Utility of NucleoCounter for the chondrocyte count in the collagenase digest of human native cartilage

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Abstract In cartilage tissue engineering, viable cell numbers should be correctly counted in the collagenase digest of the biopsied cartilage. However, this is a difficult task due to the presence of matrix debris, cell ghosts and their aggregates. To search for the correct cell counting method in this situation, we evaluated the utility of an automatic cell counting device, the NucleoCounter, and compared it with conventional staining using the LIVE/DEAD® kit. We first measured the cell numbers of a standard chondrocyte sample by the NucleoCounter, which showed a high accuracy ($R^2 = 0.9999$) and reproducibility (%CV: 2.00–8.66). We then calculated the cell numbers and viability in some collagenase digests of native cartilage using either the NucleoCounter or LIVE/DEAD® kit, revealing that the total cell numbers, viable ones and viability were highly correlated between them ($R^2 = 0.9601$, 0.9638 and 0.917 , respectively). However, both the intrapersonal and interpersonal variabilities in the NucleoCounter

was significantly decreased to about 1/20–1/5, compared to that of the LIVE/DEAD® kit. The NucleoCounter was regarded as a useful tool for simple, rapid, and highly reproducible cell counts, which may not only provide constant experimental data in a certain laboratory, but also contribute to the high reproducibility of the clinical results of cartilage tissue engineering among multiple institutions.

Keywords Cartilage · Chondrocytes · Cell count · Isolation · Collagenase

Abbreviations

ACI Autologous chondrocyte implantation
PI Propidium iodide
 R^2 The coefficient of regression
%CV The coefficient of variation

Introduction

Cartilage tissue engineering has been clinically applied as ACI for the treatment of focal joint defects (Brittberg et al. 1994) or augmentation of nasal cartilage (Yanaga et al. 2006). This cell-based therapy involves the following procedures: a harvesting of a small cartilage biopsy, the isolation of chondrocytes by collagenase digestion, culturing of the isolated chondrocytes in vitro, and the subsequent

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transplantation of proliferated autologous chondrocytes into the lesions. In the ACI, the correct counting of viable cell numbers in the collagenase digest of the biopsied cartilage is one of keys leading to a successful culture, eventually producing fair clinical results for ACI. Therefore, the establishment of chondrocyte counting is significantly demanded in the field of cartilage tissue engineering.

However, the correct cell count in the collagenase digest of the native cartilage is a difficult task due to the presence of cells with a variety of sizes or shapes, and various degrees of viability. In addition, the cartilage digest also includes abundant fragments of extracellular matrices or cell aggregates in which the cells are in direct contact with each other or mediated by the fragmented matrices (Fig. 1). Therefore, the establishment of a cell counting method with a high reliability, consistency and accuracy even in the collagenase digest of the native cartilage is required for safe and stable cartilage tissue engineering.

Up to now, some methods, including the classical method using a hemocytometer with trypan blue staining, or the fluorescent system of the LIVE/DEAD® Viability/cytotoxicity kit for mammalian cells (Invitrogen) that distinguishes viable cells from dead ones, have been commonly applied for the counting of viable cells (Yamaoka et al. 2006; Papadopoulos et al. 1994). As the trypan blue dye only enters the walls of impaired plasma membranes, it can distinguish the dead cells from the viable ones. The number of unstained cells should be evaluated by light microscopy for the viable cell count. However, if more than 5 min have passed since the addition of

the dye, the trypan blue exclusion becomes inaccurate because the number of blue staining cells increases with time (Kenneth et al. 1985). Otherwise, if the fragmented matrices are not stained by trypan blue, it may not be distinguished from the viable cells that are similarly unstained by this dye.

In contrast, the LIVE/DEAD® kit provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of viable and dead cells using two probes (calcein AM and ethidium homodimer). These probes can directly detect both viable and dead cells, respectively, which would increase the accuracy of the cell viability measurement. However, the fragmented matrices or cell debris contained in the collagenase digest of the native cartilage occasionally emit an autofluorescence, thus interfering with the exact and reproducible cell counting (Gareau et al. 2004).

