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Cell diameter measurements obtained with a handheld cell counter could be used as a surrogate marker of G2/M arrest and apoptosis in colon cancer cell lines exposed to SN-38

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ABSTRACT

In vitro assessment of chemosensitivity are important for experiments evaluating cancer therapies. The Scepter 2.0 cell counter, an automated handheld device based on the Coulter principle of impedance-based particle detection, enables the accurate discrimination of cell populations according to cell size and volume. In this study, the effects of SN-38, the active metabolite of irinotecan, on the colon cancer cell lines HCT116 and HT29 were evaluated using this device. The cell count data obtained with the Scepter counter were compared with those obtained with the ^{3}H-thymidine uptake assay, which has been used to measure cell proliferation in many previous studies. In addition, we examined whether the changes in the size distributions of these cells reflected alterations in the frequency of cell cycle arrest and/or apoptosis induced by SN-38 treatment. In our experiments using the Scepter 2.0 cell counter, the cell counts were demonstrated to be accurate and reproducible measure and alterations of cell diameter reflected G2/M cell cycle arrest and apoptosis. Our data show that easy-to-use cell counting tools can be utilized to evaluate the cell-killing effects of novel treatments on cancer cells *in vitro*.

1. Introduction

Various methods, such as the colony formation assay [1], the MTT (3-[4,5-dimethylthiazol-2-yl],5-diphenyltetrazoliumbromide) assay [2,3], the trypan blue assay [4], the BrdU (5-bromo-2′-deoxyuridine) incorporation assay [5] and the ^{3}H-thymidine incorporation assay [6,7], have been used to assess *in vitro* cell proliferation as a means of evaluating the treatment effects of chemotherapeutic agents, ionizing radiation or other cell-killing agents. The colony formation assay is considered to be the standard method for evaluating the effects of such treatments on cell proliferation *in vitro*, but it takes a relatively long time (about 2 weeks), and it is sometimes difficult to evaluate colony formation via microscopic observations. The MTT assay is a colorimetric assay that is used to determine the activity of cellular enzymes that reduce tetrazolium salts to yield insoluble formazan dyes, and the WST (water soluble tetrazolium salt) assay is a colorimetric assay that uses a water soluble tetrazolium salt and yields a water soluble formazan dye [8]. These colorimetric assays measure the cellular metabolic activity of NAD(P)H-dependent oxidoreductase enzymes, so their results indirectly reflect cell viability. These assays are relatively simple and quick, however, their results can sometimes be misleading. For example, it has been reported that soluble formazan assays can be affected by the medium used, serum albumin and fatty acids [9]. In the ^{3}H-thymidine incorporation assay, the radioactivity of DNA-incorporated ^{3}H-thymidine is measured with a scintillation beta-counter. This assay is an accurate indicator of DNA synthesis, but has to be performed in a laboratory equipped with a microplate scintillation counter.

Direct cell counting using a microscope and hemocytometer is laborious and time-consuming and so cannot be used as a routine examination method for evaluating the cell-killing effect of a novel compound. Various automated cell counters have been developed as bench-top systems based on the principles of conventional

Abbreviations: MTT, 3-[4,5- dimethylthiazol-2-yl],5-diphenyltetrazoliumbromide; BrdU, 5-bromo-2′-deoxyuridine; WST, water soluble tetrazolium salt; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PI, propidium iodide; CV, coefficients of variation; MSI, microsatellite instability.

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image analyzers and/or flow cytometry. The Merck Millipore Corporation have developed a handheld automated cell counting tool, the Scepter 2.0 cell counter, which uses the Coulter principle of impedance-based particle detection and displays reliable accuracy [10]. Furthermore, this system can also assess the size and volume of cells and obtains more information than other cell counting tools.

Irinotecan is a major chemotherapeutic drug that acts as an inhibitor of topoisomerase I, which is involved in breakage-reunion reactions during the DNA replication and translation of DNA [11]. Irinotecan is a prodrug that is converted to SN-38 by the action of carboxylesterase (EC 3.1.1.1) *in vivo* [12,13]. SN-38 is the active metabolite of irinotecan and induces double stranded DNA breaks, cell cycle arrest at G2/M and apoptosis in colon cancer cells [14,15].

Here, we evaluated the treatment effects of SN-38 on colon cancer cell lines using the Scepter 2.0 cell counter. The obtained data were compared with those derived from other assays such as the ^{3}H-thymidine incorporation assay; a cell cycle assay measuring the nuclear fluorescence intensity of propidium iodide (PI); and apoptosis assays using annexin V, calcein AM and PI staining. The aim of this study was to examine the correlation between cell diameter and nuclear DNA content and/or apoptosis and to validate whether the Scepter 2.0 cell counter can be used to assess the effect of a novel compound *in vitro*.

