests that (−)-epigallocatechin-3-gallate, which is one of the major

flavonoids contained in green tea, induces apoptosis of osteoclasts.[12] This inhibits bone resorption, which may lead to increased BMD.

In this study, however, subjects were recruited only at one osteoporosis outpatient clinic and interviewed not about their history of green tea consumption but about the current level of their green tea consumption. Hence, this study did not indicate that green tea drinking increased BMD among a general Japanese population, but that green-tea drinkers had higher BMD than non-green-tea drinkers among subjects at one osteoporosis outpatient clinic. To clarify the effect of green tea drinking on BMD, a prospective randomized study regarding the quantity of green tea drinking and BMD among population-based cohorts is necessary.

In this study, BMD was measured at the lumbar spine. Degenerative spinal diseases are reported to be associated with increased lumbar spine BMD measurements in the elderly.[13] Lumbar spine BMD is currently the gold standard for estimating osteoporosis.

There are many kinds of Japanese green tea that may have different effects on bone metabolism, but this study was not performed on the basis of different types of green tea. However, the results of multiple regression analysis showed that the effect of green tea on BMD was independent of age, BMI, and other variables. Up to now, there have been no reports relating green tea drinking and BMD, so this is the first study to indicate the possible effect of green tea drinking on BMD.

Conclusions

This cross-sectional study was performed at an osteoporosis outpatient clinic. The results indicated that patients with the habits of alcohol drinking, green tea drinking, and physical activity had significantly higher BMD and those who were smokers had significantly lower BMD than patients without each habit after adjusting for age, BMI, and other variables regarding lifestyles.

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The effects of radial shock waves on gene transfer in rabbit chondrocytes *in vitro*

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Summary

Objective: The purpose of this study was to develop a new technique of gene transfer utilizing radial shock waves. The effects of radial shock waves on gene transfer in rabbit chondrocytes were examined by varying the parameters of exposure conditions *in vitro*.

Methods: Chondrocytes were obtained from New Zealand white rabbits and cultured in a monolayer. A luciferase-encoding gene expression vector, or vector alone, was added to chondrocyte cell suspensions, and the cells were then exposed to radial shock waves. Parameters such as pressure amplitude, number of pulses, frequency, and DNA concentration were varied, and luciferase activity was measured 48 h after transfection. Transfection efficiency of radial shock waves was compared with the FuGENE6 transfection method using a green fluorescence protein (GFP)-encoding gene vector by fluorescent-activated cell sorter (FACS) analysis.

Results: Radial shock wave exposure significantly increased luciferase activity over 140-fold as compared to the control under the optimal exposure conditions. Both pressure amplitude and number of pulses were relevant to transfection efficiency and cell viability, but frequency was not. Transfection efficiency increased in a dose-dependent manner with DNA concentration. FACS analysis showed 4.74% of GFP-encoding gene using radial shock waves. FuGENE6 transfection was almost similar in transfection efficiency to radial shock wave.

Conclusion: In spite of certain degree of cell disruption, radial shock waves significantly augmented reporter gene transfection in rabbit chondrocytes *in vitro*. Radial shock waves may potentially contribute to the treatment of the cartilage morbidities by enhancing the potency of tissue healing and gene transfection of growth factors.

Key words: Radial shock waves, Gene transfer, Transfection efficiency, Cell viability, Gene therapy, Chondrocyte.

Introduction

Cartilage morbidities such as osteoarthritis and traumatic cartilage defect do not always respond to conventional conservative treatment. Surgical procedures such as mosaic plasty or microfracture have shown stable clinical results in many cases. Additionally, autologous chondrocyte transplantation has been developed as a novel, improved treatment of cartilage defect[1] and gradually has become popular. However, some drawbacks of these modalities including, unsatisfactory results for larger defects, or elderly cases of surgical procedures, high cost and adverse events of autologous chondrocyte transplantation[2], still remain unsolved. Gene therapy can be considered a new treatment candidate for cartilage lesions because it enables patients to synthesize the medicative gene products endogenously, and potentially in a prolonged and regulated manner.

Although clinical trials of gene therapy have been attempted for cartilage morbidities, none of them have produced satisfactory results. The main reason for the delay in development of gene therapy in this field seems to be the lack of appropriate gene delivery systems for clinical use. Successful gene therapy requires delivery systems that are safe, easy to apply and that allow for efficient transgene expression. Viral methods have been shown to deliver efficient, uniform and continuous gene transfection *in vitro* and *in vivo*[3–5]. Additionally, gene transfer of growth factors using lentiviral or adeno-associated viral vectors has been reported to improve the repair of articular cartilage defects *in vivo*[6–9]. However, the viral recombinant vectors still have some disadvantages, including the need for specific cell culture conditions[10] and immune or inflammatory responses that adversely affect the cellular phenotype[11–13].

Meanwhile, non-viral plasmid DNA transfer has certain advantages over viral methods. The mass-production and quality control of non-viral vectors are substantially easier than those of viral vectors. In addition, they can be transferred into quiescent cells without producing proteins that raise immunogenic responses, while also being capable of carrying a large molecule including plasmid DNA[14–16]. Among many non-viral methods of transfection, ultrasound[17–19] and focused extracorporeal shock waves[20–23] have been investigated as physical means of improving gene delivery. Since these new techniques suffer from lower transfection efficiencies compared to viral methods,

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optimization of the condition is still needed to achieve their full potential and overcome the adverse effects of conventional viral techniques.

Ultrasound-mediated gene transfer is basically a safe, easy and low-cost method. Addition of microbubble agents has been reported to distinctly increase the efficiency of gene transfer by ultrasound[18,24,25]. Although the elevation of temperature in the target tissue is considered to be a possible adverse side effect of this method[26], the clinical relevance is controversial despite these studies[27,28]. Meanwhile, focused extracorporeal shock waves have shown relatively high cytotoxicity and tissue damage[22,23] as well as a lower efficiency of gene transfer as compared to ultrasound[29]. The bulk and expense of the device used to generate focused extracorporeal shock waves are suggested to be considerable disadvantages in research and clinical applications.

