CELL MORPHOLOGY CHANGE BY THE ULTRAVIOLET RAY IRRADIATION

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The effect of low doses of ultraviolet (UV) irradiation on morphology changes of cell has been studied based on the observation of the cell length. It was shown that UV-irradiated cell has different behavior in comparison with non-irradiated cell. From the histogram of cell-length distribution, it was confirmed that cell cycle of non irradiated cell was 28 hours, and that cell cycle of irradiated cell with dose of 20 Jm⁻² was delayed (39 hours), while irradiated cell with 40 Jm⁻² and 60 Jm⁻² did not divide and kept growing continuously.

It was supposed that in case of 20 Jm^{-2} of irradiation dose, the cell cycle was delayed because the checkpoint worked in order to repair DNA damage induced by generation of pyrimidine dimer, reactive oxygen species and so on. It was also supposed that in case of 40 Jm^{-2} and 60 Jm^{-2} of irradiation dose, overgrowth was induced because the checkpoint was not worked well. The morphology of overgrown cell was similar to that of normally senescent cell. Therefore, it was considered that cell senescence was accelerated by UV irradiation with irradiation doses of 40 Jm^{-2} and 60 Jm^{-2} .

Keywords : Ultraviolet Irradiation, Living Cell, Cell Morphology, Cell Length, Cell Senescence

1. INTRODUCTION

Cell damage induced by UV irradiation is one of the factors that cause ageing and diseases [1]. Various researchers have been investigated the mechanism of cell damage, especially by UVA and UVB which reaches to the surface of the earth [2]. On the other hand, UVC with shorter wavelength than UVA and UVB is absorbed by upper atmosphere such as ozone layer, and do not reach to the surface of the earth. However, it is possible to examine the effect of UVC irradiation on cell in a short time because of its high energy. Therefore, UVC has been widely used as a source of radiation for acceleration experiment on cell damage [3,4].

It has been reported that DNA damage is one of the main factors of cell damage, in which cyclobutane pyrimidine dimers (CPD) and 6-4 photoproduct were generated TT array in DNA [5-7]. It was reported that reactive oxygen species (ROS) generated from protein and

lipid also damage DNA and lipid [8,9]. Effects of UVC irradiation on gene expression (p16, p21) and protein (cyclin-Cdk) in which controlled cell cycle have been studied based on the idea that cell cycle is affected by DNA damage [10,11]. It was confirmed that chromosome which was increased with procedure of cell cycle consists of DNA, and stagnation of cell cycle by UVC irradiation using the flow-cytometry in which DNA content can be detected by fluorescence intensity [12,13]. However, most of these studies are for cell damage with high irradiation dose in which survival rate of cell is low.

In contrast, there are few reports which investigate damage of the living cell with low irradiation doses. It is supposed that damage of living cell causes ageing or various diseases rather than cellular death. And, it is supposed that damaged living cell contributes to ageing based on the report that PDLs (population doubling levels) of human cell decreases with age [14,15]. On the other hand, there is a report that cancer cell which forms tumor has high proliferation capacity than normal cell because of shorter cell cycle [16].

On the basis of above-mentioned backgrounds, we studied the effects of UVC irradiation on living cell. When

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we estimate the effect of low irradiation dose of UVC on the living cell, there is a problem that it is difficult to find the significant effects on DNA expression relating to the cell cycle by DNA analysis. In order to detect the change in the cell cycle by UVC irradiation of low dose, we focused attention on the cell morphology change. Cell morphology changes with cell cycle progress; it starts from G1 phase until the cell attains enough size to start to grow, and the next state is extension from S to M phase, and then reaches cell division at the end of M phase [17]. Cell morphology change differs depending on the kind of the cell. By observation of morphology change corresponding to cell cycle, it is possible to estimate the response of living cell to UVC of low irradiation dose.