Recently, an automatic cell counting device, i.e., the NucleoCounter (ChemoMetec) has been developed, which is equipped with a fluorescence microscope and CCD camera for determining the cell number and viability. In this system, the cell sample is first mixed with a lysis buffer to produce free nuclei. The solution containing the free nuclei is then loaded into the NucleoCassette (ChemoMetec), which is internally coated with PI. After the cassette is inserted into the NucleoCounter chamber, the total cell number is automatically calculated by the software NucleoView (ChemoMetec). The number of dead cells can also be counted by the direct load of the cell suspension without adding the lysis buffer, because the cells with damaged membranes would be

Fig. 1 Phase contrast images of chondrocytes in the collagenase digest of native cartilage (*left*) and cell suspension obtained during subculture (passage 2, *right*)

stained by the PI. The subtraction of the dead cell number from the total cell number provides the viable cell numbers. According to the standardized protocol, all these procedures can be automatically completed within a few minutes, which would improve the accuracy and reproducibility of the cell counting. It has already been reported that this apparatus was useful for the counting of mammalian suspension cells including the mouse-mouse hybridoma TB/C3, NS0 myeloma, CHO 320 (Dimpalkumar et al. 2006), or hematopoietic progenitor cells (Mascotti et al. 2000). However, the usefulness of this counting system for the determination of the cell count of adherent mammalian cells that are embedded within extracellular matrices, like human chondrocytes, has not yet been examined.

The purpose of the present study is to confirm the utility of the NucleoCounter for the counting of human chondrocytes and to evaluate its reliability, consistency, or accuracy for the chondrocyte count in collagenase digest of human native cartilage. For this purpose, we treated human cartilage with collagenase, counted the cell numbers by both the NucleoCounter and a conventional method using the LIVE/DEAD® kit, and compared the specificities of both methods.

Materials and methods

Cell preparation

All procedures of the present experiments were approved by the ethics committee of the University of Tokyo Hospital (ethics permission number 622). Remnant auricular cartilage from microtia patients was obtained during surgery in adherence to the Helsinki Principles. The chondrocytes were isolated with digestion by treatment with 0.15% collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 24 h. In order to obtain the standard sample, the human chondrocytes were seeded in a 100-mm plastic tissue culture dish at a density of 2,500 cells/cm² and cultured in Dulbecco's modified Eagle's medium/F12 containing 5% human serum supplemented with fibroblast growth factor-2 (FGF-2, 100 ng/mL) and insulin (5 µg/mL), as previously reported (Takahashi et al. 2005). Passaging was

performed three times using a trypsin–EDTA solution (Sigma, St. Louis, MO).

Cell counting

The staining by the LIVE/DEAD® kit (Invitrogen, Carlsbad, CA) was according to the manufacturers's protocol. Briefly, the assay reagent including calcein AM and ethidium homodimer was added to an aliquot of the cartilage digest. After incubation at room temperature for 30 min, the sample was placed in the hemocytometer and observed by fluorescent microscopy (DMI4000B, Leica, Wetzlar, Germany). The fluorescent filter conditions were calcein AM:N2.1 (FITC) and ethidium homodimer: L5 (Texas Red). The number of viable cell (green fluorescence) and dead ones (red fluorescence) were then measured. Viability = $C_v / (C_v + C_d) \times 100$, where C = Cell numbers; v = viable; d = dead.

For the NucleoCounter (ChemoMetec, Allerød, Denmark), the procedure for the total cell count involved sample preparation and sample analysis. During the sample preparation, the cell suspension or the tissue digest (100 µL) was mixed with an equal volume of lysis/disaggregation buffer and vortexed. A volume of stabilization buffer equal to the initial sample (100 µL) was added to the cell lysate and vortexed. This cell lysate (50 µL) was loaded into a NucleoCassette, in which the nuclei were stained with PI. The NucleoCassette was then placed in the NucleoCounter for analysis. The total cell concentration in the NucleoCassette was displayed on a personal computer using the NucleoView software. By measuring a cell sample without pretreatment, only cells with pre-impaired plasma membranes were PI-stained, which gave an estimate of the dead cell number. By using these two measurements, the viability can be automatically calculated and displayed via the NucleoView software.

Statistics

Scatter diagram was used to analyze the association of two different parameters that were obtained from cell counts by the NucleoCounter or LIVE/DEAD® kit. Regression line and coefficient of correlation R² were calculated using Microsoft Office Excel 2007 software (Microsoft, Redmond, WA, USA).

