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Effects of Ascorbic Acid on the Proliferation of Subcultured Canine Chondocytes in Monolayer and Alginate Beads Culture

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Abstract : Ascorbic acid has been used widely as a medium supplement to stimulate cell proliferation, but its effects on cell proliferation have not yet been elucidated, and no reports have analyzed effects on subcultured chondrocytes. Subcultured canine chondrocytes of passage one, two and four were cultured in monolayer and alginate beads with and without ascorbic acid. Cell proliferation was examined by 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) (XTT) colormetric assay. Ascorbic acid stimulated cell proliferation significantly in both culture methods ($p<0.05$). The increased cell numbers by stimulation with ascorbic acid were significantly high in passage one cells compared to that of other passages. Differences in cell proliferative capacity by subculturing were not determined. These results suggest that ascorbic acid stimulated the proliferation of subcultured canine chondrocytes and enhanced it more in low-passage cells than in the other cells tested.

***Key words* :** Ascorbic acid, Cell proliferation, Chondrocytes, Dog.

Introduction

Over the last decade, autologous chondrocyte transplantation (ACT) has been reported to be an ideal method of accelerating the regeneration of damaged cartilage (5,11). Many studies on cell transplantation were described in animals on research basis. Chondrocytes were isolated from biopsy of cartilage and cultured for proliferation. Proliferated cells were implanted by the injection of free or encapsulated cells into the defect covered with a periosteal graft. A lot of cells that maintain properties of chondrocytes is required for ACT to repair damaged cartilage (7).

Ascorbic acid (Asc), an antioxidant nutrient, was reported to inhibit the progression of osteoarthritis in guinea pigs and human (19,21,24). The growth of chondrocytes and the production of extracellular matrix (ECM) were influenced by Asc under different culture conditions (1,4,6,8,16,20,23). Ascorbic acid was reported to stimulate the growth of chondrocytes in many studies, although inhibition was also reported (7). The effects of Asc on cell proliferation and ECM production have not yet been elucidated, and no reports have analyzed Asc's effects on subcultured chondrocytes.

The proliferation and properties of chondrocytes have been investigated in a variety of culture systems. Alginate has been applied as a cell scaffold for the transplantation of cells in plastic and reconstructive surgery (9,22).

The purpose of this study is to evaluate Asc's effects on subcultured canine chondrocytes in monolayer and alginate gel cultures.

Materials and Methods

Chondrocyte isolation

Articular cartilage was harvested from clinically healthy beagles. The cartilage was washed with sterile physiological saline containing 50 ug/ml gentamicine and was then cut into small pieces. They were digested with 1 mg/ml collagenase (Sigma Chemical Co., USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene, Korea) containing 100 units/ml penicillin and 100 ug/ml streptomycin for 18 hours at 37°C with gentle stirring. Isolated chondrocytes were filtered through sterilized surgical gauze and centrifuged at 200 g for five minutes. The pellet were resuspended and cultured at a density of 1×10^6 cells/75 cm^2 plate with DMEM containing 10% fetal bovine serum (FBS).

Monolayer culture

Cultures of chondrocytes were performed at 37°C in a 95% air and 5% CO^2 humidified atmosphere. The culture medium was changed twice each week. At confluence, chondrocytes were harvested after 0.25% trypsin treatment, and cells were subcultured to passage 4. Every passage's cells were suspended in DMEM containing 10% FBS and 10% dimethyl sulfoxide and were cryopreserved in liquid nitrogen for cell proliferation examinations. Cryopreserved cells were resuspended in DMEM

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containing 10% FBS and were cultured with and without 50 ug/ml of Asc (L(+)-Ascorbic Acid, Junsei, Japan) in a 75 cm^2 plate. Trypsinized cells were cultured at a density of 2×10^4 cells/well on a 96-well culture plate for five days. The culture medium was changed every two days. Cell proliferation assay was performed at intervals of 1, 3 and 5 days. Cell viability was estimated by a trypan blue test.

