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The Effect of Polysaccharide from *Angelica Gigas* Nakai on Controlling the Differentiation of Human Embryonic Stem Cells

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ABSTRACT : **It was found that the purified extract from *A. gigas* Nakai (polysaccharide, M.W., 25 kD) controled differentiating human ES cells. Its optimal supplementation concentration was decided as 0.8 (μg/ml) to efficiently control the differentiation. It also enhanced the cell growth, compared to the control. However, most widely used and commercially available differentiating agent, Leukemia Inhibitory Factor (LIF) negatively affected on the cell growth even though it controls the differentiation of ES cells, down to 40-50 % based on morphological observation and telomerase activity. It was presumed that the extract first affected on cell membrane and resulted in controlling signal system, then amplify gene expression of telomere, which enhanced the telomerase activity up to three times compared to the control. LIF only increased the enzyme activity up to two times. It was confirmed that the extract from *A. gigas* Nakai could be used for substituting currently used differentiation controlling agent, LIF from animal resources as a cheap plant resource and not affecting the cell growth. It can broaden the application of the plants not only to functional foods and their substitutes but also to fine chemicals and most cutting-edge biopharmaceutical medicine.**

Key words : *Angelica gigas* Nakai, differentiation, telomerase activity, human embryonic stem cellIntroduction

INTRODUCTION

There have been many reports on cultivating embryonic stem (ES) cells derived from many resources such as human, mouse and rabbit, etc. (Evans et al., 1981 ; Andrews 1981 ; Fong & Bongso 1999). Among them, the results about human ES cells have been greatly focused because they can be widely used for transplantation medicine, human developmental biology and drug discovery, etc even though the work on human ES cells has still faced on ethical conflict (Brower 200 ; Marshall 1998). However, the knowledge of human ES cells should be expanded in terms of founding fundamental mechanisms of *in vitro* and *in vivo* differentiating embryonic cells and contributing medicinal development as far as human ES can be used for clinical treatments. Human ES cells, so-called, pluripotent stem cells have the ability of proliferating on the feeder cells while remaining karyotypically and phenotypically stable. These cells can eventually *in vivo* and/or *in vitro* differentiate into a various types of cells on the feeder layers. Human ES cells could be used to identify polypeptide factors involved in differentiation and proliferation of committed embryonic progenitor cells. (Li et al., 1999 ; Thomson et al., 1998). These implications must require to maintain large quantities of undifferentiated ES cells *in vitro*. So far, ES cells can grow only on feeder layer cells, human primary fibroblast cells and need cytokines such as

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Leukemia Inhibitory Factor (LIF) in the cultures to maintain undifferentiately proliferation (Ling & Neben 1997 ; Thomson et al., 1995 ; Rose et al., 1994). But LIF is very expensive and may not be much effective on controlling differentiation of human ES cells in growing on human fibroblast cells (Kallos 1998). There has never been reported the attempt of using plant extracts for controlling the cell proliferation and differentiation of ES cells even though they are most inexpensive and safe resources for human clinical trials.

Angelica gigas Nakai is very well know medicinal plants in treating gynecological diseases, anti-tumor and anti-complementary activities, and immunostimulation activation activity, etc since ancient ages (Ahn et al., 1996 ; Kumazawa et al., 1982). It contains many kinds of coumarin such as decrusinol, decrusin, nodakeentin, umbelliferon and beta-sistosterol, etc, which can control blood circulation and other age-related deseases (Wang et al., 1994 ; Ahn et al., 1998). However, most of the work has been focused on screening and identifying biological activities of roots and other parts of *A. gigas*, not expanding the possibility of using its extracts as an biological modulator on molecular and cell levels. Therefore, in this work, the possibility of new types of biologically active chemicals from plant resources, *A. gigas* Nakai will be demonstrated for the use of mammalian culture systems to control the differentiation of human ES cells *in vitro*.

MATERIALS AND METHODS

Sample preparation

Root of *A. gigas* Nakai produced in Pyungchang, Kangwondo, Korea, 2001 was dried in shade area, and extracted by adding 5 times volume of mixed solvent, water : ethanol (1 : 1 v/v) at 80°C for 10 hours. The extract was evaporated by a rotary vacuum evaporator, and freeze-dried. The extract was first precipitated by adding three volumes of ethanol at 4°C for 24 hours. The precipitated was dissolved in water and separated by DEAE cellulose column at the rate of 5 ml/min at 280 absorbance. The solution was centrifuged at 5000 rpm for 25 min, then washed with Tris buffer (pH 7.0) and dissolved in 15% NaCl. Then, it was dialyzed and freeze-dried. The sample was stored at -4°C before use. Molecular weight of the sample was estimated as 25k dalton by HPLC (waters) analysis, which is a polysaccharide composed of fructose, galactose and glucose including uronic acid.

