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NOTES

High Inoculation Cell Density Could Accelerate the Differentiation of Human Bone Marrow Mesenchymal Stem Cells to Chondrocyte Cells

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Abstract

The effects of the density of human mesenchymal stem cells (MSCs) on their differentiation to chondrocytes in a differentiation medium supplemented with dexamethasone, TGF β 3, and IGF-1 were investigated for the regenerative therapy of cartilage. The increase in the initial density of MSCs from 0.05×10^4 to 0.9×10^4 cells/cm² accelerated the increase in the expression level of aggrecan mRNA during the differentiation culture for 7 d. The conditioned medium harvested at 7 d from the differentiation culture with an initial MSC density of 0.3×10^4 cells/cm² accelerated the initial increase in the expression level for 3 d in the differentiation culture with an initial MSC density of 0.3×10^4 cells/cm², whereas the conditioned medium harvested at 7 d in the differentiation culture with an initial MSC density of 0.05×10^4 cells/cm² did not. The differentiation culture after 14 d with an initial MSC concentration of 0.3×10^4 cells/cm² showed an expression level 1.7-fold that in the case of the culture with an initial MSC concentration of 0.05×10^4 cells/cm². Thus, a high MSC inoculum density might be appropriate for the rapid differentiation of MSCs to chondrocytes.

Combined addition of insulin-like growth factor (IGF)-1, transforming growth factor (TGF)- β 3 and dexamethasone was effective for the differentiation of mesenchymal stem cells (MSCs) in adult bone marrow to chondrocyte cells (1). In general, cytokines produced by cultivated cells other than externally added cytokines to the culture, might affect cell differentiation. Thus, cell density should have some affect on cell differentiation because cytokine production rate depends on cell density. For example, an increase in initial stromal cell concentration from 0.4×10^5 to 2.7×10^5 cells/ml increased progenitor concentration during a three-dimensional coculture of murine bone marrow hematopoietic cells with murine bone marrow stromal cells (2). Moreover, initial cell density might be one of the easily adjustable operational variables.

Besides generally added cytokines such as TGF- β 3 and IGF-1, cytokines secreted by MSCs have been considered to regulate the differentiation of MSCs to chondrocyte cells. However, it remains unclear how MSC density influences the differentiation of MSCs to chondrocytes. Consequently, the effects of MSC density on MSC differentiation were investigated in this study.

MSCs were isolated from bone marrow aspirate obtained by routine iliac crest aspiration from human donors (age: 65-73) as previously reported (1). The content of CD105⁺ CD45⁻ cells among the cells analyzed by flowcytometer was approximately 90% (data not shown) (3)

The growth medium used was DMEM-LG (Gibco, NY, USA) supplemented with 10% FCS (Gibco), 2500 U/l penicillin, and 2.5 mg/l streptomycin.

The differentiation medium was DMEM-HG (Gibco) supplemented with 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, 50 μ g/ml L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka), 100 μ g/ml sodium pyruvate (Wako), and 40 μ g/ml proline (Wako). Growth factors, namely, 10 ng/ml TGF- β 3 (Peprotech), 39 ng/ml

dexamethasone (ICN Biomedicals), and 100 ng/ml insulin-like growth factor- I (IGF- I ; Peptotech), were added.

The cells were cultured on a multiwell dish (9.6 cm²; Sumitomo Bakelite, Tokyo) at densities of 0.05×10^4 to 0.9×10^4 cells/cm² employing the growth medium and allowed to attach for 1 d at 37°C in 5% CO₂. Then, the medium was replaced with the differentiation medium and differentiation culture was started. Cell density was determined by trypan blue dye exclusion after trypsinization.

The mRNA expression ratio of aggrecan, which is a typical extra cellular matrix produced by chondrocytes, to actin was determined by previously reported method employing real-time RT-PCR (1) and employed as an index of differentiation of MSCs to chondrocytes.