Recently, radial shock wave was developed as a new type of extracorporeal shock wave. Radial shock waves were utilized in the veterinary field first, especially for equine musculoskeletal disorders[30]. It has been shown that radial shock waves have therapeutic effects when applied to the lateral humeral epicondylitis, or so called "tennis elbow"[31]. The conventional devices for focused extracorporeal shock waves have an exposure field which is rotationally symmetric around the axis, perpendicular to the surface of the generator, and the maximum energy is generated in the center of the exposure area. On the other hand, the exposure area of the radial shock waves radiates from the surface of the applicator and the energy gradually decreases depending on the distance from the applicator. For the use of gene transfer, the wide exposure area could be an advantage in covering a larger area of target cells or tissues. The differences in the size of the exposure field and the property of energy transmission between focused extracorporeal shock waves and radial shock waves may have some impacts on cytotoxicity and tissue damage as well as transfection efficiency.

The purpose of this study was to develop a new technique of gene transfer in chondrocytes utilizing radial shock waves. We examined, for the first time, the effects of radial shock waves on transfection efficiency and cell viability by varying the parameters of exposure conditions.

Materials and methods

PREPARATION OF PLASMID DNA

To test the efficiency of gene transfer, an expression vector with a Cytomegarovirus (CMV) promoter encoding the luciferase gene (pGV-C2, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and encoding green fluorescence protein (GFP; pAcGFP1-N1, BD Biosciences, Mountain View, CA, USA) were used. The DNA plasmids were propagated in *Escherichia coli* (HB101, Wako Pure Chemical Industries, Ltd., Osaka, Japan). From the bacterial culture media, large quantities of plasmid DNA amenable for transfections were prepared using a chromatographic column from an EndoFree™ Plasmid Kit (Qiagen Inc., Chatsworth, CA, USA) as recommended by the manufacturer. Both expression vectors were prepared at 1 mg/ml in the buffer solution (Transfection efficiency (TE) buffer pH 8.0; 10 mM Tris–HCl, 1 mM EDTA: Ethylenediaminetetraacetic acid) and stored at −20°C until use.

CELL PREPARATION

The experimental protocol was conducted in accordance with the guidelines for animal experimentation of the Ethics Review Committee of Chiba University. Chondrocytes were obtained by enzymatic digestion of the sliced cartilage from the knee joints of New Zealand white rabbits at 4 weeks of age. The chondrocytes were cultured in Dulbecco's modified eagle medium/Ham's nutrient mixture F12 (DMEM/F12), containing 10% fetal bovine serum, 50 μg/ml gentamicin, 360 μg/ml L-glutamine and 25 μg/ml ascorbic acid on six-well plates until the cells reached confluency. Cells were trypsinized into 5 ml of medium with a trypsin-EDTA solution (Sigma–Aldrich, St. Louis, MO, USA) containing 5 μg/ml porcine trypsin and 2 μg/ml EDTA. Ten micrograms of plasmid DNA encoding luciferase was added to each well, 5 min before exposure to the radial shock waves. The cell number of the cell suspension was maintained at a constant 5.0 million per well in 5 ml of medium for all experiments.

RADIAL SHOCK WAVE EXPOSURE

Radial shock waves were generated with a newly developed device, Swiss Dolorclast® (Electro Medical Systems SA, Switzerland). The applicator was placed in direct contact with the prepared cell suspension from the upper side of each well during the exposure period. Three parameters were examined; exposure amplitude, number of pulses, and frequency, to determine the optimal conditions of gene transfer. The values of exposure amplitude used in this study were 0.1, 0.2, 0.3 and 0.4 MPa. The number of pulses was varied at 200, 500, 1000, 2000 and 5000. The effect of frequency was tested at 5, 10 and 15 Hz. For each experiment, the cell suspensions were kept at room temperature (23–25°C) during the shock wave exposure period. For the control groups, the plasmid DNA encoding luciferase was added to the cell suspension without the following exposure to shock waves (RSW(−) group). Additionally, vector alone was added to the cell suspensions without shock wave exposure (pLuc(−) group). Aliquots of the cells were stained with 0.4% trypan blue to assess their viability immediately after the exposure to shock waves at each condition. The other cells were allowed to recover for 48 h after the exposure in a 37°C humidified incubator (5% CO_2, 95% air).

EXAMINATION OF DNA CONCENTRATION

The effect of DNA concentration for the transfection efficiency had been also examined. The amount of DNA plasmid was varied at 5, 10, 20, 50 and 100 μg per sample. The experiment was conducted under identical conditions to the former investigation as to the parameters of exposure.

LUCIFERASE ASSAY

Luciferase gene expression was assessed by a standard luciferase assay procedure as follows: after the removal of culture media, cells were rinsed twice with phosphate-buffered saline, followed by treatment with cell lysis buffer (Toyo Ink Mfg. Co., Ltd., Tokyo, Japan). The samples were stored at −80°C until evaluation. After brief centrifugation (12,000*g* for 5 s), 20 μl of the supernatant (cell extract) was mixed with 100 μl of luciferase assay reagent (Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) at room temperature. Luciferase activity was measured with a luminometer (ADVANTEC, Tokyo, Japan) and expressed as relative light units (RLU). The obtained data were normalized by total protein content of each sample and used as the measure of transfection efficiency (relative luciferase activity: RLU/mg).

FLOW CYTOMETRY: DIRECT COMPARISON WITH FuGENE6 TRANSFECTION

For a direct comparison between the effects of radial shock waves and another non-viral method of gene transfer, we used the FuGENE6 Transfection Reagent (Roche, Indianapolis, IN, USA). In the radial shock wave group (RSW group), the cells were transfected with DNA encoding GFP at the determined optimum condition of exposure and DNA concentration. FuGENE6 transfection of DNA encoding GFP for the chondrocytes was performed according to the manufacturer's instruction (FuGENE6 group). Cell counts were conducted on the live cells by fluorescent-activated cell sorter (FACS) analysis. The GFP used in this experiment has a maximum excitation of 490 nm and emits light in the 520-μm range (according to the manufacturer). In the FACS, the cells were exposed to a 488-nm light source and were detected at 530 ± 15 nm. The background level of fluorescence was determined by assaying cells that had not been experimentally manipulated and setting a threshold of fluorescence above which cells were defined to be transfected (<0.5% of control cells). This threshold was used to determine the transfection rate of living cells. In all cases, 10,000 cells were counted.

