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RESEARCH ARTICLE

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Effects of malondialdehyde on growth and proliferation of human bone marrow mesenchymal stem cells *in vitro*

Abstract Malondialdehyde (MDA) is a well known inducer of carbonyl stress in a variety of human cells, however, its effects on human bone marrow mesenchymal stem cells (hMSCs) have not been documented. In this study, the effects of MDA concentration on the growth rate and proliferation of hMSCs *in vitro* were assessed. Under high concentrations of MDA, the cell count was decreased and the population doubling time (PDT) was lengthened. Flow cytometry (FCM) demonstrated that MDA triggered cells to undergo apoptosis, in parallel with the findings in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay which showed that it can also impair cellular viability. Surprisingly, FCM also determined that the percentage of hMSCs in G_2/M- and S- phases also increased in a dose-dependent manner with respect to MDA concentration. These results strongly suggest that even though hMSCs were severely impaired by high concentrations of MDA, they were still able to send signals that resulted in accelerated cellular proliferation process. This study provided important insights on how carbonyl stress affects cell cycle and proliferation of hMSCs.

Keywords malondialdehyde, mesenchymal stem cells, carbonyl stress, replicative senescence

1 Introduction

Human bone marrow mesenchymal stem cells (hMSCs) are crucial in that they have the unique capacity to regenerate and to give rise to several specialized cell types. Numerous studies demonstrate that hMSCs might be involved in the process of self-renewal and endogenous-repair of damaged tissues inherent in some pathological and physiological conditions. However, the body's pool of hMSCs is limited and decreases with age.

Recently, studies have shown that a variety of age-related diseases, such as Alzheimer's disease, diabetes, and atherosclerosis occur due to the accumulation of crosslinks produced by reactive carbonyl species (Markesbery 1997; Slatter et al., 2000). Lipid peroxidation is known to produce reactive carbonyl intermediates such as malondialdehyde (MDA). MDA has two conjugated carbonyl (C=O) groups similar to those found in 4-hydroxynonenals, 3-deoxyglucosone, and glyoxal; all of which are known to be genotoxic and cytotoxic (Esterbauer et al., 1999). In this study, the effects of MDA on proliferation, survival rate, apoptosis, and senescence of hMSCs were investigated.

2 Materials and methods

2.1 Isolation, cell culture and characterization of hMSCs

Human bone marrow mesenchymal stem cells (hMSCs) were obtained from the human ribs during chest operation according to procedure of Wakitani(Wakitani et al., 1995). Briefly, hMSCs were collected by flushing out the bone marrow with Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, USA). Mononuclear cells were isolated by density gradient centrifugation, and cells were rinsed three times with PBS and seeded (5×10^6 cells/cm^2) in DMEM supplemented with 15% fetal calf serum (FCS). The hMSCs were cultured at 35℃ in 5%CO_2 , ambient oxygen levels and saturating humidity.

The culture medium was replaced every 3 days and non-adherent cells were discarded. When the culture flasks became nearly confluent (80%~90%) and the adherent cells (first-passage) were detached and collected with 2.5g/L trypsin in 1.0 mM sodium ethylenediaminetetraacetic acid (Na_2-EDTA) (Gibco Laboratories, Grand Island, NY). The

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hMSCs were rinsed twice and re-seeded at a density of 2 × 10^3cells/ cm^2 onto a fresh culture flask. The above mentioned manipulation was repeated twice up to the 3rd passage in order to obtain a high purity hMSCs cell line. To validate the identity of the cells, FCM flow cytometry was performed to detect the presence cell surface antigen markers that are specific for hMSCs. Mouse anti-human CD19, CD29, CD34, CD38, CD45, CD71, and CD90 antibodies were used to characterize the cells(Conget and Minguell 1999).

2.2 Preparation of MDA

A fresh MDA stock solution (50 mM) was prepared by hydrolyzing 1,1,3,3-tetramethoxypropane (TMP) (Fluka Chemie AG) was prepared with some modifications according to the method described by Kikugawa, et al. (Kikugawa et al., 1980). The concentration of 50 mM for the stock solution was decided to avoid possibility of MDA self-polymerization during preparation. Briefly, 0.423 mL (2.5 mmol) TMP was mixed with 2.0 mL 1.0 M HCl and shaken at 40°C to release MDA upon acidic hydrolysis. When the solution became homogeneous, which is indicative of successful TMP hydrolization, the pH of the solution was adjusted to 7.2 using 6.0 M NaOH, and the final volume was brought to 50 mL using PBS. The MDA concentration in the solution was confirmed by the taking spectrophotometric readings at 267 nm (ε =31 500).