Results

At first, we verified the usefulness of the NucleoCounter for the cultured chondrocytes that are adherently growing cells. We prepared the standard sample in which the chondrocyte suspension (2×10^4 cells/mL, 5.2×10^5 , 1.28×10^6 , and 3.08×10^6) had been exactly prepared by the triple measurement of the cell density using the LIVE/DEAD® kit followed cautious dilutions. The cell numbers of the standard samples were then counted by the NucleoCounter. When one examiner measured the cell numbers three times for each sample, the results of the NucleoCounter showed $2 \times 10^4 \pm 1.73 \times 10^3$ (%CV, 8.66), $5.27 \times 10^5 \pm 1.05 \times 10^4$ (%CV, 2.00), $1.29 \times 10^6 \pm 9.01 \times 10^4$ (%CV, 7.01), and $3.03 \times 10^6 \pm 2.35 \times 10^5$ (%CV, 7.75), suggesting a high accuracy ($R^2 = 0.9999$) and reproducibility (each %CV: 2.00–8.66) (Fig. 2).

We next attempted to use the NucleoCounter for cell counting of the collagenase digests in the native cartilage. The solutions ($n = 18$) in which the native cartilage from 18 patients was individually digested by 0.15% collagenase were measured for cell counts using both the NucleoCounter and LIVE/DEAD® kit at one time by one examiner. The total cell numbers,

Fig. 2 Cell count for standard chondrocyte samples by NucleoCounter standard samples containing human chondrocytes cultured at passage 3 (2×10^4 cells/mL, 5.2×10^5 , 1.28×10^6 and 3.08×10^6) were measured three times by one operator using the NucleoCounter. Data are expressed as mean (bars) $\pm$ S.E. (error bars)

the viable cell numbers and the viability were highly correlated between the NucleoCounter and LIVE/DEAD®, with R^2 values of 0.9601, 0.9638 and 0.917 (Figs. 3a, 4a and 5a). Regarding the intrapersonal variability in the three tests per person (for three evaluated operators in total), the %CV of the total cell numbers was 2.09 for the NucleoCounter, and it

b

	NuceloCounter	Live/Dead®
Average	288,000	223,000
SD	6,000	26,300
%CV	2.09	11.8

c

	NuceloCounter	Live/Dead®
Average	294,000	242,000
SD	7,500	105,000
%CV	2.55	43.4

Fig. 3 Comparison of the total cell numbers in collagenase digests between NucleoCounter and LIVE/DEAD® kit. **a** One operator measured total cell numbers in 18 samples using both the NucleoCounter and LIVE/DEAD® kit. Each sample was measured only once. **b** Total cell number in one collagen digest sample was measured three times using both the NucleoCounter and LIVE/DEAD® kit by one operator. **c** Total cell number in one collagen digest sample was individually measured only once using both the NucleoCounter and LIVE/DEAD® kit by three different operators

increased to 11.8 for the LIVE/DEAD® method (Fig. 3b). Also, for the viable cell numbers, the intrapersonal variability of the NucleoCounter was 2.04, thus lower than that of the LIVE/DEAD® method (17.5) (Fig. 4b). The %CV of the viability calculated by the LIVE/DEAD® method (7.95) was significantly higher than when compared to that of the NucleoCounter (0.581) (Fig. 5b). When we

examined the interpersonal variability of the three operators per method, the %CV of the total cell numbers, viable cell numbers and viability of the NucleoCounter (2.55, 2.85 and 0.840, respectively, Figs. 3c, 4c and 5c) were not significantly changed, compared with those from the intrapersonal measurement (2.09, 2.05 and 0.581, respectively, Figs. 3b, 4b and 5b). However, the %CV of all three

	NuceloCounter	LIVE/DEAD®
Average	199,000	143,000
SD	4,160	26,300
%CV	2.04	17.5

	NuceloCounter	LIVE/DEAD®
Average	200,000	169,000
SD	5,690	74,800
%CV	2.85	44.3

Fig. 4 Comparison of the viable cell numbers in collagenase digests between NucleoCounter and LIVE/DEAD® kit. **a** One operator measured viable cell numbers in 18 samples using both the NucleoCounter and LIVE/DEAD® kit. Each sample was measured only once. **b** Viable cell number in one collagen digest sample was measured three times using both the NucleoCounter and LIVE/DEAD® kit by one operator. **c** Viable cell number in one collagen digest sample was individually measured only once using both the NucleoCounter and LIVE/DEAD® kit by three different operators

	NuceloCounter	Live/Dead®
Average	67.8	64.0
SD	0.00394	5.08
%CV	0.581	7.95

	NuceloCounter	Live/Dead®
Average	67.8	70.5
SD	0.569	12.4
%CV	0.840	17.5

Fig. 5 Comparison of the viability in collagenase digests between NucleoCounter and LIVE/DEAD® kit. **a** One operator measured viability in 18 samples using both the NucleoCounter and LIVE/DEAD® kit. Each sample was measured only once. **b** Viability in one collagen digest sample was measured three times using both the NucleoCounter and LIVE/DEAD® kit by one operator. **c** Viability in one collagen digest sample was individually measured only once using both the NucleoCounter and LIVE/DEAD® kit by three different operators

parameters for the LIVE/DEAD® kit increased 2–4-fold (intrapersonal trials vs. interpersonal trials: 11.8 vs 43.4, 17.5 vs. 44.3, and 7.95 vs. 17.5), when the three different operators did individual measurements (Figs. 3, 4 and 5b, c).