STATISTICAL ANALYSIS

All numerical data, with the exception of the FACS data, were represented by mean ± standard error. The statistical significance of the data was determined with Tukey's test of equal sample variance with a confidence level greater than 95% ($P < 0.05$).

Results

EXPOSURE AMPLITUDE

The effect of the radial shock wave exposure amplitude on transfection efficiency and cell viability was examined at a constant frequency of 10 Hz and 500 pulses (Fig. 1). Relative luciferase activity showed a tendency to increase with pressure amplitude (0.1 MPa: 3.42 ± 0.06 RLU/mg; 0.2 MPa: 7.72 ± 1.56 RLU/mg; 0.3 MPa: 16.41 ± 2.16 RLU/mg; 0.4 MPa: 16.58 ± 1.68 RLU/mg) [Fig. 1(A)]. The relative luciferase activities at the conditions of 0.3 and 0.4 MPa were significantly higher than those of the control groups (RSW(−) group: 3.47 ± 0.21 RLU/mg; pLuc(−) group: 3.66 ± 0.15 RLU/mg). Cell viability slightly decreased by the addition of plasmids (RSW(−) group: 89.4 ± 0.6%; pLuc(−) group: 90.4 ± 0.8%), and significantly decreased with increasing exposure amplitude up to 0.3 MPa (0.1 MPa: 65.6 ± 0.6%; 0.2 MPa: 57.4 ± 0.1%; 0.3 MPa: 43.0 ± 0.7%; 0.4 MPa: 38.6 ± 0.3%) [Fig. 1(B)].

Amplitude of 0.3 MPa was used for subsequent experiments with variation of number of pulses and frequency, since relative luciferase activity was considered to be saturated at this condition.

NUMBER OF PULSES

Relative luciferase activity showed a tendency to increase with increasing number of pulses and decreased at the condition of 5000 pulses (200 pulses: 6.37 ± 0.32 RLU/mg; 500 pulses: 16.41 ± 2.16 RLU/mg; 1000 pulses: 23.89 ± 3.53 RLU/mg; 2000 pulses: 78.39 ± 4.73 RLU/mg; 5000 pulses: 16.21 ± 0.74 RLU/mg) [Fig. 2(A)]. Cell viability sharply decreased to 47.0 ± 0.8% at 200 pulses, and

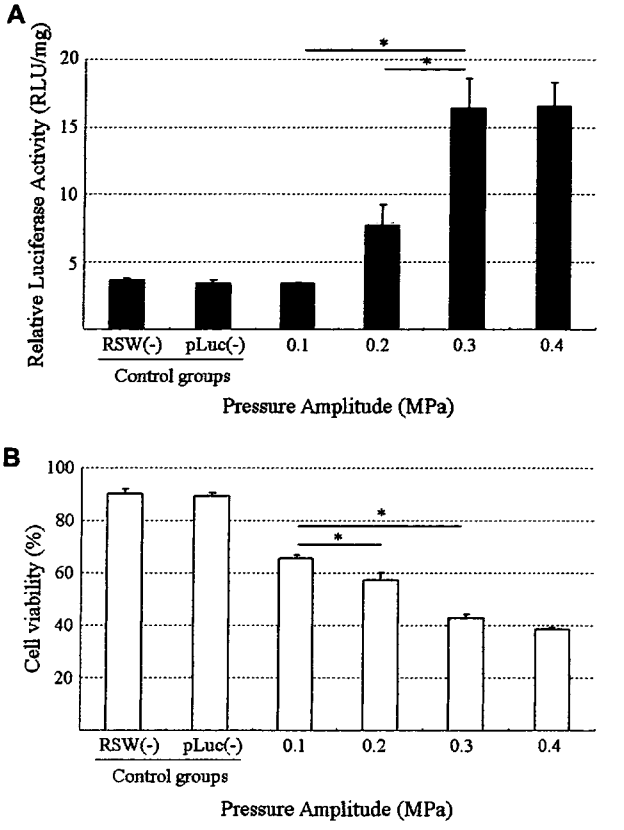

Fig. 1. The relationship of pressure amplitude and relative luciferase activity (A) and cell viability (B). The cells were exposed to radial shock waves at 500 pulses and a frequency of 10 Hz. Mean + SE ($n = 6$), $^*P < 0.05$.

remained steady with increasing number of pulses through 2000 (500 pulses: 43.0 ± 0.7%; 1000 pulses: 45.0 ± 1.4%; 2000 pulses: 39.4 ± 0.3%). At 5000 pulses, cell viability decreased dramatically to 6.2 ± 1.5% [Fig. 2(B)].

FREQUENCY

Relative luciferase activities of the shock wave-treated groups were significantly higher than those of the control groups (RSW(−) group: 3.47 ± 0.21 RLU/mg; pLuc(−) group: 3.66 ± 0.15 RLU/mg), which increased with increasing frequency up to 10 Hz and slightly decreased at 15 Hz (5 Hz: 136.9 ± 17.8 RLU/mg; 10 Hz: 164.1 ± 21.6 RLU/mg; 15 Hz: 105.3 ± 7.9 RLU/mg), although the differences among these groups were not statistically significant [Fig. 3(A)]. Cell viability slightly increased with frequency (5 Hz: 41.0 ± 0.4%; 10 Hz: 43.0 ± 0.7%; 15 Hz: 46.0 ± 0.8%) [Fig. 3(B)].