2.3 Assay of the effect of MDA on cultured hMSCs

MDA was added to the culture medium to final a concentration of 10^{-3}, 10^{-4}, and 10^{-5} M; for the control, PBS was used instead of MDA. The culture medium was replaced with fresh medium every three days, and supplemented with the desired MDA concentration. All the cells used in these experiments were in their 3rd passage.

2.4 Measurement the growth curve of hMSCs

HMSCs (2×10^3 cells/cm^2) were plated and cultured in 24-well plate (500 μL for each well). Cell number was counted every 24 hours in three wells, and the mean values were used to draw growth curves. Population doubling time (PDT) was calculated on the seventh day from the start of the experiment.

2.5 Tests of the viability of cells by MTT

HMSCs (2 × 103 cells / cm^2) were plated and cultured in 24-well plate (500 μL for each well). After 7 days, 50 μL MTT solution (5.0g/L) was added to each well and incubated further for 4 hours. The supernatant was discarded and 375 μL dimethyl sulfoxide (DMSO) was added to dissolve any crystals that formed during the addition of MTT. The difference of absorption between 490 nm and 630 nm (as the reference wavelength) representing the viability of hMSCs were measured with Microplate Analyzer (Sun and Wang 2000) (Bio-Tek, USA).

2.6 Assessment of cell cycle by FCM

8.0 mL hMSCs were seeded (2×10^3 cells/cm^2) in a cell culture flask. After 7 days, the cells were harvested with 0.25% trypsin and rinsed twice. The cells were PI stained and the cell cycle was assessed by FCM(Gregory et al., 2005).

2.7 Estimation of Apoptosis of hMSCs

8.0 mL hMSCs were seeded (2×10^3 cells/cm^2) in cell culture flask. After 7 days, the cells were harvested with 0.25% trypsin and the concentration of cell suspension was adjusted to 1 × 10^6 cells / ml. The number of apoptotic cells were measured using FCM after labeling with Annexin V-FITC (Zheng et al., 2005).

2.8 Statistical analysis

Data were presented as mean ± standard deviation (s. d.). One-way analysis of variance (One-way ANOVA) was used to test the differences of multi-groups and the comparison of samples was performed using *t*-test.

3 Results

3.1 Morphological characteristics of hMSCs in culture

The mononuclear cells from bone marrow were plated in cell culture flask. After 3 days, the primary cells adhered to

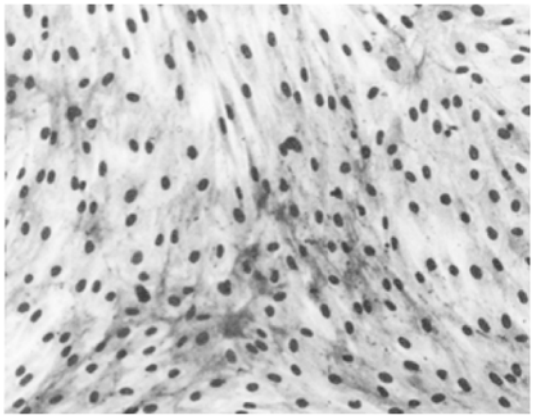


Fig. 1 Normal morphological characteristics of hMSCs (Swiss-Giemsa stain, 10 × 40)

the wall of the flask. Under the microscope, they mainly round and/or short-spindle in shape with a discernable nucleus at the center. After 10~15 days, cell clones increased in size and grew to reach confluence. These cells now became spindle and triangular in shape. After passage culture, hMSCs grew in a diffused manner instead of forming clones (Fig.1). Haematopoietic cells and other cells were eliminated in the course of the replacement of culture medium. Typical mesenchymal stem cells adhere to the wall of flask and reach full confluence within 6~12 days in culture.