Alginate beads culture

A solution of low-viscosity sodium alginate (2.4%, Sigma Chemical Co., USA) in 0.15 M sodium chloride was sterilized by filtering through a 0.45-um pore size filter. Cells were resuspended in DMEM at a density of 1×10^6 cells/ml and mixed (1:1) with a 2.4% solution of sodium alginate. The cell suspension in alginate solution (1.2%) at a density of 5×10^5 cells/ml was dropped slowly using a yellow pipette (1-200ul) into a 102 mM calcium chloride solution agitated gently.

After instantaneous gelation, beads were washed three times with 0.15 M sodium chloride and then cultured in a 6-well culture plate in DMEM containing 10% FBS with and without 50 ug/ml of Asc. The culture medium was changed every two days for 15 days. Every five days, beads were resolved in a 1.5 ml centrifuge tube with 1 ml of a solution containing 50 mM ethylenediamine tetraacetic acid and 10 mM 2[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, and were used for the cell proliferation assay.

Cell proliferation assay

The proliferation of cells was assayed by 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt)(XTT) (Welgene, Korea). Forty ul of XTT and phenazine methosulfate mixture reagent were added to each well of 96-well culture plate. The cells and the mixture reagent were incubated four hours, and the sample's absorbance was measured with a spectrophotometer (Emax, Molecular Deivce, USA) at a wavelength of 450 nm.

Statistical analysis

Data of cell proliferation were means ± standard deviation. Statistical analysis of comparison between cells cultured with and without ascorbic acid by using unpaired t-test. Comparisons of the proliferation between three passages were determined by one-way ANOVA with Scheffe's test.

Results

The viability of isolated chondrocytes and cryopreserved cells was always more than 90%. The cell showed fibroblast-like cells in monolayer culture. Differences in cell shape were not noted between cells cultured with and without Asc. Ascorbic acid stimulated cell proliferation significantly in monolayer culture ($p<0.05$). Cell were cultured in 96-well plate at an initial density of 2×10^4 cells/well, and cell numbers were determined by the standard curve with an XTT colormetric assay (Table 1). At passage 1, the number of cells cultured with Asc had increased approximately two fold compared to that without Asc, and this increase was significantly different from that of other passages at three days ($p<0.05$). At this time, cells were almost confluent.

The number of chondrocytes cultured without Asc increased about 3.8-fold over those on day 0 in all passages. Cell proliferation in the absent of Asc was not influenced by subculturing.

Cells were cultured in alginate beads at an initial density of 1.25×10^4 cells/gel. Cells observed were often solitary cells with round to oval shapes and formed small clusters after five days (Fig 1). Ascorbic acid stimulated the cell proliferation significantly in alginate beads culture at passage one ($p<0.05$), and increasing rate was high in low passage cells (Table 2), although cell number had decreased in all passages on day five.

Discussion

Ascorbic acid has the ability to enhance cell proliferation, but also to alter cells' phenotype (10,14). However, the mechanisms have not been clearly explained. This study analyzed Asc's effects on the proliferation of subcultured canine chondrocytes in monolayer and alginate beads cultures. Ascorbic acid stimulated the proliferation of subcultured chondrocytes in both culture methods. This result was the same as the reports that Asc stimulates the growth of chondrocytes cultured in monolayer and collagen cultures. However, the increasing rate of cell proliferation in culture with Asc differed by subculturing. Ascorbic acid stimulated more cell proliferation in low-passages cells than it did in other cells. Differences in cell pro-

Table 1. The effect of ascorbic acid on the proliferation of canine subcultured chondrocytes in a monolayer culture