DNA CONCENTRATION

The optimal exposure condition of this experimental system was determined from the former results; pressure amplitude of 0.3 MPa, 2000 pulses, and a frequency of 10 Hz. This set of conditions was employed during the examination of DNA concentration. Relative luciferase activity showed a tendency to increase with increasing DNA concentration within the limit of our experiment (5 μg: 4.66 ± 2.79 RLU/mg;

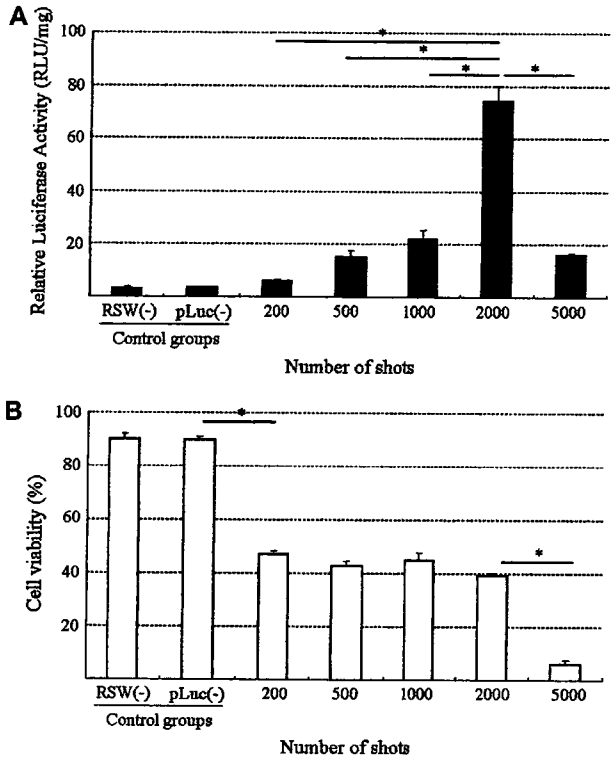

Fig. 2. The relationship of number of pulses and relative luciferase activity (A) and cell viability (B). The cells were exposed to radial shock waves at an amplitude of 0.3 MPa and a frequency of 10 Hz. Mean + SE ($n = 6$), $^*P < 0.05$.

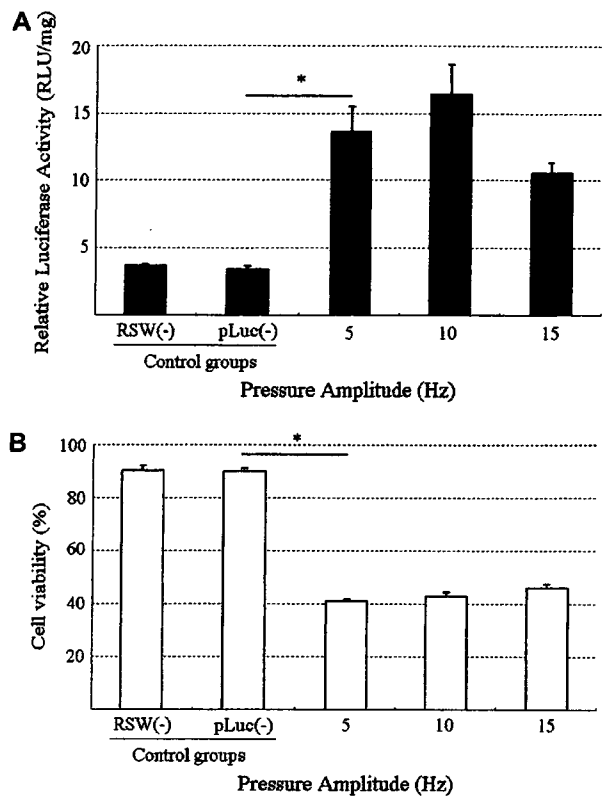

Fig. 3. The relationship of frequency and relative luciferase activity (A) and cell viability (B). The cells were exposed to radial shock waves at an amplitude of 0.3 MPa and 500 pulses. Mean + SE ($n = 6$), $^*P < 0.05$.

10 μg: 78.39 ± 4.73 RLU/mg; 20 μg: 59.42 ± 11.75 RLU/mg; 50 μg: 88.39 ± 24.18 RLU/mg; 100 μg: 525.85 ± 76.79 RLU/mg) [Fig. 4(A)]. At the concentration of 100 μg, the transfection efficiency was more than 140-fold as compared to the control groups (the RSW(−) group: 3.47 ± 0.21 RLU/mg; the pLuc(−) group: 3.66 ± 0.15 RLU/mg), and it was significantly higher than that in any other condition that was tested in the current study. Cell viability showed a tendency to decrease with DNA concentration over the concentration of 10 μg, but it was not statistically significant [Fig. 4(B)].

FLOW CYTOMETRY

For the RSW group, the conditions of optimal exposure and DNA concentration determined by former experiments were used as reference; a pressure amplitude of 0.3 MPa, 2000 pulses, a frequency of 10 Hz, and DNA concentration of 100 μg per sample. The RSW group showed a considerable uniform transfection pattern and a transfection efficiency of 4.74% [Fig. 5(A)]. Meanwhile, the FuGENE6 group also showed uniform transfection and a transfection efficiency of 3.87% [Fig. 5(B)].

Discussion

This study demonstrated that radial shock waves induced uniform gene transfer of a reporter gene to rabbit articular chondrocytes. Transfection efficiency was augmented by about 140-fold compared to the non-exposed control in particular condition with a certain degree of cell disruption [Fig. 4(A)]. The optimal numerical value of transfection efficiency, which was determined by flow cytometric analysis showed 4.74% [Fig. 5(A)]. Recent studies utilizing focused extracorporeal shock waves, and ultrasound, reported transfection rates of up to 0.5–15%[18,21,25,32] using naked DNA, and 3- to 70-fold enhancements in reporter gene expression[24,26,33]. Despite the difference of the reporter gene and the method of evaluation, the effect of radial shock waves on gene transfer seems to be comparable to that of focused extracorporeal shock waves, as well as ultrasound. On the other hand, FuGENE6 transfection resulted in a transfection efficiency of 3.87%. This value is lower than that has been expected since several methods of non-viral gene transfer including lipofection and electroporation have been reported to show higher transfection efficiency than our results[34,35]. We should be cautious to discuss the merits of these methods of gene transfer considering many factors, i.e., transfection efficiency, costs, safety. In spite of cell disruption and relatively low efficiency, radial shock wave may have the advantage of feasibility over other methods since it has been applied to clinical subjects.