3.2 Surface markers of hMSCs

FCM data confirmed that the cells obtained were hMSCs. Surface marker antigens that are specific for hMSCs like CD29, CD71, and CD90 reacted positively, while other markers such as CD19, CD34, CH38, and CD45 did not (Fig. 2). The results indicate that the cells obtained have the typical characteristics and cytochemical traits of hMSCs (Conget and Minguell 1999).

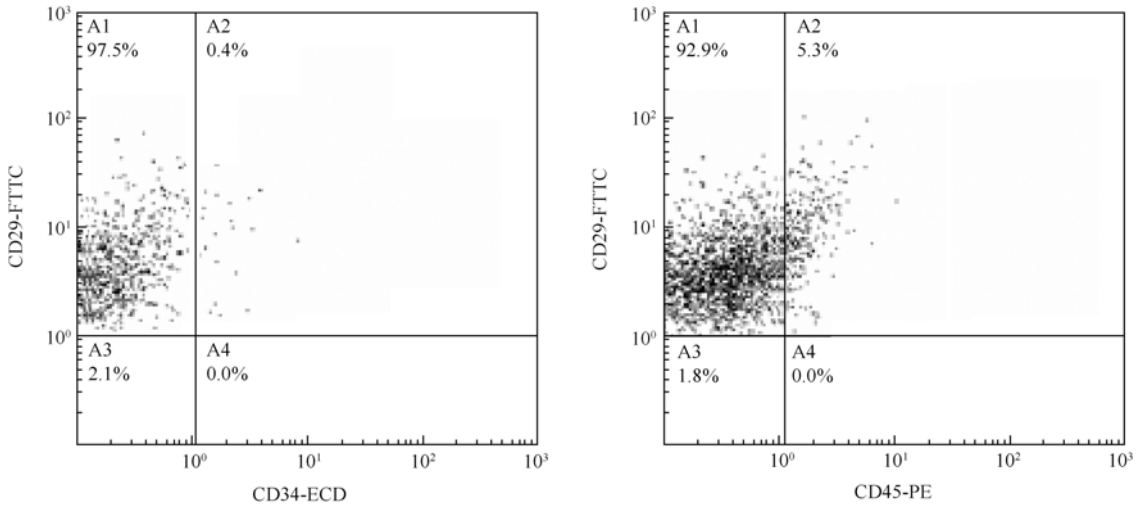


Fig. 2 Partial surface markers of hMSCs (by FCM)

3.3 Effect of MDA on growth of hMSCs

When the growth curve of the 4th passage cells was compared with that of the control, the cells treated with 10^{-5} M MDA grew normally, whereas those treated with higher concentrations of MDA were markedly suppressed (Fig. 3).

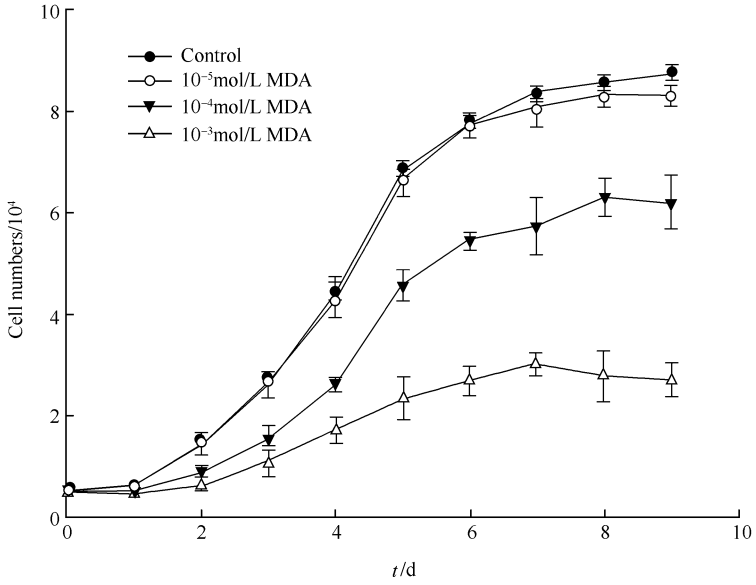


Fig. 3 Effect of MDA on growth of hMSCs

The data from all groups were gathered to calculate PDT. The PDTs of control, cells treated with 10^{-3}, 10^{-4}, and 10^{-5} M MDA are: (41.32 ± 0.19), (41.87 ± 0.73), (47.78 ± 1.84) and (64.58 ± 3.42) hours respectively. The PDTs of cells treated with 10^{-3} either 10^{-4} M MDA were significantly lower compared to that of the control. No significant statistical difference exists between the cells treated with 10^{-5} M MDA and the control.