2. MATERIALS AND METHODS

2.1 Cell Sample

Adipose tissue derived stromal cells (ADSCs) from rabbit were used as experimental samples. These ADSCs were provided by Kaneda laboratory, Department of medicine and gene therapy science, Osaka University. ADSCs are easy to distinguish the states of formation of focal adhesion, cell extension and cell division. It is reasonable to investigate the change in cell cycle via growing process with ADSCs. ADSCs were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine with sodium pyruvate (Nakarai tesque Co.). The DMEM were prepared by mixing 500 ml of culture media and 50ml of antitoxin (10% FBS: Fetal bovine serum, Biowest) and 5 ml of antibody (1 % penicillin, Nacalai tesque Co.). The ADSCs were cultivated with these culture media in culture dishes (100 × 20 mm, BECTON DICKINSON) for 2 days (310 K, 5 % CO₂). After incubation, ADSCs adhered on the bottom of dishes were washed with phosphate-buffer saline (pH=7.4, Nakarai tesque Co.). The washed ADSCs were exfoliated from the bottom of dishes by DMEM (Nakarai tesque Co.). The resultant ADSCs were incubated for 5 min at 310 K and this subculture procedure was repeated four times.

2.2 Cell Cycle Synchronization

To examine the effect of UVC irradiation onto cell cycle, it is necessary to synchronize the cell cycle to G0/G1 phase before UVC irradiation. Serum-starved cultivation is generally used for synchronization of cell cycle [18,19]. There are also other methods such as using reagent [20] or temperature control [21,22]. These treatments shift the cell to dormant state called G0 phase. Among these methods, serum starvation is the method in which the cell can return to G1 phase, and can start DNA replication and cell division

again by addition of serum or growth factor [18,23]. Thus, we aligned the cell cycle by serum starvation, and then observed the influence on cell cycle by irradiation. There are various kinds of the mammal cell, and adequate serum concentration required to align the cell cycle varies by species. Before examining the effect of UVC, we cultivated ADSCs under various serum concentrations, and observed whether cell cycle of the ADSCs was aligned at G0/G1 phase. In the method of serum-starved cultivation, we added the serum and growth factor to mammal cell after the cultivation of serum starvation, and then evaluated whether the cell cycle is synchronized or not from cell density and temporal change in cell length.

2.2.1 Change in cell density of cultured ADSCs in various serum concentration.

The ADSCs after four-times of subculture were cultivated with medium containing 0.5, 2, 10 % FBS. The cell density was estimated using 0.5 % of trypan blue stain solution and cell-counter (hematocytometer) in order to confirm whether ADSCs is stopping division briefly. The ADSCs were exfoliated using 0.25 % of trypsin-1 mM EDTA solution after cultivation for 24 h, 48 h and 72 h, and then were suspended into 2 ml of phosphate-buffer saline (PBS). 20 µl of suspended ADSCs were diluted by 0.5 % of trypan blue (20 µl) /PBS solution (160 ml). Then, 200 µl of diluted solution were inserted to cell-counter (hematocytometer), and living cells were counted by using phase-contrast microscope (Nikon, 200 × objective). Because the counted number of cells are sufficiently large, we statistically estimated the standard deviation of number of cells as $\sigma = \sqrt{n}$, in which n represents the number of counted number of cells.

2.2.2 Temporal change in cell length at serum starvation condition.

The cell cycle of ADSCs used in this experiment is 28 h. To minimize the serum starvation period, ADSCs were cultivated in medium containing 0.5 % FBS for 28 h at 310 K, CO₂ 5 %. To confirm whether the cell cycle was synchronized keeping the proliferating ability, 28 h-starved cells were cultivated in medium containing 10 % FBS, and temporal change in cell length for 50 h cultivation was examined. In order to track the morphology change in the identical cell, cells were cultivated in Petri dish marked with grids (1.5 mm \times 1.5 mm, 36 grids). The cells were observed by using phase-contrast microscope with CCD camera (infinity1, Arugo, 200 × objective) during 50 hours of incubation time and the temporal change in cell morphology in the same certain grid was monitored. Change in length of the identical cell was observed every 3-5 hours of incubation time. Cell length was estimated by using