Extracorporeal shock waves, and ultrasound, induce the physical phenomenon of cavitation, which is defined as the movement of newly formed and pre-existing gas bubbles containing gas or vapor in a fluid[36,37]. A shear force generated by cavitation leads to a transient permeabilization of the cell membrane and enables uptake of exogenous molecules, including plasmid DNA, into the target cells or

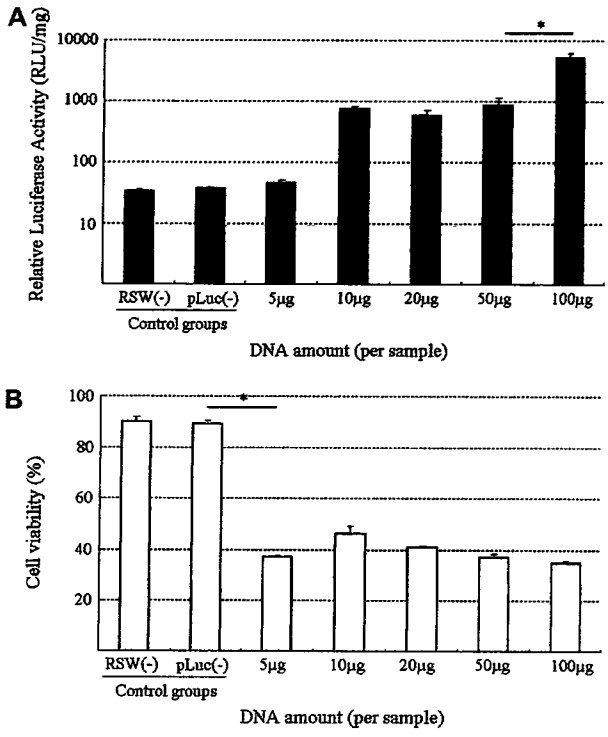

Fig. 4. The relationship of DNA concentration and relative luciferase activity (A) and cell viability (B). The cells were exposed to radial shock waves at an amplitude of 0.3 MPa, 2000 pulses, and a frequency of 10 Hz. Mean + SE ($n = 6$), $^*P < 0.05$.

tissues[38]. Cavitation is also known to be a cause of unfavorable cytotoxicity and tissue damage. The main mechanism of gene transfer by radial shock waves might be cavitation, similar to other physical methods. Decreased cell viability indicates that a certain level of cells was damaged by the exposure to radial shock waves. Conversely, a sublethal dose of cavitation may be responsible for permeabilization of the cell membrane and successful gene transfer. For the expression of a reporter gene, exposed cells have to take up plasmid DNA and possess the capacity to actually express the reporter gene[25]. These results might suggest that the cell-specific property of the shearing force might play an important role in gene transfer as a physical method induced by the radial shock waves. Cell-specific optimization which produces high transfection efficiency and simultaneously spares cell viability may be determined for each cell line, or for each employed device generating various types of ultrasound and shock waves.

The biological effect of cavitation exhibits a strong dependence on both pressure amplitude and exposure time, which is equivalent to the number of pulses[28,29]. We found that transfection efficiency and cell viability depended on pressure amplitude and number of pulses, which is consistent with previous findings. However, the change in transfection efficiency and cell viability did not demonstrate a simply proportional pattern with pressure amplitude and number of pulses. In the current study, there seemed to be thresholds around the conditions of 0.3 MPa and 2000 pulses in which a prominent change of relative luciferase activity and cell viability was observed (Figs. 1 and 2). The cavitation threshold for transfection efficiency has been shown in previous studies[21,22,29]. Furthermore, the number of pulses is considered to be more relevant than pressure amplitude in transfection efficiency, because the relative luciferase activity at 2000 pulses showed the sharpest increase compared to any other level of pressure amplitude (Figs. 1 and 2). Considering the uniform pattern of transfection shown by FACS analysis, the decrease of transfection efficiency at 5000 pulses was most likely caused by severe cell disruption. The sharp decrease in cell viability observed between 2000 and 5000 pulses is difficult to interpret. Several papers examining physical methods of gene transfer have observed decreases in cell viability proportional to increases in the number of pulses[25,27,28]. With respect to the optimal number of pulses, transfection efficiency would not likely continue to significantly increase once the number of pulses starts to exceed 2000 because of resultant decreases in cell viability. Thus, pulse numbers exceeding 2000 shots should be avoided because of the likelihood of resultant severe cell disruption and consequent low transfection efficiency.

We could not find apparent involvement of frequency in transfection efficiency and cell viability (Fig. 3). Pressure frequency has been reported to increase the number of cavitation events but has no influence on the cavitation bubble lifespan and size[39]. Clinical tissue damage in lithotripsy decreases with frequency[40]. On the other hand, some previous studies have demonstrated that the biological effect did not depend on frequency[28,41]. These conflicting findings might be due to the difference of methodology, including target cell or tissue, or the employed device. Our experimental protocol could not prove any contribution of frequency on gene transfer.

Our results suggested that transfection efficiency was increased with the concentration of DNA. However, transfection efficiency has been reported to be limited by more than DNA concentration[18,25,42]. The decrease in the cell membrane permeability would be determined by the exposure conditions of the radial shock waves (i.e., the amount of cavitation). Since passive induction of exogenous molecules into the cell is caused by the decrease of permeability, the allowable value of the transferred exogenous molecules may be limited regardless of the concentration of the exogenous molecules. Although the inflammatory effect of DNA on the applied cell or tissue has been reported[19,43], our results did not show such an effect, as in a decrease of cell viability with DNA concentration. The cell disruption or inflammatory effect of DNA itself can be negated, although a small number of cell disruptions (cell viability of the RSW(−) group: 89.4 ± 0.6%) was observed by the addition of DNA.

The effect of extracorporeal shock waves on chondrocytes or cartilage tissue has not been sufficiently elucidated. The potency of extracorporeal shock wave treatment has been partly demonstrated in several findings, including the expression of genes for osteogenesis[44], the differentiation of bone-marrow stromal cells[45] and the significant healing of the tendon–bone interface in animal studies[46,47]. Gene transfer using radial shock waves can provide an opportunity for enhancing these mechanisms when we supply the gene coding for appropriate growth factors or medicative proteins. On the other hand, macroscopic, radiological and histological analyses after exposure of focused extracorporeal shockwave have shown no pathological changes in the joint cartilage of growing rabbits[48]. This study suggested that effective exposure of extracorporeal shockwave to articular cartilage was prevented by some unknown mechanisms. The difference of acoustic impedance between soft tissue and cartilage tissue and the heterogeneous