2. Materials and methods

2.1. Cell lines and culturing

The human colon cancer cell lines HCT116 and HT29 were purchased from the American Type Culture Collection (ATCC; Summit Pharmaceutical International, Tokyo, Japan). The cells were cultured in RPMI1640 medium (Life Technologies Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Japan, Yokohama, Japan) and 100 μg/ml kanamycin (Sigma–Aldrich, St. Louis, Mo, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Drug

SN-38 (Sigma–Aldrich) stock solutions were prepared by dissolving SN-38 in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, and stored at −20 °C.

2.3. Cell count assay using the Scepter 2.0 cell counter

Cells were trypsinized and harvested during the exponential growth phase, and a total of 1–3 × 10^4 cells were plated in 24-well culture plates (500 μl/well). After 24 h, the cells were exposed to various concentrations of SN-38 for the indicated time periods. In the cell counting experiments using the Scepter 2.0 cell counter (Merck Millipore Japan, Tokyo, Japan), the cells were trypsinized and resuspended in culture medium. After the resultant cell suspensions had been transferred to 1.5 ml microcentrifuge tubes, the cells were counted with a Scepter 2.0 cell counter equipped with a 60 μm sensor tip, according to the manufacturer's recommendation. Cell counts and cell size distributions were shown as histograms on the monitor of the Scepter 2.0 cell counter, and these data were analyzed with the Scepter 2.0 Software Pro computer software. Before the cells were counted, the upper and lower gates of the counter were adjusted manually to eliminate small particles. Data regarding cell diameter are presented as the mean ± standard deviation (SD) values of triplicated experiments.

2.4. ^{3}H-thymidine incorporation assay

A total of 2–4 × 10^3 cells in 100 μl of culture medium were plated in 96-well culture plates. After 24 h, the cells were exposed to various concentrations of SN-38 for 48 h. ^{3}H-thymidine (1 μCi/well) was added to each well, and the plates were incubated at 37 °C for 2 h. Then, the ^{3}H-thymidine containing medium was discarded, and each well was washed twice with 100 μl of PBS. For cell fixation, 100 μl of 5% trichloroacetic acid (TCA) were added to each well, and then the plates were incubated on ice for 10 min. After discarding the 5% TCA, 50 μl of 1 N NaOH were added to each well, and the plates were incubated at 37 °C for 10 min. Next, 30 μl of the cell lysate were transferred to 96-well LumaPlates (Perkin-Elmer, CA, USA) and air-dried. Finally, the radioactivity of ^{3}H-thymidine was assessed using the TopCount NXT microplate scintillation counter (Perkin-Elmer). Data are expressed as mean ± SD values for replicated wells (n = 6).

2.5. Analysis of DNA content using the IN Cell Analyzer 1000

The DNA contents of the cell nuclei were measured by PI staining using the IN Cell Analyzer 1000 (GE Healthcare Japan, Tokyo, Japan). A total of 2–4 × 10^3 cells in 100 μl of culture medium were plated in 96-well culture plates. After 24 h, the cells were exposed to various concentrations of SN-38 for 48 h, before being washed twice with 100 μl of PBS. For cell fixation, 100 μl of 70% ethanol were added to each well, and the plates were incubated at 4 °C for 60 min. The ethanol was then discarded, and 100 μl of PBS containing 0.2% Triton X-100, 1 μl of PI (BioVision, PA, USA), and 1 μl of RNase A (Worthington Biochemical Co., Lakewood, USA) were added to each well, before the plates were incubated at room temperature for 60 min. The PBS containing Triton X-100, PI, and RNaseA was discarded, and 100 μl of PBS were added to each well. The IN Cell Analyzer 1000 scanned images of 9 fields from each well, and the intensity of the fluorescence produced by PI was calculated with the IN Cell Investigator software (GE Healthcare Japan).

2.6. Apoptosis assay using fluorescence microscopy

Cell apoptosis was evaluated using the Annexin V-Biotin Apoptosis Detection Kit (BioVision). A total of 5 × 10^5 cells were resuspended in 200 μl of the binding buffer together with 2 μl of annexin V-biotin and 2 μl of PI, before being incubated at room temperature for 5 min. The cell suspensions were centrifuged, and the resultant pellets were resuspended in 200 μl of the binding buffer supplemented with 0.1 μl of calcein AM stock solution (1 mM) (Life Technologies Japan) and 0.2 μl of Cy5-labeled streptavidin (GE Healthcare Japan), before being incubated at room temperature in the dark for 10 min. The cell suspensions were centrifuged, and the resultant cell pellets were resuspended in 100 μl of PBS. The cell suspensions were then dropped onto slides and covered with cover slips. Microphotographs were acquired using a BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan).