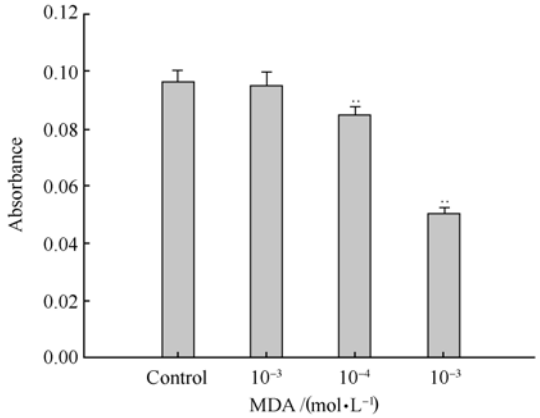


Fig. 4 Effect of MDA on cell viability of hMSCs by MTT

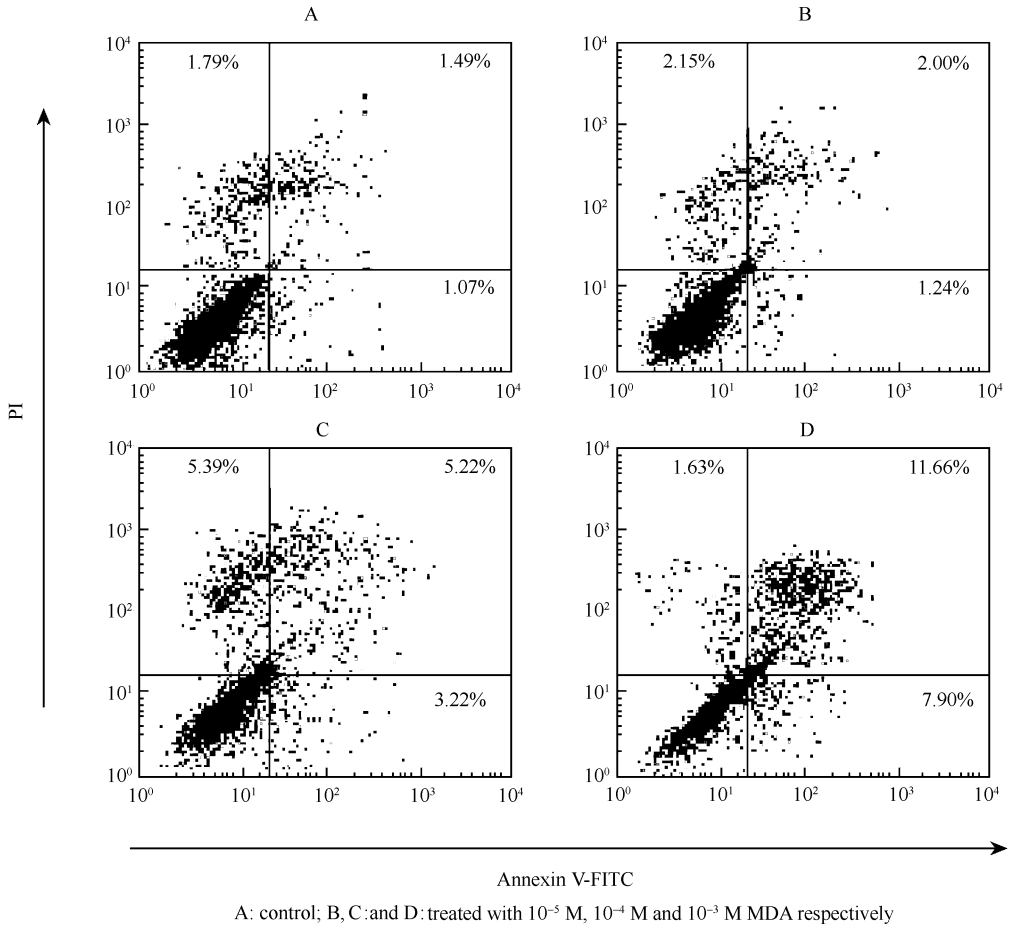
3.4 Effect of MDA on cell viability of hMSCs

The results of MTT assay showed that the growth of hMSCs treated with 10^{-3} M MDA was markely suppressed, and the suppression was dose-dependent (Fig. 4). This suggests that MDA could cause damage to mitochondria, and thus a consequent decrease in hMSC viability.