		Day 0	Day 1	Day 3	Day 5
Passage 1	Asc+	20000	25030±6400	84550±8182^{Aa}	117923±11787^{Bd}
	Asc-	20000	23888±6439	43150±5089^{a}	75922±14614^{d}
Passage 2	Asc+	20000	25569±4820	67763±6741^{Ab}	100934±11760^{Be}
	Asc-	20000	23570±3581	43681±3680^{b}	76525±15817^{e}
Passage 4	Asc+	20000	26453±5736	65718±8166^{Ac}	98055±13118^{B}
	Asc-	20000	24428±3973	46678±8042^{c}	77942±10710

Data of cell proliferation are means±standard deviation (n=5). Asc+: with ascorbic acid; Asc-: without ascorbic acid.
A, B Cell number of passage one is significantly different from passages two and three ($p<0.05$).
a, b, c, d, e Significantly different when compared to the number of cells cultured without Asc on the same day ($p<0.05$).

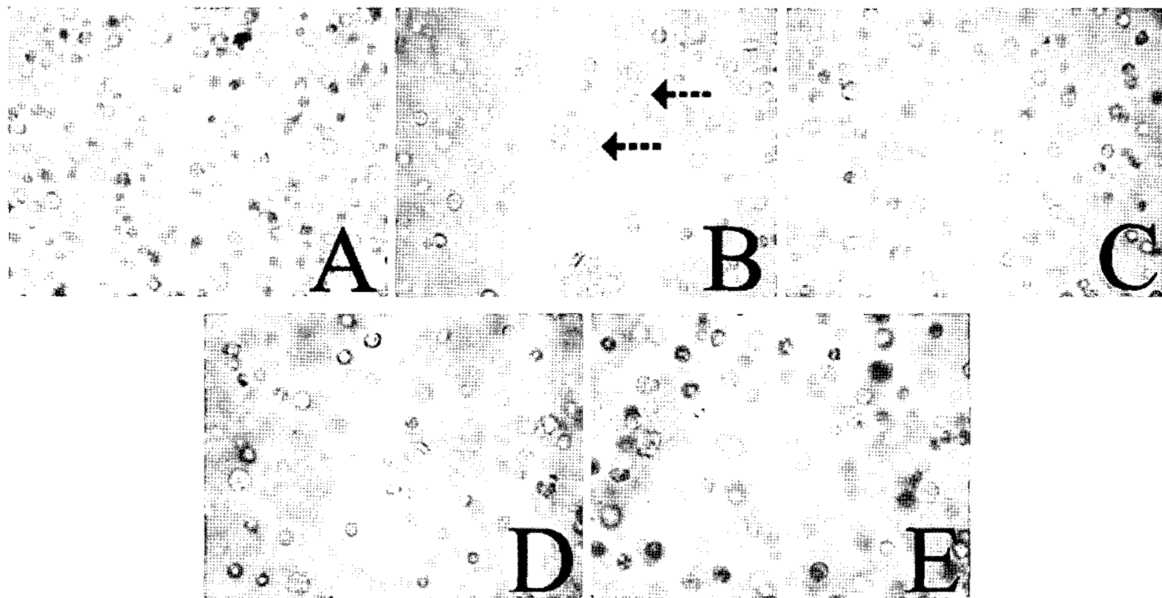

Fig 1. Canine chondrocytes were round and often exhibited intensive proliferation cells (arrows) in alginate beads. Ascorbic acid stimulates cell proliferation in all passages (B, D). A: Cells in alginate beads on day 0. B, C: Passage one cells on day 15. D, E: Passage four cells on day 15.

Table 2. The effect of ascorbic acid on the proliferation of canine subcultured chondrocytes in an alginate beads culture

		Day 0	Day 5	Day 10	Day 15
Passage one	Asc+	12500	9652±2382	15866±1176	21429±2005[Aa]
	Asc-	12500	10043±1394	14421±2497	17292±2337[a]
Passage two	Asc+	12500	9141±1342	12570±1999	16368±2007[A]
	Asc-	12500	9327±1035	12457±2169	16163±2336
Passage four	Asc+	12500	9336±2253	15777±2014	17214±1436[A]
	Asc-	12500	9705±2024	13779±3758	16883±4221

Data of cell proliferation are means±standard deviation (n=4). Asc+: with ascorbic acid; Asc-: without ascorbic acid.
A: Cell number of passage one is significantly different from passage two and three ($p<0.05$).
a: Significantly different when compared to the number of cells cultured without Asc on the same day ($p<0.05$).

liferative capacity by subculturing were not determined in this study. The increasing rate of passage one became lower on day five, because cells in the presence of Asc were almost confluent by day three.