2.7. Statistical analysis

Data are expressed as mean ± SD values. The significance of differences in cell diameter, SN-38 exposure time, and/or SN-38 concentration was analyzed by two-way repeated measures ANOVA and the square of the correlation coefficient (r^2) was analyzed using Pearson's correlation test to assess the correlation between the Scepter cell count and ^{3}H-thymidine uptake or between the percentage of cells in the high diameter region and the percentage of tetraploid (4n) cells. All statistics were calculated using the GraphPad Prism V5.0 software (GraphPad Software, Inc., San Diego,

CA, USA). *P*-values of <0.05 were considered to be statistically significant.

3. Results

In this study, we used two colon cancer cell lines, HCT116 and HT29, to examine the chemosensitivity of colon cancer to SN-38, the active metabolite of irinotecan. At various concentrations of SN-38 (0, 2, 4, and 8 nM), cell proliferation was analyzed by counting the number of cells using the Scepter 2.0 cell counter (Fig. 1A and B). The HCT116 cells were more sensitive to SN-38 than the HT29 cells. The cell counts and diameter measurements obtained with the Scepter 2.0 cell counter were reproducible, displaying the coefficients of variation (CV) less than 5% in all samples (Table 1A and B). By adjusting the upper and lower diameter ranges of its gates, the Scepter 2.0 cell counter can also be used to assess the size distributions of cell populations. In both cell lines, the mean cell diameter of the gated fraction increased in a time- and dose-dependent manner (time course: $P < 0.0001$, drug concentration: $P < 0.0001$, interaction: $P < 0.0001$) (Supplementary Fig. 1A and B). Increases in mean cell diameter and the percentage of cells belonging to the small particle fraction were observed at lower SN-38 concentrations in the HCT116 cells than in the HT29 cells (Supplementary Fig. 1A and B).

To determine whether cell number as assessed by the Scepter 2.0 cell counter could be used as an index of cell proliferation, we evaluated the correlation between the cell counts obtained with the cell counter and the results of the ^{3}H-thymidine uptake assay, which is considered to be the standard assay for assessing DNA synthesis and cell proliferation, at various SN-38 concentrations (Fig. 1C–E) [6,7]. The dose-response curves produced by the Scepter 2.0 cell counter and ^{3}H-thymidine uptake assay were well correlated with each other in both cell lines ($r^2 = 0.9761$, $P = 0.0002$ in HCT116; $r^2 = 0.9968$, $P < 0.0001$ in HT29) (Fig. 1D and E). The half maximal inhibitory concentrations (IC_{50}) of SN-38 determined by the Scepter 2.0 cell counter and ^{3}H-thymidine uptake were 1.14 nM and 0.72 nM in the HCT116 cell line, and 3.25 nM and 2.63 nM in the HT29 cell line, respectively (Fig. 1D). Thus, the IC_{50} values of SN-38 measured by Scepter 2.0 cell counter were higher than those measured by the ^{3}H-thymidine uptake assay in both cell lines.

To examine the correlation between cell diameter and the frequency of cell cycle arrest, we analyzed DNA content using the IN Cell analyzer 1000. The IN Cell Analyzer 1000 is an automated imaging cytometer with a charge-coupled device (CCD) image sensor that can estimate DNA content by evaluating the intensity of PI fluorescence. PI staining is one of the standard methods for measuring nuclear DNA content [16]. On being exposed to various concentrations of SN-38, the diameter and DNA content of the HCT116 cells increased in a dose-dependent manner, resulting in DNA tetraploidy and G2/M arrest (Fig. 2). The increase in cell diameter as measured by the Scepter 2.0 cell counter was closely correlated with the increase in the amount of tetraploid DNA as measured by the IN Cell Analyzer 1000 (Fig. 2E, $r^2 = 0.9143$, $P = 0.0028$).