Differentiation culture was performed for 7 days employing several inoculation cell densities (0.05 , 0.15 , 0.3 , 0.6 , 0.9×10^4 cells/cm²) and the expression level of aggrecan mRNA was determined (Fig. 1). The cells grew exponentially in all the cultures and there was almost no difference in specific cell growth rate between cultures that used different inoculum cell densities.

There was almost no expression of aggrecan mRNA at the initial time of differentiation culture (Fig. 1). Thereafter, the expression level increased monotonically until 168 h in all the cultures. At 72 h, the culture with the highest inoculation cell density showed the highest expression level. The aggrecan expression level in the culture with the lowest inoculum cell density was markedly lower than that of the other cultures at 168 h.

To confirm the presence of soluble factors produced by cells during the differentiation culture and the acceleration of the MSC differentiation, the conditioned media harvested on day 7 during the differentiation culture with the respective inoculum cell

densities of 0.05 and 0.3×10^4 cells/cm² were employed for other differentiation cultures with the inoculum cell density of 0.3×10^4 cells/cm² for 3 d.

The aggrecan expression level in the culture using the conditioned medium from the culture with the initial cell density of 0.3×10^4 cells/cm² was markedly higher than that in the culture using the conditioned medium from the culture with the initial cell density of 0.05×10^4 cells/cm² (Fig. 2). It was also higher than the aggrecan expression level in the culture using the fresh medium.

It was revealed in previous experiments that a higher inoculum cell density results in a faster increase in the expression level of aggrecan mRNA during short-term culture such as 7 d. To confirm whether a higher inoculum cell density leads to a higher expression level even in long-term culture, differentiation cultures with the inoculum cell densities of 0.05 and 0.3×10^4 cells/cm² were performed for 14 d, with weekly medium changes.

The cells continued to grow until the end of culture (336 h) in both cultures with the inoculum cell densities of 0.05 and 0.3×10^4 cells/cm² (Fig. 3); there was no marked difference in growth rate between the cultures.

The aggrecan expression level at 169 h in the culture with the inoculum cell density of 0.3×10^4 cells/cm² was 1.69-fold that in the culture with the inoculum cell density of 0.05×10^4 cells/cm². Aggrecan expression level also continued to increase up to 336 h in both cultures. The expression level at 336 h in the culture with the higher inoculum cell density was also 1.69-fold that in the culture with the lower inoculum cell density.

The addition of TGF- β 3 and IGF-1 initiated the differentiation of MSCs to chondrocytes, because almost no expression of aggrecan mRNA at 0 h of differentiation culture and a marked expression at 72 h were observed (Fig. 2). However, the expression level at 72 h differed between cultures with several inoculum cell densities in

spite of the same concentrations of TGF- β 3 and IGF-1 used. Thus, there might be factors other than TGF- β 3 and IGF-1 affecting the expression of aggrecan mRNA in the culture. The other factors may not contain direct contact between cells, because the cultures did not reach confluence even at 168 h (data not shown). Moreover, the possible soluble factors may not affect cell growth rate, because the cell density employed in this study did not affect specific cell growth rate (Fig. 1).

The expression level of aggrecan mRNA reached 0.045 at 72 h with the use of fresh differentiation medium (Fig. 2), which was apparently lower than the expression level (0.26) at 72 h in the culture with the inoculum cell density of 0.3×10^4 cells/cm² obtained in an earlier experiment whose results are shown in Fig. 1. The reason for this difference may contain the difference in the condition of employed cells, because the expression level (0.022) at 0 h in the earlier experiment (Fig. 1) was higher than the expression level (0.008) in the later experiment (Fig. 2). However, the expression level in the culture using the conditioned medium from the culture with an initial cell density of 0.3×10^4 cells/cm² was markedly higher than those in both cultures using fresh medium and the conditioned medium from the culture with the initial cell density of 0.05×10^4 cells/cm² (Fig. 2). Consequently, the conditioned medium with the initial cell density of 0.3×10^4 cells/cm² might contain some soluble factors accelerating the increase in the expression level of aggrecan mRNA and the concentration of the possible soluble factors might be higher in the culture with the higher inoculum cell density. Not only the secretion of the soluble factors by cells but also the decomposition and inactivation of added factors such as TGF- β 3 might occur during the culture. The later change may be dominant in the conditioned medium from the culture with the initial cell density of 0.05×10^4 cells/cm² and resulted in lower expression level compared with that with fresh medium. These may be the reason the expression level increased

faster in the culture with the higher inoculum cell density (Fig. 1).