The effects of Asc on cell proliferation and ECM production have not yet been elucidated. It is important to note differences between Asc's direct and matrix-related effects on cellular proliferation (15). Various cells whose proliferation was stimulated by Asc synthesize mainly collagen type I, except fresh chondrocytes. The synthesis of collagen type II in chondrocytes was shifted to that of collagen type I by an Asc supplement (6,16). Subculture of chondrocytes is also one of factors that shifts the synthesis of ECM (1,18). These studies raise the possibility of Asc's direct effects on cellular proliferation, because chondrocytes in this study had ECM of collagen type I.

A concentration of 50 ug/ml of Asc was applied to stimulate cell proliferation in this study. From other studies of ascorbic acid, this concentration appeared to be ideal to stimulate cell proliferation with minimal negative effects. Monolayer-cultured chondrocytes attach to the culture plate and dedifferentiate rapidly into spindle-shaped cells. A three-dimensional culture of chondrocytes in agarose or alginate has become popular for the maintenance of chondrogenic properties (2,3,12,13) and the redifferentiation of dedifferentiated chondrocytes (17,18). Chondrocytes can be cultured in ionically gelled alginate beads with calcium ions or other multivalent ions, and alginate beads can be rapidly solubilized by the addition of a calcium chelator. However, the proliferation of cells in alginate beads was slow, and thus prolonged evaluation of the cell proliferation in alginate beads is required to elucidate clearly the Asc's effect. Cell number was reduced on day five in this study. It is reported that stimulation of cell proliferation by Asc is associated with increased chondrocyte apoptosis, and some cells were affected during gelation of the alginate solution.

We concluded that the proliferation of subcultured chondro-

cytes was stimulated in monolayer and alginate beads culture by Asc. The increasing rate of cell proliferation in culture with Asc differed by subculturing, and Asc stimulated more cell proliferation in low-passages cells than in other cells. Differences in cell proliferative capacity by subculturing were not determined in culture without Asc. Further studies are needed to elucidate the direct effect of Asc on cellular proliferation.

Acknowledgments

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단순배양과 알긴산배양에서 개 연골세포의 증식에 있어 계대배양에 따른 아스코빅산의 영향

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요 약 : 아스코빅산은 세포 증식촉진을 위해 배지에 첨가하여 사용되나, 증식 촉진 작용기전에 대해서는 구체적으로 밝혀져 있지 않으며, 연골세포의 계대배양에 따른 효과에 대해서는 보고된바 없다. 제 1, 2 그리고 4계대배양한 개 연골세포의 단순배양과 알긴산 배양을 아스코빅산 첨가배지와 무첨가 배지에서 실시하였다. 세포증식은 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) (XTT)검사법을 이용하였다. 아스코빅산 첨가에 의해 세포증식 촉진효과가 모든 계대배양 세포에서 관찰되었으나, 제 1계대배양 연골세포에서 아스코빅산의 세포증식 촉진 효과가 제 2, 4계대배양 연골세포 보다 유의차 있게 높게 나타났다 ($p<0.05$). 아스코빅산 무첨가 배지에서 계대배양에 따른 세포 증식에 차이가 관찰되지 않았다. 따라서 아스코빅산은 낮은 계대배양단계의 연골세포에 있어 더욱 효과적인 세포증식 촉진효과가 있는것으로 판단된다.

주요어 : 아스코빅산, 세포증식, 연골세포, 개