image analysis software (Infinity software-v4.0.2). Cell population density of serum starved ADSCs was 250 cells per 14 blocks. Cell length was defined as a distance along with long axis of a cell, since cell grows up in one direction. Distribution of cell length was represented as histogram as shown in Fig. 2. Since, in this histogram, we express the cell population of every 15 µm, the accuracy of horizontal axis also corresponds to about 15 µm. The median length of cells and its FWHM (full width at half maximum) were calculated from the histogram, and temporal change in cell length of serum-starved ADSCs was examined. From the result, we confirmed synchronization and proliferation ability of ADSCs after serum-starvation. The result of serum-starved ADSCs was compared with the temporal change in cell length of control ADSCs (Subculture of usually operation). Cell density of control ADSCs was 300 cells per 15 blocks.

2.2.2.1 Cell number change rate at serum starvation condition.

As described in 2.1 of experimental section, the number of living cell in serum-starved ADSCs $(1 \times 10^5 \text{ cellml}^{-1})$ were counted by using phase-contrast microscope with CCD camera (infinity1, Arugo, 200 × objective) during 50 hours of cultivation, and cell density rate was estimated from the number of cell which was counted every 3-5 hours of cultivation. Because the counted number of cells are sufficiently large, we statistically estimated the standard deviation of number of cells as $\sigma = \sqrt{n}$. Based on the results of temporal change in cell length and cell density, ADSCs cultured in medium containing 0.5 % FBS for 28 h were used as UVC irradiation sample.

2.3. Effect of UVC Irradiation on ADSCs

The UVC irradiation to ADSCs $(2.5 \times 10^5 \text{ cellml}^{-1} \text{ for} 3-4,5\text{-dimethyl-2-thiazoly-2,5-diphenyltetrazolium Bromide} (MTT) measurement, and <math>1 \times 10^5 \text{ cellml}^{-1}$ for cell number counting) was performed using the UV lamp of the ultraviolet rays irradiation device (TOSHIBA Co., Ltd) with the center wavelength of 254 nm. The irradiation dose was 1.0 Jm^{-2} s. Total irradiation dose for MTT measurement of irradiated cells were in the range of 20-160 Jm⁻², while those for observation of cell length and cell number were 20 Jm⁻², 40 Jm⁻² and 60 Jm⁻². After UVC irradiation, ADSCs were cultivated at 310 K, 5 % of CO₂. MTT measurement was performed after 5 hours of incubation time, and cell length and cell number were measured till to 50 hours of incubation time after irradiation.

2.3.1 MTT measurement (cell survival ratio) after UVC irradiation.

MTT method is one of the techniques to estimate the

survival rate of cells by detection of formazan dye formed by the reaction between mitochondrial dehydrogenase of living cell and MTT reagent. After UVC irradiation, 1ml of MTT solution was added to each ADSCs samples cultured for 5 h, and were placed in the incubator for 3h to induce color reaction. After these processes, DMEM was removed using aspirator, and 1ml of DMSO (kishida Co.,Ltd) was added to each sample in order to dissolve formazan dye. The optical density (OD) of each sample was measured by using a micro plate reader (IMMUNO-MINI, NJ-2300, Nalge Nunc International) at a wavelength of 570 nm which is maximum absorption wavelength of formazan. Each measurement was performed four times repeatedly in order to estimate the average and standard deviation of OD. The survival rate of irradiated cells was derived from normalized absorbance at 570 nm by that of non-irradiated sample.