To examine the correlation between cell diameter and the frequency of apoptosis, we analyzed the cell surface expression of phosphatidylserine residues using annexin V and fluorescence microscopy. Annexin V has high affinity for the phosphatidylserine residues expressed on the surfaces of apoptotic cells [17]. Early

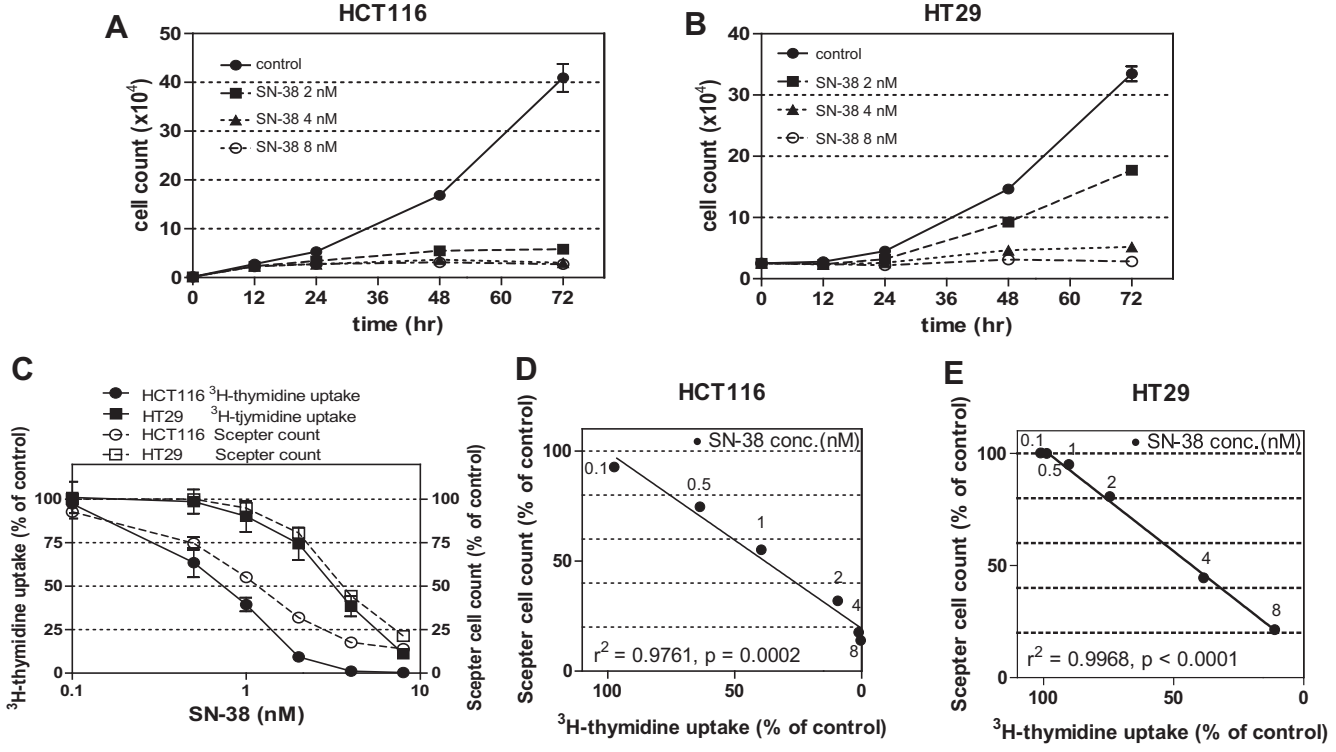

Fig. 1. Effects of SN-38 treatment on HCT116 and HT29 cells and comparison of cell proliferation between the Scepter 2.0 cell counter and ^{3}H-thymidine uptake assays. Cell proliferation assays of HCT116 (A) and HT29 (B) cells treated with SN-38 (0, 2, 4, or 8 nM) for the indicated time periods (0, 12, 24, 48,or 72 h). The cells were counted in triplicate using the Scepter 2.0 cell counter, and the resultant data are expressed as mean ± SD values. (C) Correlation between the results obtained with the Scepter 2.0 cell counter and those derived from ^{3}H-thymidine uptake in HCT116 and HT29 cells treated with SN-38 (0, 0.1, 0.5, 1, 2, 4, or 8 nM) for 48 h. ^{3}H-thymidine uptake and Scepter cell counts are expressed as percentages relative to the value for the control cells, which were not treated with SN-38. The SN-38 concentration is expressed on a $\log_{10}$ scale. Each data point represents the mean ± SD ($n = 6$ in the ^{3}H-thymidine uptake assay and $n = 3$ in the Scepter cell counting assay). (D, E) Correlation between the Scepter cell count (%) and ^{3}H-thymidine uptake (%) in the HCT116 (D) and HT29 (E) cells ($r^2 = 0.9761$, $P = 0.0002$ in HCT116 cells; $r^2 = 0.9968$, $P < 0.0001$ in HT29 cells).

Table 1
Mean diameter of (a) HCT116 cells and (b) HT29 cells.