The concentration of soluble factors rather than cell density at each time during culture may affect the expression as mentioned above. Initial cell density might directly affect the time course of the concentration of the soluble factors. This may be the reason why the initial cell density apparently affected the increasing rate of expression level.

The concentration of cytokines produced in the cell culture was normally less than 1 ng/ml (2, 4). Thus, the possible soluble factors may be factors other than TGF β 3 and IGF1, whose concentrations in the fresh medium were 10 and 100 ng/ml, respectively.

The expression levels at 7 d (169 h) in the cultures with inoculum cell densities of 0.05 and 0.3×10^4 cells/cm² (1.19 and 2.02 in Fig. 3) were not so different from those at 7 d (168 h) in Fig. 1 (1.41 and 2.36), respectively. The fold increases in expression level due to the increase in the inoculum cell densities from 0.05 to 0.3×10^4 cells/cm² were 1.67 in Fig. 1 and 1.69 in Fig. 3, respectively. Thus, the effect of initial cell density on the increase in the expression level of aggrecan mRNA was quantitatively reproducible.

The fold increase in the expression level of aggrecan mRNA due to the increase in inoculum cell density from 0.05 to 0.3×10^4 cells/cm² was 1.69 at both 7 d (168 h) and 14 d (336 h) in Fig. 3. This shows that the effect of a higher inoculum cell density on the increase in the expression level was maintained for a long term such as 14 d. Besides, high inoculum cell density could reach to the same expression level (2.02 in Fig. 3) at 7 d as that (2.07 in Fig. 3) at 14 d in the culture with lower inoculum cell density. So, high inoculum cell density could decrease the length of culture period to the half that of lower inoculum cell density.

In conclusion, a higher inoculum MSC density accelerated the increase in the

expression level of aggrecan mRNA in the differentiation medium supplemented with TGF β 3 and IGF-1. The fold increase caused by the higher inoculum cell density was quantitatively reproducible and maintained for at least 14 d. This effect may be due to some soluble factors produced by MSCs. Consequently, a higher inoculum MSC density might be appropriate for the differentiation of MSCs to chondrocyts for the regenerative therapy of cartilage.

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FIG. 1. Effect of initial cell density on expression level of aggrecan mRNA.

Differentiation culture was performed for 7 d employing several inoculation cell densities of 0.05 (open circles), 0.15 (squares), 0.3 (triangles), 0.6 (inverted triangles), and 0.9 (closed circles) $\times 10^4$ cells/cm². The average of triplicate determinations is shown.

FIG. 2. Effect of conditioned medium on differentiation. Conditioned media were harvested at 7 d during the differentiation culture with inoculum cell densities of 0.05 and 0.3×10^4 cells/cm², respectively. Then, other differentiation cultures with an inoculum cell density of 0.3×10^4 cells/cm² for 3 d were performed employing fresh medium, and conditioned media from the cultures with inoculum cell densities of 0.05 [CM (0.05)] and 0.3×10^4 cells/cm² [CM (0.3)], respectively. Each bar indicates the standard deviation of triplicate culture. $P < 0.05$.

FIG. 3. Effect of inoculum cell density during long-term culture. The differentiation cultures with inoculum cell densities of 0.05 (squares) and 0.3×10^4 cells/cm² (circles) were performed for 14 d. Medium was changed weekly. The average of triplicate determinations is shown.