2.3.2 Temporal change in cell length by UVirradiation

Cell morphology was observed by using phase-contrast microscope with CCD camera (infinity1, Arugo, $200 \times$ objective) during 50 hours of incubation time. In order to track the morphology change in the identical cell, the experiment was performed as described in experimental 2.2. Cell population densities of UVC irradiated samples were 250 cells per 16 blocks (20 Jm^{-2}), and 250 cells per 18 blocks (40 Jm^{-2} , 60 Jm^{-2}). The median length of the cells and its FWHM (full width at half maximum) were calculated from the histogram of the cell length (Fig. 2). Cell cycle of UVC irradiated ADSCs was estimated from temporal change in cell length.

2.3.3 Cell number change rate by UVC irradiation.

Change in number of survival cell of UVC irradiated ADSCs was estimated during 50 hours of incubation time as described in experimental 2.1. ADSCs were released using the 0.25 % trypsin-1 mM EDTA solution at the same elapsed time as cell length measurement, and then the cell density was counted using 0.5 %-trypan blue stain solution and cell-counter (hematocytometer). Because the counted number of cells are sufficiently large, we statistically estimated the standard deviation of number of cells as $\sigma = \sqrt{n}$.

2.3.4 The ratio of cell division, overgrowth and apotosis by UVC irradiation.

The ratio of cell division, overgrowth and apotosis were estimated using the same method as the measurement of the cell length. The ratios were estimated from the photomicrographs of cells inside the same grid which was observed at 5 and 50 hours of incubation time. Cell morphology after 5 h of incubation time was compared with morphology change of identical cell after 50 h, and those identical cells were discriminated as cell division, overgrowth and apotosis. Numbers of these cells were divided by number of the whole cells to calculate each ratio. Cells that became flat and enlarged over the length in normal cell division (110 μ m) were classified as overgrowth. Cells floating on the culture fluid as a result of losing the focal adhesion and with diameters around 20 μ m were classified as apotosis. Increase in cell number in the initial position was assumed as cell division. The ratio of cell division, overgrowth and apotosis against the total cell number in 5 h were calculated respectively.

3. RESULTS

3.1 Synchronization of Cell Cycle by Serum Starvation3.1.1 Change in number of cultivated ADSCs in various serum concentration

To investigate the effect of UVC irradiation, it is necessary to find a suitable serum concentration for synchronize the cell cycle of ADSCs to G0/G1 phase. ADSCs were cultivated in various serum concentration (10, 2, 0.5 % FBS). Change in cell population density in each serum concentration is shown in Fig. 1. Whether cell is stopping division briefly or not was checked by change in number of cells. From the result in Fig. 1, it was confirmed that ADSCs undergo division in the culture medium containing 10 % and 2 % FBS. Cell hardly undergo division in the culture medium containing 0.5 % FBS. It was confirmed that cell division of ADSCs was stopped briefly in culture medium containing 0.5 % FBS and the cell cycle stays at G0/G1 phase.

3.1.2 Temporal changes in cell length and cell density under serum starvation state.

To confirm whether ADSCs cultivated in serum starvation (0.5 % FBS) were synchronized and maintain their proliferating ability, temporal changes in cell length and cell density during additional cultivation were investigated. Cell cultivated in FBS starvation (0.5 % FBS) and control cell (continuously-cultivated) were cultivated further in 10 % FBS. The temporal change in cell length was estimated by morphology change in each identical cell. The temporal changes in cell length and cell number during further cultivation were shown in Fig. 3 (A, B). In this figure, closed squares and dotted lines indicate the median length and is FWHM derived from Fig. 2, respectively. As the cell cycle of ADSCs used in this experiment was 28 h, to apply a minimum serum starvation state, cell cultivated in 0.5 % FBS for 28 h were used as the sample for UVC irradiation. The temporal changes in the cell length and cell number of serum starved ADSCs were shown in Fig. 3 (A), and those of control ADSCs were shown in Fig. 3 (B).