	SN-38 exposure time									
	0 h		12 h		24 h		48 h		72 h	
SN-38 (nM)	Mean cell diameter (μm)	CV (%)	Mean cell diameter (μm)	CV (%)	Mean cell diameter (μm)	CV (%)	Mean cell diameter (μm)	CV (%)	Mean cell diameter (μm)	CV (%)
(A) Mean diameter of HCT116 cells										
0	18.4 ± 0.21*	1.12	17.9 ± 0.18*	1.01	17.7 ± 0.63*	3.55	16.6 ± 0.48**	2.90	14.5 ± 0.02**	0.11
2			18.1 ± 0.47*	2.56	19.9 ± 0.16*	0.80	21.2 ± 0.22*	1.04	22.3 ± 0.15*	0.66
4			18.2 ± 0.43*	2.35	20.7 ± 0.3*	1.45	22.5 ± 0.41*	1.81	24.1 ± 0.31*	1.27
8			18.2 ± 0.28*	1.54	20.9 ± 0.28*	1.35	22.8 ± 0.04*	0.17	23.8 ± 0.35*	1.47
(B) Mean diameter of HT29 cells										
0	16.6 ± 0.17*	0.99	17.4 ± 0.12*	0.71	17.6 ± 0.34*	1.91	15.9 ± 0.08**	0.48	15.1 ± 0.23**	1.52
2			17.7 ± 0.44*	2.46	19.5 ± 0.14*	0.70	17.8 ± 0.2*	1.13	17.5 ± 0.24*	1.35
4			18 ± 0.28*	1.58	20.7 ± 0.17*	0.83	21 ± 0.10*	0.50	20.8 ± 0.49***	2.35
8			17.8 ± 0.07*	0.41	21.6 ± 0.12*	0.53	23.3 ± 0.35*	1.52	23.4 ± 0.24***	1.02

Time course: $P < 0.0001$, drug concentration: $P < 0.0001$, interaction: $P < 0.0001$.
Diameter range (μm): *11.61–33, **6.82–33, ***13.03–33.