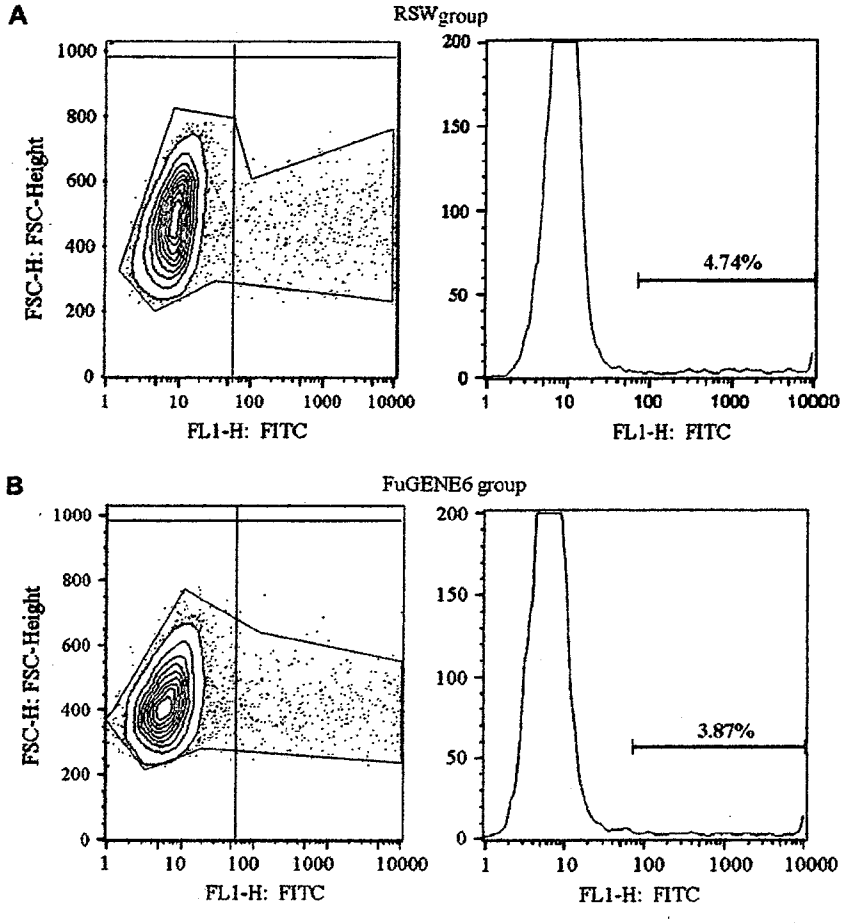

Fig. 5. Flow cytometric analysis of GFP expression in transfected cells by radial shock waves or FuGENE6 methods. The cells treated by radial shock waves (RSW group: A) and FuGENE6 (FuGENE6 group: B) are shown. The dot plot of each group is shown as fluorescence of GFP (horizontal axis) and forward scatter (vertical axis). The cells within the sphere enclosed with a kinked line were analyzed and displayed in the histograms, respectively. In each histogram, the horizontal axis shows relative fluorescent intensity and the vertical axis shows cell numbers.

distribution of joint fluid, which is essential for the presence of cavitation, may account for this phenomenon.

An ultrasound contrast agent consisting of microbubbles was reported to dramatically enhance transfection efficiency combined with ultrasound[24,43]. We did not include this factor in this study since we intended to clarify the effect of radial shock waves alone. The optimization of these factors, which may be the subject of future investigation, is required to develop a gene transfer method using radial shock waves.

The optimal conditions of this study may not be directly applied to *in vivo* or clinical subjects. However, a comparison has demonstrated that *in vivo* pressure amplitude is only 15–25% lower than the one measured *in vitro*[49]. The attenuation of radial shock waves *in vivo* may be compensated by increasing the output level of the device. Furthermore, the extent of tissue damage occurring *in vivo*, or in clinical subjects is expected to be much less than that observed *in vitro*[50–53]. Of further interest would be *in vivo* studies involving use of animal models and transfection of medicative genes, which may elucidate the prospects of gene transfer using radial shock waves. However, the clinical relevance of gene transfer by radial shock wave cannot be determined at that time. The current study indicates that low transfection efficiency and the potential for cell or tissue damage are problems which remain as equally challenging for radial shock wave therapy as for other non-viral methods. At the same time, the study findings provide encouragement that in the future, application of radial shock wave-mediated gene therapy may help mitigate cartilage morbidities.

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ORIGINAL ARTICLE

Irregularity of medial femoral condyle on MR imaging serves as a possible indicator of objective severity of medial-type osteoarthritic knee—a pilot study

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Abstract Irregularly described contour of the femur and the tibia on magnetic resonance (MR) imaging is commonly seen in osteoarthritic (OA) knees. The aim of this study is to examine the relationship between irregularity of contour of medial femoral condyle (tentatively named I-index) and severity of OA. Twenty-six medial-type OA knees with a mean age of 63.8 were studied. All patients had undergone MR imaging to measure the I-index using image analysis software, and its relationship to Lysholm score was examined. The I-index negatively correlated with Lysholm score ($r=-0.55$, $p<0.01$). The I-index for each Kellgren and Lawrence grade was significantly different. We have concluded that the I-index is a potent indicator to objectively describe the severity of OA especially for the advanced stage OA.

Keywords Irregularity · Knee · Magnetic Resonance Image · Medial femoral condyle · Osteoarthritis · Subchondral bone

Introduction

Objective assessment of the severity of osteoarthritic (OA) knee is fundamental in selecting treatment options as well as for assessing clinical results of various interventions. X-ray examination, as well as physical examination, plays an important role in objectively assessing the severity of OA of the knee joint. Joint space narrowing, osteophyte formation, and deviation of mechanical axis are typical findings detected by X-ray examination. Those findings and the clinical severity of the disease are known to be relatively well correlated [1–3], but on the other hand, discrepancies between these two factors are also observed [4–9]. Moreover, Spector et al. [6] and Spector and Hart [10] reported only about half of patients with radiographic knee OA had symptoms. In that sense, X-ray findings help to assess disease severity, but they cannot be relied on exclusively, especially when making decisions regarding surgical treatment. Obtaining a reliable index that can objectively indicate disease severity would be helpful in selecting treatment options. With this goal in mind, we attempted to establish a new system by which severity of OA would be indicated more objectively. We focused on magnetic resonance (MR) imaging because our previous study showed a correlation between knee scores and MR imaging findings. (1) Partial thinning of subchondral bone, (2) formation of multiple small cysts, and (3) low intensity lesions in the bone marrow adjacent to subchondral bone are the factors correlated with knee score [11]. We had also showed that irregularity of contour of medial femoral condyle (MFC) on MR imaging had potent utility as a predictor for the clinical outcome of surgical treatment. We classified the patient of medial-type OA knee into smooth or irregular groups using MR imaging findings. The improvement in clinical outcome after arthroscopic surgery was less for the irregular group than the smooth [12]. In this paper, we measure the irregularity of contour of MFC on MR imaging using image analysis software and examine its relationship to clinical severity of OA as well as X-ray examination.