Among the temporal change in cell length, the period in which median length increases corresponds to cell growth process, and the period in which median length decreases corresponds to cell division process. In spite of synchronizing the cell cycle, the cell growth rate is not uniform during cultivation because of individual difference of each cell. Thus, it can be considered that the broad distribution of the cell length corresponds to the cell extension process. On the basis of this hypothesis, median length and FWHM of ADSCs cultivated in starvation state (0.5 % FBS) followed by cultivation in usual condition (10 % FBS) was used as samples. The result indicates that median length increased for 18 hours, and then decreased for 11 hours. It was also found that FWHM increased by incubation time during initial 18 hours. From the median



Fig. 1. Time-dependent cultivation behavior of cell density with the concentration of FBS (\bullet : 10 %, \blacktriangle : 2 %, \bullet : 0.5 %).



Fig. 2. Cell length distribution after 5 h incubation of non-irradiated cell.

length and FWHM calculated from histogram, it was confirmed that ADSCs (0.5 % FBS) grows up for initial 18 hours, and then divides during subsequent 11 hours. Cell division was also confirmed from cell number change; the cell number started to increase from 18 hours, and became 1.65 times of the initial number after 29 hours.

Median length of control ADSCs increased until 18 hours, and then decreased for following 10 hours, but the change was more slowly than serum starved ADSCs. FWHM of control cells were larger than that of serum starved cells in the identical incubation time. The cell number increased 1.67 times after 28 hours, and we confirmed that increase in cell number of control cells is approximately equal to that of serum starved cells. However, decrease in median length of control cells was slower than that in serum starved ADSCs, because control cells were gradually divided in the long time. Slow increase in median length from 12 to 18 hours indicates that some of cells were divided after 12 hours of incubation time.

We confirmed that serum starved cells show more obvious change than control cells in cell extension and



Fig. 3. Median length and FWHM calculated from cell length distribution and cell density as functions of cultivation time. Closed square is median length and dotted line is FWHM. Open diamond is cell density. (A): synchronized culture in 10 % FBS after 28 h of FBS starvation (0.5 %FBS), (B) subcultured in 10 % FBS.

division. In addition, median length and FWHM of serum starved cells after initial 8 h corresponds to initial 5 h of control cells. It indicates that the cell cycle of serum starved ADSCs delayed about 3 hours in the initial phase than control ADSCs. It is considered that cell division in serum starved ADSCs requires longer time than in control ADSCs because most of them are in G0/G1 phase which is out of cell cycle before cultivation. It is supported by the report that the time to get into S phase from G0 phase is longer than that from G1 phase in cell cycle [24].

The medium length of serum starved ADSCs once increased, and started to decrease after 29 hours. It suggests that the cell cycle of serum starved ADSCs is 29h. From the change in cell length and cell number, we confirmed that serum starved ADSCs is more synchronized than control. It was also confirmed that serum starved ADSCs maintains the proliferating ability, because increase in the cell number is equal to control. In the following experiment, ADSCs cultured for 28 h (310 K, 5 % of CO₂) in nutrient medium containing 0.5 % FBS were used as irradiation sample.

3.2 The Effect of UVC Irradiation on ADSCs3.2.1 Change in cell survival ratio by UVC irradiation

Change in cell survival rate of ADSCs by UVC irradiation was shown in Fig.4. Cell survival rate decreased with increase in irradiation dose of UVC; survival rate at 20 Jm⁻² and 120 Jm⁻² of irradiation dose were 90 % and 25 %, respectively. In order to ensure enough statistical reliability in observing the effect to living cell under low irradiation dose, a lot of living cell is necessary corresponding to high survival rate. On the other hand, in case of near 100 % of survival rate under low irradiation doses, it was assumed that we cannot find any effects in the living cell. Therefore, in following experiment, we investigated into UVC



Fig. 4. Survival rate of synchronized cells as a function of ultraviolet irradiation dose.

irradiation effect of the living cell with irradiation doses of 20 Jm⁻², 40 Jm⁻² and 60 Jm⁻² in which cell survival ratio exceeds 50 %.