Discussion

Cartilage matrices are principally composed of collagen and proteoglycan of which the former one consists for two-thirds of the dry weight of adult cartilage (Eyre 2004). The collagen in cartilage mainly includes type II collagen (>80%) and other ones, such as type IX or XI collagens. The collagen molecules are composed of a stable triple helix crosslinked with each other, forming the fibril networks in the native cartilage (Eyre 2004). These stable structures can hardly be degraded by pepsin, trypsin or gelatinase, but are effectively hydrolyzed by collagenase (Brittberg et al. 1994, Yanaga et al. 2006). Actually, some matrix remnants are usually observed in the collagen digest of native cartilage, as shown in Fig. 1. Moreover chondrocytes are able to express many kinds of adhesion molecules for both the cell-to-matrix contacts and cell-to-cell interactions including integrins $\alpha 1$, $\alpha 6$, αv , and $\beta 1$ or cadherins-1 and 11 (Takahashi et al. 2007). After collagenase digestion, the isolated chondrocytes that express abundant adhesion molecules probably interact with the remnants of the degraded matrices or are attached to each other, forming the aggregates of cells and/or matrices. The contamination of these aggregates or the matrix remnants in the collagenase digest of the native cartilage is inevitable during the chondrocyte isolation. These contaminants interfere with the correct cell counting. The complete digestion of the native cartilage and better isolation of naked chondrocytes could be achieved if a combination of enzymes were used instead of collagenase alone. For example, the sequential use of hyaluronidase, pronase and collagenase may enable complete digestion of the matrix in a short time, facilitating the cell count. However, when chondrocytes are thoroughly deprived of the surrounding extracellular matrices, the cell–matrix interaction may be downregulated.

The cell–matrix interaction is often mediated by integrins, whose signals are involved in not only proliferation, and differentiation, but also survival of

chondrocytes (Svoboda 1998). Because the chondrocyte isolation procedure from the native cartilage interrupts the signal of the cell-to-matrix interactions, we can not deny the possibility that the chondrocytes after the complete digestion of the matrix are more or less impaired with respect to their viability. The decrease in cell viability is speculated to influence cell shape and cell sizes, as well, which may also make the correct cell count a more difficult task. Therefore, the sequential use of various enzymes will possibly result in complete digestion of the matrix, but further study is needed to conclude whether the usage of a combination of enzymes improves the maintenance of cell viability as well as the preciseness of the cell count or not.

On the other hand, the NucleoCounter could exactly and reproducibly measure the chondrocyte number even in the collagen digest. The %CV of the cell numbers or cell viability in the NucleoCounter was much lower than that in the conventional fluorescent cell counting system, i.e., the LIVE/DEAD® kit, either when three different operators counted the same sample or when one person evaluated a given sample three times. These results clearly showed that both the intrapersonal and interpersonal variabilities were reduced when using the NucleoCounter. The NucleoCounter may not only provide constant experimental data in a given laboratory, but also contribute to a high reproducibility in the clinical results of cartilage tissue engineering among multiple institutions. The accuracy and consistency of the NucleoCounter are due to the characteristics of the counting system for which all procedures of cell disaggregation, cell staining and recognition of viable cells are carried out according to a standard protocol.

For the operation time, we found that it took approximately 60 min for the conventional assessment of the cell number and viability using the LIVE/DEAD® kit. This may be altered by the cell density of the samples, the number of sample lots, the experience of the operator and many other factors (Al-Rubeai et al. 1997). In comparison, the NucleoCounter measurement was composed of only two pipetting steps, followed by the analysis which took approximately 1 min. In conclusion, we have shown that the NucleoCounter is a useful tool for simple, rapid, and highly reproducible cell counting, even for collagenase digests of native cartilage containing

abundant contaminations of cell ghosts, matrix debris and aggregates of cells and/or matrices.

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