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Materials and methods

Patients and knee function

This study included 26 consecutive patients with medial side knee pain attended by our department from April to October 2003. Twenty-six knees from 26 patients with medial-type OA were diagnosed in anteroposterior (AP) radiographs of their knees. None of them had received surgical treatment at the time of the study. Their age, knee score (Lysholm score), and varus deformity assessed by standing AP X-rays and X-ray grading using Kellgren and Lawrence (K/L) system were recorded.

Calculation of irregularity of femoral condyle

MR imaging of each knee was obtained using a 1.5 T magnet (Signa; GE Medical Systems, Milwaukee, WI) equipped with a commercial knee-surface coil. Sagittal and coronal imaging was performed with proton-weighted sequences (fast spin echo repetition time: 2000, echo time: 12 ms, echo train length: 4, time: 4 min 24 s). To assess the irregularity of the contour of the MFC, a combination of image analysis software was used. First, data from sagittal MR images of the MFC were captured into a computer (PowerMac G3; Apple, Tokyo, Japan) using an image scanner (GT-7600; Epson, Tokyo, Japan), and converted into black and white images (Photoshop 5.5; Adobe, Tokyo, Japan) (Fig. 1). Then, the irregularity of the contour was calculated using software that was originally developed to assess surface irregularity of grafted osteochondral pegs. With this software, width of contour was measured at each pixel, and root mean square (RMS) of the measured values was calculated. Originally, Hacker et al. [13] named this RMS as surface roughness. Two serial images that represent the center of the medial compartment were evaluated. The average of the calculated values was tentatively called the I-index (or irregularity index).

Statistical analysis

Spearman's rank correlation coefficient was used to assess the relationship between the I-index and knee score (Lysholm score). One-way analysis of variance associated with Tukey multiple comparisons was used to identify differences among I-index scores in each K/L group. Statistical significance was demonstrated with p values less than 0.05.

Results

Twenty-six patients consented to examination, 18 of whom were women, and 8 were men. Mean age of patients was 63.8 (range 49–82) years old. Seven, nine, and ten knees were graded II, III, and IV on the K/L system, respectively. I-index did not correlate with deviation of mechanical axis detected with standing AP X-ray (correlation coefficient of 0.155). Figure 2 shows representative relationships between extracted contours and I-index. I-index negatively correlated with Lysholm score (correlation coefficient of −0.55; Fig. 3). Mean score of I-index was 0.25±0.09 (mean ± SD)

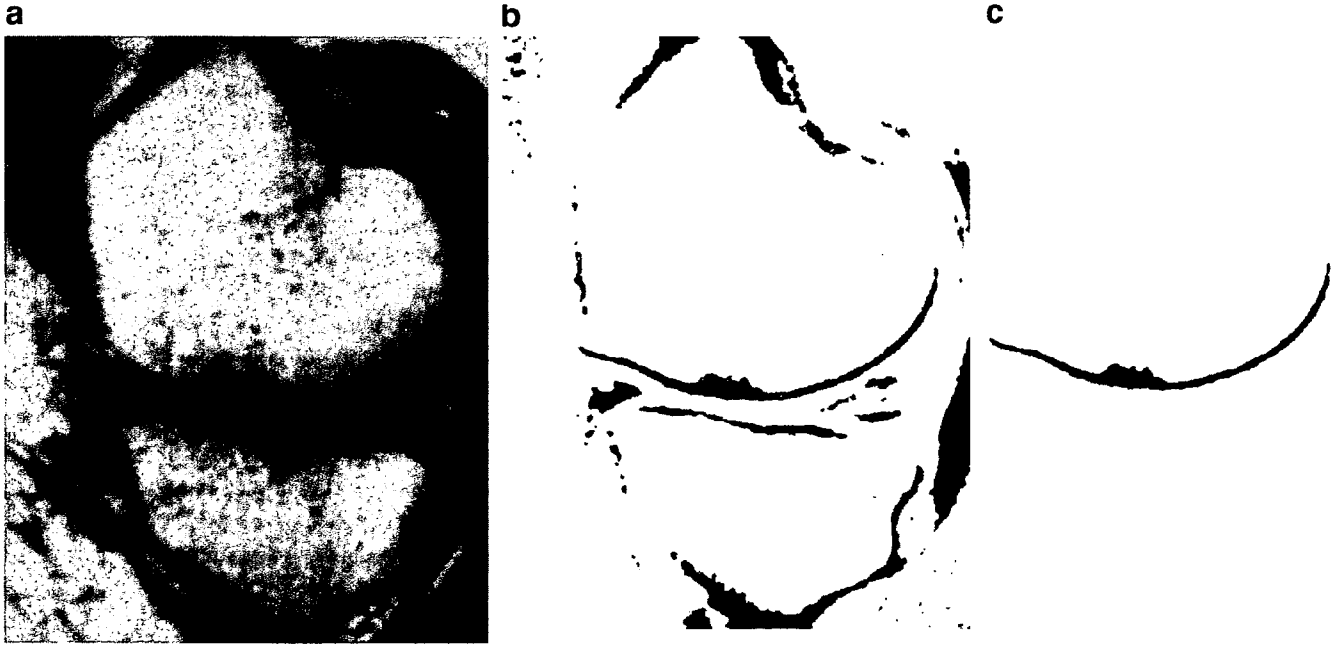

Fig. 1 Extraction of contour of medial femoral condyle. **a** Proton-weighted sagittal image of the center of the medial compartment. **b** Conversion into a black-and-white image using imaging software. **c** Contour of the medial femoral condyle

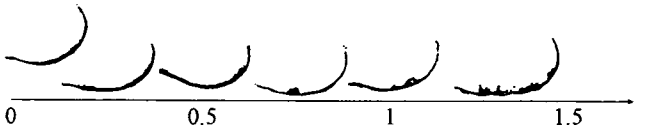

Fig. 2 Representative relationship between the irregularity index (I-index) and extracted contour. As the I-index becomes high, irregularity of contour becomes obvious

for grade II, 0.47±0.27 for grade III, and 0.82±0.41 for grade IV; I-index for grades II and III, as well as grades III and IV was significantly different (Fig. 4).