3.2.2 Change in cell morphology by UVC irradiation

In order to investigate the effect of UVC irradiation on the living cell with irradiation doses of 60 Jm⁻² in which cell survival ratio exceeds 50 %, morphology change of the identical cell was observed in certain period of incubation time. The photomicrographs of the identical cells (nonirradiated (A~C), 20 Jm⁻² (D~F), 40 Jm⁻² (G~I) and 60 Jm⁻² dose (J~L)) at each incubation time (A, D, G, J: 11 h, B, E, H, K: 33 h, C, F, I, L: 50 h) was shown in Fig.5. In case of non-irradiated ADSCs, almost all the cells undergo division within 33 hours, and next cell division occurs after 50 hours. Cells after irradiation of 20 Jm⁻² showed tendency of extension than division even after 33 hours. Cell extension was also observed after 50 hours. ADSCs at irradiation doses of 20 Jm⁻² were confirmed that cell division and proliferation rate more decrease than nonirradiated ADSCs.



Fig. 5. Photomicrographs of synchronized cells with and without ultraviolet irradiation.

A~C: non-irradiated, D~F: 20 Jm⁻², G~I: 40 Jm⁻², J~L: 60 Jm⁻² A, D, G, J: 11 hour of culture time

C, F, I, L: 50 hour

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In contrast, ADSCs with irradiation dose of 40 Jm⁻², 60 Jm⁻², almost all the cells did not undergo division even after 50 hours. In addition, cell morphology change into flat and enlarged shape was observed compared with non-irradiated and 20 Jm⁻² irradiated ADSCs. From theses results, it is supposed that there is a difference in extension and division progress between irradiated and non-irradiated living cells.

Cell growth and division are controlled by cell cycle [25]. It consists of four phases, namely, G1 (preliminary step toward mitotic division and nuclear DNA replication), S (nuclear DNA replication), G2 (RNA synthesis and protein synthesis) and M (mitotic phase). Cell grows up through G1, S and G2 phases, and then reaches cell cleavage in M phase [17,25]. As a result stated above, because of difference to extension and division progress of cell was shown, it is supposed that UVC-irradiation is exerted influence on the cell cycle of living cells.

Cell length was measured at regular time intervals, and then the median length of cells and its FWHM were calculated from the histogram of cell length distribution. Change in cell cycle by UVC irradiation on living ADSCs was investigated by temporal change in cell length. In addition, influence of UVC irradiation on cell proliferation rate was investigated by tracking change in cell number.

3.2.3 Temporal change in cell length and cell number changed by UVC irradiation

Figure 6 shows FWHM and the median length calculated from histogram of cell length distribution with UVC irradiation ((A): 20 Jm⁻², (B): 40 Jm⁻², (C): 60 Jm⁻²) and cell number change. From the result in 1.2, non-irradiated ADSCs have cell cycle of 29 hours, including the time required to shift from G0 to G1 phase. FWHM increased with increase in incubation time (~18 hours, median length was 110 µm) during the time period of cell extension. In addition, it was stated that cell number increase from 18 hours to 29 hours of cultivation time when cell indicates division tendency. In contrast, median length increased to 110 µm after 29 hours, and then decreased till 39 hours at 20 Jm⁻² of irradiation dose. From the result, we presumed that the cell cycle duration at 20 Jm⁻² of irradiation dose is approximately 39 hours. FWHM was narrow till 11 hours while obvious change was observed in median length, but became large from 11 and 29 hours while median length increased. Cell number started to increase from 18 hours, and continued gradual increase to 1.69 times until 39 hours. The increase of cell number was not observed after 39 hours while median length increased again. Also, increase of cell number was almost as same as that of non-irradiated cell after 29 hours of cell cycle (1.65 times). It was also confirmed that cell cycle of ADSCs irradiated with 20 Jm⁻² was 39 hours.