3.5 Effect of MDA on hMSCs apoptosis

Annexin V, a sensitive indicator of apoptosis. Can be used to detect changes in membrane phospholipid composition characteristic of the early-phase apoptotic cells (Zheng et al., 2005). Annexin V-FITC and PI (a nucleic acid dye) were utilized to distinguish between dead or the late-phase apoptotic (Annexin V^+/PI^+), early phase apoptotic (Annexin V^+/PI^-), normal (Annexin V^-/ PI^-), and mechanically damaged (Annexin V^+/PI^+) cells. FCM results demonstrated that MDA induced apoptosis of hMSC significantly in a dose-dependent manner (Fig. 5).

Fig. 5 Effect of MDA on hMSCs apoptosis



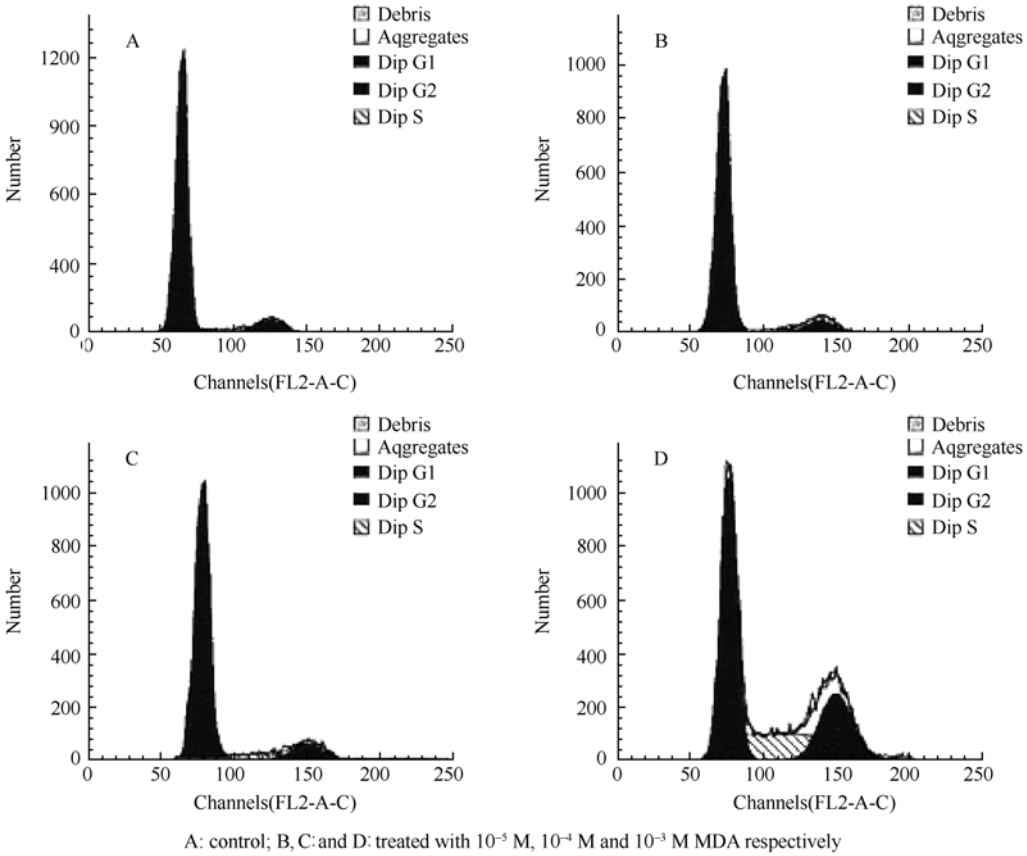
A: control; B, C and D: treated with 10^{-5} M, 10^{-4} M and 10^{-3} M MDA respectively

3.6 Effect of MDA on the cell cycle of hMSCs

The FCM results revealed that the percentage of hMSCs in G_2/M- and S- phase increased with respect to MDA concentration. (Table 1, Fig. 6). The results indicate that MDA boosted the rate of cell division.