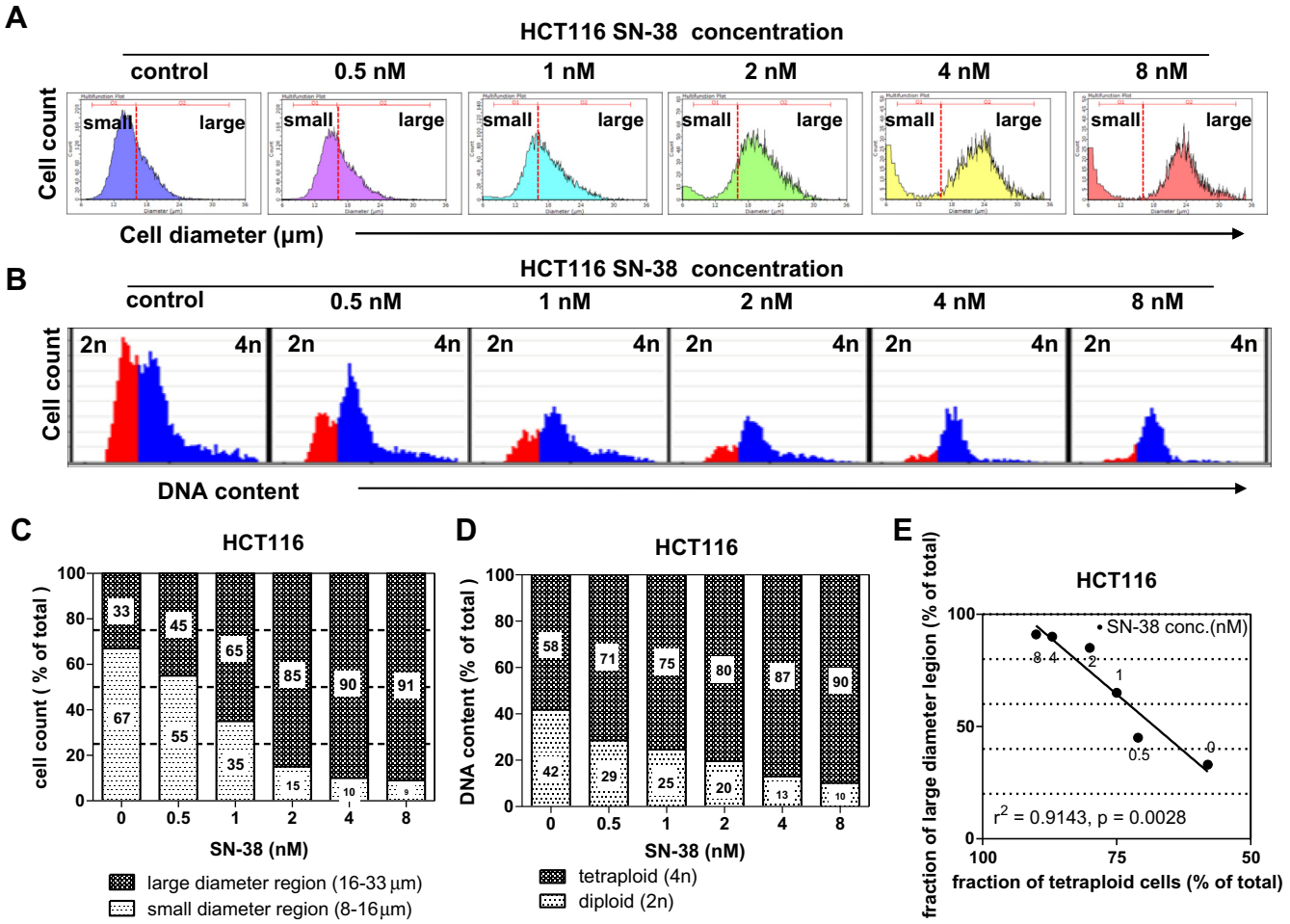

Fig. 2. Comparison of cell diameter and DNA content in HCT116 cells treated with SN-38. (A) Diameter distributions of HCT116 cells treated with SN-38 (0, 0.5, 1, 2, 4,or 8 nM) for 48 h according to the Scepter 2.0 cell counter. Cell size was classified as small (diameter: 8–16 μm) or large (diameter: 16–33 μm) depending on the cell gating range. (B) DNA content of HCT116 cells treated with SN-38 (0, 0.5, 1, 2, 4, or 8 nM), as measured using PI staining and the IN Cell Analyzer 1000. Each data point represents the total cell count for 6 wells in each column of a 96-well plate. The cell count distribution exhibited two peaks: the red peak represents diploid (2n) cells, and the blue peak represents tetraploid (4n) cells. (C) The proportions of cells in the small and large diameter fractions are shown in bar graphs in Fig. 3A. Data are expressed a mean values of triplicated experiments. (D) The proportions of diploid (2n) and tetraploid (4n) cells are shown as bar graphs in Fig. 3B. (E) Correlation between the proportion of large diameter HCT116 cells as measured by the Scepter 2.0 cell counter and that of tetraploid HCT116 cells as measured with the IN Cell Analyzer 1000 ($r^2 = 0.9143$, $P = 0.0028$).

apoptotic cells are stained with annexin V and calcein AM, while late apoptotic cells are stained with annexin V and PI. The exposure of HCT116 cells to a higher concentration of SN-38 resulted in an increased percentage of small particles compared with the control (Fig. 3A and B). Using fluorescence microscopy, we determined that the number of apoptotic cells increased among the HCT116 cells