B, E, H, K: 33 hour

On the other hand, the median length of ADSCs irradiated with 40 Jm⁻² and 60 Jm⁻² kept increasing even after 50 hours. It indicates that most of cell irradiated with 40 Jm⁻² and 60 Jm⁻² is not divided, and extends till 50 hours even if the length of cell attains the length in which normal cell is divided. On the other hand, FWHM of ADSCs irradiated with 40 Jm⁻² increased until 50 hours, and the number of cells also increased to 1.22 times. This suggests that most of ADSCs irradiated over 40 Jm⁻² keeps growing continuously, whereas a portion of cell is divided. The



Fig. 6. Median length and FWHM calculated from cell length distribution and cell density as functions of cultivation time. Closed square is median length and dotted line is FWHM. Open diamond is cell density.

(A~C): 28 h of FBS starvation followed by ultraviolet irradiation in 10 % FBS. (A) $- 20 \text{ Jm}^2$, (B) $- 40 \text{ Jm}^2$, (C) $- 60 \text{ Jm}^2$.

FWHM of ADSCs irradiated with 60 Jm⁻² extended more remarkably compared with irradiated with 40 Jm⁻². The cell number increased 1.08 times even after 50 hours. It indicates that the cell can hardly increase. It is considered that almost all the cells kept growing continuously because both the FWHM and median length of ADSCs irradiated with 40 Jm⁻² and 60 Jm⁻² increased.

Consequently, the cell cycle of ADSCs irradiated with 20 Jm⁻² was 39 hours, which was longer than that of nonirradiated ADSCs. Almost all ADSCs irradiated with 40 Jm⁻² and 60 Jm⁻² were not divided over the length which nonirradiated cells divided, and kept growing continuously. In addition, increase in FWHM with increase in irradiation dose suggests large individual difference induced by irradiation.

3.2.4 The percentages of cell division, overgrowth and apotosis by UVC irradiation

To consider the ratio of division, overgrowth, and apotosis during cultivation, we tracked the grow process of each cell with photomicrographs after 5 and 50 hours. We discriminated division, overgrowth and apotosis by change in cell length.

As a result, 91 % of non-irradiated ADSCs divided. In case of irradiated doses of 20 Jm⁻², 71 % underwent cell division and 22 % underwent overgrowth. In case of irradiated doses of 40 Jm⁻², 20 % underwent cell division and 71 % underwent overgrowth. In case of irradiated doses of 60 Jm⁻² 9 % underwent cell division, 70 % underwent overgrowth, and 14 % underwent apoptosis. From these results, we confirmed that the ratio of cell division decreased but that of overgrowth increased with increase in irradiation dose. We also confirmed that the ratio of set. However, it was suggested that overgrowth is a main factor in cases of 40 Jm⁻² and 60 Jm⁻².



Fig. 7. The percentages of apotosis, over-growth, growth and division of synchronized cells after ultraviolet irradiation followed by 50 h cultivation.

4. DISCUSSION

As the reason that the cell cycle of ADSCs irradiated with 20 Jm⁻² was delayed, it was considered that the main cause is stagnation until 11 hours in the early period of growth. This is supported by the result that extension and division behavior after stagnation corresponds to that of non-irradiated ADSCs. In addition, median value and FWHM during the stagnation corresponds to result after 5 hours of incubation time of non-irradiated ADSCs (Fig. 3(A)). It indicates that the stagnation time in G1 phase is longer than that required to shift from G0 to G1 phase in the cell cycle. Mammalian cell shows a distinguishable specific morphology in each phase in cell cycle; formation of focal adhesion, cell extension, and cell cleavage [17]. Observation of cell morphology change in this study was started from the state of formation of focal adhesion after UVC irradiation. Because focal adhesion was formed in G1 phase, the stagnation period corresponds to G1 phase. In case that there is trouble or defect in cell, the cell cycle checkpoint works in order to maintain a normal activity. The complex composed of cyclin-dependent kinase (CDK) and cyclin plays a central role in the cell cycle checkpoint [26,27]. Some researchers reported that cyclin which plays important roles in each phase of cell cycle [28-30]. The shift from G1 to S phase is controlled by cyclin D1 [28]. Based on this, the stagnation of cell cycle of irradiated ADSCs under irradiation dose of 20 Jm⁻² was caused by the function of cyclin D1 to repair cell damage by UVC irradiation. On the other hand, the function to repair cell damage by UVC irradiation did not work in case of 40 Jm⁻² and 60 Jm⁻², because the stagnation was never observed. It was reported that cell damages by UVC irradiation are caused by DNA damage by formation of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproduct generation, or DNA and lipid damages by reactive oxygen generated through protein and lipid [5,9]. The above-mentioned cell damage is the research under high irradiation dose which causes cell death, and the research for low irradiation dose is hardly conducted. Whereas, in this study, it is considered that even low irradiation dose that cell death is hardly caused also induces the cell damage. Therefore, we presumed that even under irradiation dose of 60 Jm⁻² cause DNA damage. It is supposed that ADSCs irradiated with 20 Jm⁻² were suspended in G1 phase of cell cycle by the checkpoint to repair DNA damage, and the cell cycle was delayed. On the other hand, it was also supposed that irradiation of 40 Jm⁻² and 60 Jm⁻² caused overgrowth because the cell cycle checkpoint did not work well.