Table 1 Effect of MDA on the cell cycle of hMSCs

Concentration of MDA	Phases of cell cycle		
	G_0/ G_1	G_2/M	S
control	88.11	7.26	4.63
10^{-5}	86.04	8.08	5.88
10^{-4}	83.91	8.96	7.14
10^{-3}	50.45	23.89	25.66



A: control; B, C: and D: treated with 10^{-5} M, 10^{-4} M and 10^{-3} M MDA respectively

Fig. 6 Effect of MDA on the cell cycle of hMSCs

4 Discussions

Reactive carbonyl species such as MDA, 4-hydroxynonenal, glyoxal, methylglyoxal, and 3-deoxyglucosones, contain highly unstable unsaturated carbonyl groups. These reactive compounds are generated from almost au major biological macromolecule during the various posttranslational side-reactions, glycation and oxidative stress (Yin 1996). Even in the presence of carbonyl dehydrogenases and other biomolecules, scavenge free radicals (e.g. glutathione), micromolar amounts of reactive carbonyl species still exist in healthy tissues (Yin 1996). Reactive carbonyl species react readily with almost all biomolecules under physiological pH and cause a variety of fluorescent and non-fluorescent cross-linkages that consequently result in their structural and functional alterations (Yin 1996). In 1995, Yin proposed a carbonyl toxification theory of aging (Yin 1995) and suggested that reactive carbonyl species seem to play an important role in senescence-related alterations in biological systems. Among reactive carbonyl species, MDA is mostly extensively studied. However, its effects on stem cells like hMSCs have not been examined. Unsaturated carbonyls are cytotoxic and genotoxic. Many of them have already been implicated in promoting cell senescence and even cell death.

In this study, we have shown that high concentrations of MDA prolonged the PDT of hMSCs *in vitro* and decreased their viability as shown by MTT assay. The effect of carbonyl stress inducing molecule, MDA, when given at high concentrations yielded surprising results. FCM data revealed that MDA can cause hMSCs to undergo apoptosis and at the same time stimulate cells to proliferate by increasing the ratio of cells in G2/M- and S-phases.

Hydrogen peroxide and O_2^-, like MDA, are known to

accelerate the proliferation of smooth muscle cells and fibroblasts(Burdon et al., 1995; Sauer et al., 2001), their mechanism of action is not fully understood. Our studies show ed that MDA might be one of the functional intermediates that causes cellular proliferation during oxidative stress. We propose that proliferation maybe due to the alterations of functional groups on the plasma membrane as reactions between MDA and amino groups are a spontaneous biochemical process.

The FCM data indicated that higher concentrations of MDA can induce apoptosis (Moneypenny and Gallagher 2005), and is dosage dependent. The higher concentration of MDA led to the accelerated cell proliferation, but apoptosis rate of hMSCs was greatly enhanced at the same time. Our growth curve assay was consistent with these cellular retardations. hMSCs may become senescent after long periods of *in vitro* culture and repetitive cell division (Stenderup et al.,2003). Our data demonstrated that MDA accelerated cell aging in accordance with Hayflick-limit-related replicative senescence.

The senescence of organisms is an accumulative manifestation of the degeneration of different tissues/cells and organs/systems of human body (Van Zant and Liang 2003). Aging is the result of the battle between damage and defense (Yin and Chen 2005). Since stem cells are the important resource of restoration and rejuvenation of organisms, the functional decline of the stem cells following carbonyl stress may be one of the reasons for organisms' senescence. On the other hand, the content of reactive carbonyls was found to increase with human age. Our data strongly suggested that high concentrations of MDA were toxic to mitochondria, leading to apoptosis of hMSCs. However, cell division of hMSCs was also accelerated under this condition. In conclusion, reactive carbonyl species played a critical role in growth, proliferation, and metabolism of stem cells.

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