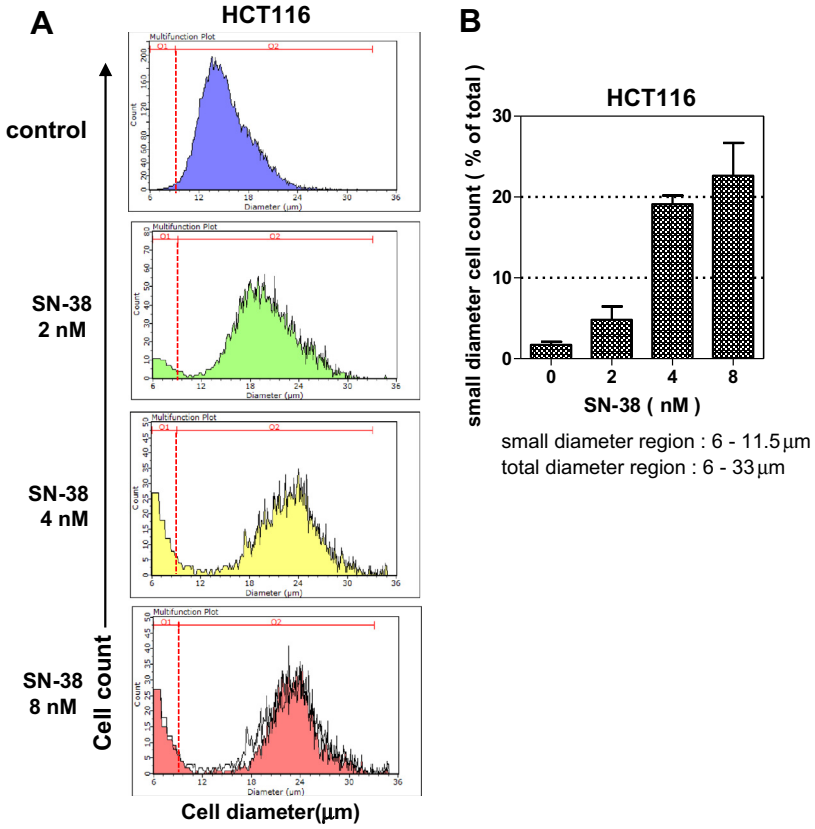

Fig. 3. Comparison of cell size distributions obtained with the Scepter cell counter and the results of annexin V-based apoptosis assay. (A) Diameter distributions of HCT116 cells treated with SN-38 (0, 2, 4,or 8 nM) for 48 h as measured by the Scepter 2.0 cell counter. The cells were classified into small (diameter: 6–11.5 μm) or large (diameter: 11.5–33 μm) fractions depending on the gating range. (B) The proportion of small diameter cells at different SN-38 concentrations is plotted on a bar graph in A. Data are expressed as the mean ± SD values of triplicated experiments. (C) Early and late apoptosis of HCT116 cells treated with or without SN-38 (8 nM) for 48 h as detected by multicolor fluorescence microscopy using annexin V, calcein AM, and PI staining. The white arrows indicate early apoptotic cells (positive staining for annexin V and calcein AM), and the yellow arrows indicate late apoptotic cells (positive staining for annexin V and PI).

exposed to 8 nM of SN-38. In this cell population, PI-stained cells were more abundant than calcein AM-stained cells (Fig. 3C).

4. Discussion

Direct cell counting using a microscope is laborious work and replaced with the simple alternatives such as the colony formation assay, the MTT assay, and the ^{3}H-thymidine incorporation assay. Our results indicate that cell counting using the Scepter 2.0 cell counter is a simple and accurate method of evaluating the *in vitro* chemosensitivity of cells to SN-38. In this method, increases in mean cell diameter are indicative of a higher frequency of G2/M cell cycle arrest (Fig. 2), and increases in the percentage of cells in the small particle fraction after treatment with relatively high concentrations of SN-38 are indicative of late cell apoptosis (Fig. 3). Thus, cell diameter analysis using the Scepter 2.0 cell counter is an informative modality for assessing the biological events elicited by SN-38 and might also be useful for assessing the effects of other chemotherapeutic agents and/or their effects on other cell lines.

The SN-38 IC_{50} values obtained by Scepter 2.0 cell counter were higher than those obtained by the ^{3}H-thymidine uptake assay in both cell lines (Fig. 1C). One possible explanation for this discrepancy is that cell cycle arrest induced by SN-38 would not result in ^{3}H-thymidine incorporation, while the cells were still countable by the Scepter 2.0 cell counter. Therefore, counting cells with the Scepter 2.0 cell counter represents different indices from DNA synthesis measured by ^{3}H-thymidine uptake assay.

Ongena et al. reported the increase of cell size when mitotic arrest was evoked in cells treated with colchicine, which is comparable with the nuclear tetraploidy state measured by flowcytometry (Application notes; URL: http://www.Millipore.com/techpublications/tech1/ps4323en00). In the present study, the effects of SN-38 on colorectal cancer cell lines were examined in more detail. Our data demonstrated that changes of cell diameter evoked by SN-38 were well compatible with cell cycle alteration and its availability as a surrogate marker for G2/M arrest.

The increase of cell size was shown by the appearance of small-sized fraction on the left of the *X*-axis of histograms, which was considered to contain the small non-viable cells. Reportedly, the percentage of small non-viable cells by the Scepter 2.0 cell counter on CHO (Chinese hamster ovary) cells treated with camptothecin were compatible with the appearance of annexin V positive cells determined by the flow cytometry (Application notes; URL: http://www.millipore.com/techpublications/tech1/an2634en00). Therefore, we compared the data of HCT116 cells exposed to SN-38 by the Scepter 2.0 cell counter with those obtained by the multicolor fluorescence microscopy using annexin V, calcein AM, and PI staining. It was likely that the increase of small particles after SN-38 treatment indicated the increase of late apoptotic cells.