Overgrowth phenomenon was also observed in photomicrographs in Figure 5, showing flat and enlarged cell shape. Similar tendency was reported in the research for PDLs (population doubling levels) of various kinds of cell.

According to some researches, cells have their specific PDLs (population doubling levels), and cell cycle gradually becomes long with approaching to PDLs, and it results in division arrest [31-33]. It was also reported that cell becomes flat-enlarged shape when proliferation stops, and the phenomenon is one of the characteristics of senescence cell [31,32,34,35]. It was also reported that senescence cell was accelerated by external stimuli, and senescence cells in this method also show flat and enlarged shape [31,36,37]. According to the reports, mRNA in senescence cell was increased, whereas protein was not increased. This fact shows that the cell became impossible to synthesize protein in G2 phase when RNA and protein are synthesized, and not able to shift to M phase when is mitotic phase. There are few previous reports investigating the effects of UV-irradiation on senescence cell, the morphology of irradiated ADSCs under 40 Jm⁻² and 60 Jm⁻² in this study was similar to that of aged cell stated above. Thus it is supposed that UVC irradiation accelerates ageing.

5. CONCLUSION

In order to investigate the effects of low dose UV irradiation on living cell, we focused attention on the change in cell morphology and cell number. It was shown that ADSCs irradiated with dose of 20 Jm⁻² were suspended in G1 phase of the cell cycle, and accordingly extension and division of cells were delayed. By observation of cell extension process and proliferation, it was confirmed that the cell cycle of irradiated ADSCs was 11 hours longer than that of non-irradiated ADSCs, and the delay corresponds to the time required to repair damages in checkpoint (G1 phase). On the other hand, ADSCs irradiated with dose of 40 Jm⁻² and 60 Jm⁻² did not divide and kept growing continuously.

It was supposed that in case of 20 Jm⁻² of irradiation dose, the cell cycle was delayed because the checkpoint worked in order to repair DNA damage induced by generation of pyrimidine dimer and reactive oxygen species. It was also supposed that in case of 40 Jm⁻² and 60 Jm⁻² of irradiation dose, overgrowth was induced because the checkpoint was not worked well. The morphology of overgrown cell was similar to that of normally senescence cell. Therefore, it was considered that senescence cell was accelerated by UV irradiation under 40 Jm⁻² and 60 Jm⁻² of irradiation dose. From the ratio of division, overgrowth and apotosis, it was shown that irradiation of low dose (20 Jm⁻²) caused suspension and delay of the cell cycle, and probability apotosis became higher with increase in irradiation dose. These results indicates that it is possible that degree of cell damage by irradiation under low dose which is hard to analyze by other methods by observation of change in cell

morphology and cell number, from a viewpoint of the change in the cell cycle.

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