Discussion

Based on our previous study that showed the irregularity of the MFC affected the clinical outcome of arthroscopic surgery for medial-type OA [12], we attempted to establish a method of expressing the irregularity objectively (I-index) using computer software. In this study, we have presented the possibility that the I-index, which represents the irregularity of MFC on MR imaging, could serve as an indicator for assessing disease severity in medial-type OA because (1) a negative correlation between I-index and knee score is observed, and (2) I-index for each X-ray grade becomes great when the grade becomes high. We also notice that standard deviation of the I-index increases when the X-ray grade is higher. This implies that a variety of patients who have a variety of severities are put together into the same X-ray grade, and they may be given the same treatment because their X-ray grades are the same.

The K/L system was the most global scale of knee OA. However, the X-ray grading system always had some discrepancies among the observers [14–17]. Scott et al. [15] reported inter- and intra-observer reliability, calculated using intraclass correlation coefficients ranging from 0.63 to 0.83 and from 0.82 to 0.95, whereas Vilalta et al. [17] studied that the reliability among the three observers was

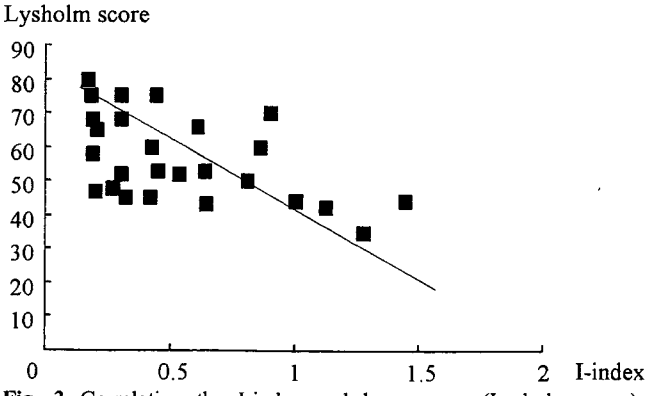

Fig. 3 Correlation the I-index and knee score (Lysholm score). Lysholm score and the I-index have negative correlation, with a correlation coefficient of −0.55 ($p<0.01$)

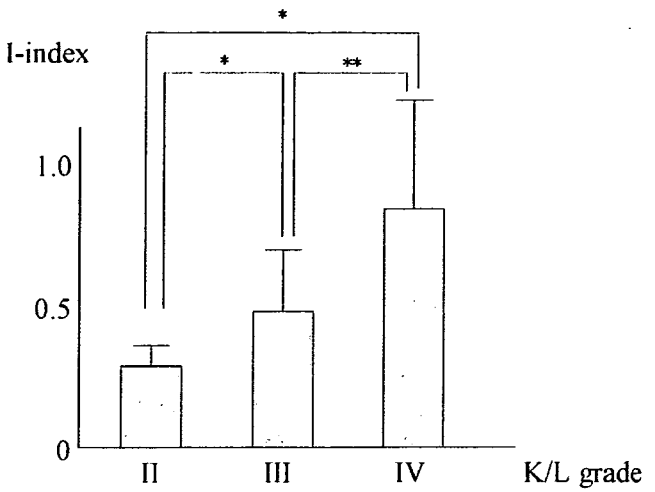

Fig. 4 Correlation of the I-index and the Kellgren and Lawrence grade. The I-index increased according to the K/L grading. *$p<0.01$, **$p<0.05$

less than 0.5. Contrary to that, because of semi-automatically calculated characters, I-index can lessen these problems. Moreover, most X-ray grading systems compulsively put all radiographs into four to five grades, whereas I-index is metric-variable. We would like to propose that treatment options should be chosen on a case-by-case basis and that I-index can provide an indication for determining what treatment should be applied to a patient. Contour of the MFC on MR imaging corresponds to the subchondral bone plate. Specimens retrieved at the time of TKA revealed that the irregularly described femoral condyles had many cystic lesions in their subchondral plate as well as derangement of the subchondral bone architecture. These results provide histological support for the correlation between irregularly described contour and severity of OA. Several limitations should be indicated for the present study. First, irregularity of only the femoral side was studied, and we did not refer to the tibial side. The major reason for this was that pathological changes occurring in the tibial side can be detected as pathological changes seen on the femoral side as a mirror lesion. Cicuttini et al. [18] pointed out that deteriorations in the femur and tibia are strongly related to one another. Assessing the femoral cartilage is advantageous compared with tibial cartilage for evaluation of knee function because the femoral cartilage articulates with patellar and tibial cartilages [19]. So we only examined the femoral side in the present study but considered that the contour of the tibia might make the I-index system more accurate. Second, the age of patients in this study was relatively inhomogenous. Age-related loss of knee function might cause errors in assessing disease severity [5, 19–21]. A more homogenous demographic background would be ideal, but consecutive outpatients were observed in the present study for practical reasons. Third, proton-weighed MR images were chosen for the present study, although a

more accurate image acquisition technique, such as spoiled gradient-echo sequencing, would make evaluation of irregularly described subchondral bone more precise. We preferred to use common MR imaging sequences for this study because 3-D techniques are overly time-consuming at present. Lastly, we did not use direct data of MR imaging but used hard copy of MR imaging as starting data to utilize the software that had been developed previously. To obtain a more accurate I-index, further development of software that calculates contour irregularity using direct data from MR images, such as DICOM data, should be pursued. The I-index system is likely to be helpful to assess only patients with a relatively advanced stage of OA. The I-index reflects the obvious pathological changes in the subchondral area, and patients who have those changes appeared to have obvious X-ray findings. Other evaluation techniques are necessary to assess patients in initial stages of OA where cartilage evaluation has priority. We conclude that the I-index can serve as an objective indicator for selecting treatment options.

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