In this study, we used the colorectal cancer cell lines HCT116 and HT29, which have different molecular backgrounds. HCT116 cells possess mutated *hMLH1* genes, which display microsatellite instability (MSI) [18]. It was reported that colorectal cancer cells showing MSI were hypersensitive to topoisomerase I inhibitors and HCT116 cells were more sensitive to irinotecan than HT29 cells [19,20]. In the present study, the results obtained with the

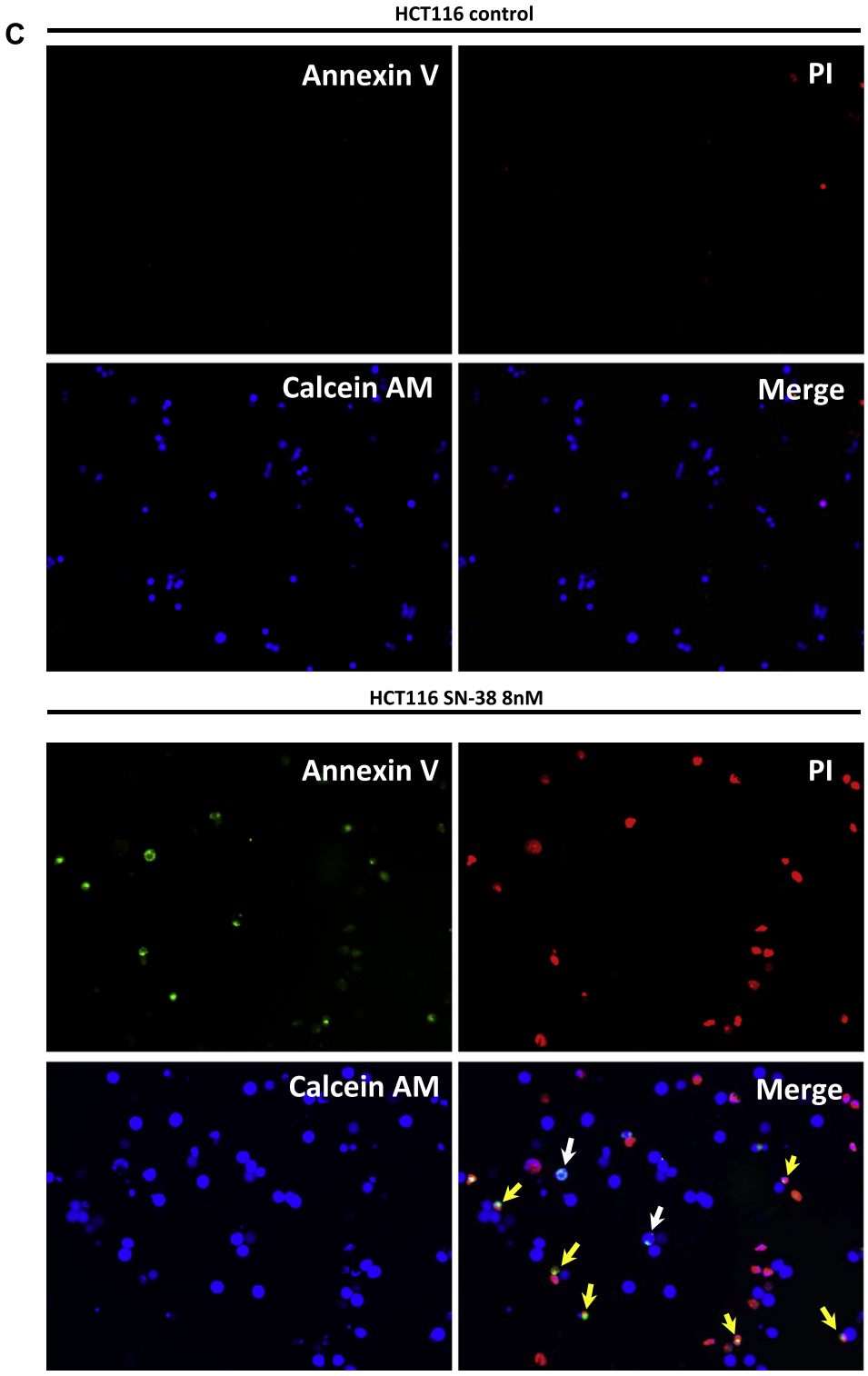

Fig. 3. (*continued*)

Scepter 2.0 cell counter and ^{3}H-thymidine incorporation assay were well correlated with each other (Fig. 1C and E). In conclusion, the results obtained with the Scepter 2.0 cell counter were accurate; reproducible; and above all, informative. This single assay can be used to obtain data about cell proliferation, cell cycle arrest, and apoptosis. This could be applicable to determine the effects of other chemotherapeutic agents on various cell lines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.128.

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