Maintenance of cell cultures

Human ES cells were generously donated by Dr. Pera (Monash Univ., Australia) and maintained in a 75 T-flask coated by mouse firbroblast feeder layer (Rose et al., 1994). Detail culture method were as follows : 5 to 9 week post-fertilization human embryos were mechanically disaggregated then incubated in 0.05% trypsin-0.5mM EDTA (GIBCO) at 37 for 5-10 min (Belldegrun et al., 2001). Cells initially were cultured and subsequently passaged on a mouse fibroblast feeder layer mitotically inactivated with 5,000 rads -radiation. Cells were grown in DMEM (GIBCO) supplemented with 20% fetal bovine serum (GIBCO) and 100μg/ml of streptomycin (GIBCO/BRL). Initially 1,000 units/ml of human recombinant leukemia inhibitory factor (LIF, Genzyme) was added to limit the differentiation of ES cells. Cultures were grown in 5% CO_2 incubator at 37°C and were routinely passaged every 7 days after disaggregation with 0.05% trypsin at 37 for 5-10min. To test the effect of the samples on differentiation, LIF or various concentrations of the purified samples were supplemented into the culture medium when the undifferentiated ES cells were first innoculated on the feeder layers.

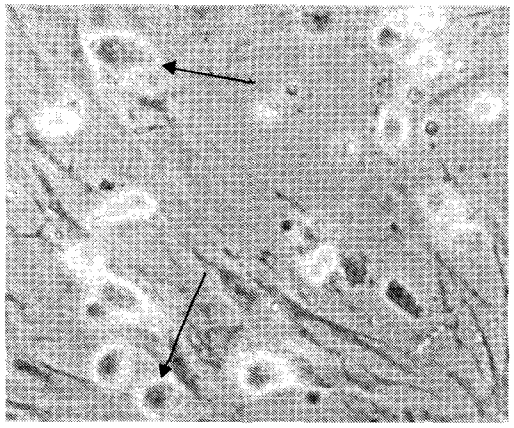
Measurement of differentiation of ES cells

The propagated ES cells grown on mouse feeder layer has been observed under an inverted microscope, to find the change of the morphology of ES cells. It is considered to be the clearest evidence whether ES cells were differentiated or not since the morphology of undifferentiated ES cells was changed when the cell started to be differentiated during proliferation (Reubinoff et al., 2000). The ratio of differentiated to undifferentiated cells was estimated by calculating the number of total cells and differentiated cells in same spot of a 75 T-flask, observing the changes of

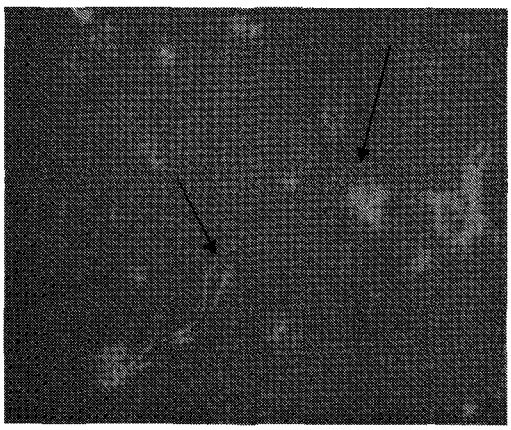
their morphology under a microscope, which can be easily identified by alkaline phosphatase. Alkaline phosphatase (AP) activity, which can be characterized as ES cells, was measured as follows : The cell was fixed by 66% acetone/3% formaldehyde solution at 4 ′ C for 30 min., and then stained with naphthol/ FRV-alkaline AP substrate (Sigma, USA) (Hogan et al., 1994). Telomerase activity, which was often used for estimating the degree of cell differentiation (Allsopp et al., 1992 ; Kim et al., 1994), was measured by telomerase repeat amplication protocol (TRAP) as follows (Kim et al., 1994) : DNA was isolated when the cells reached confluence and digested with restriction enzymes MsapI and RsapI. The fraction wa loaded onto 0 0.7 % agarose gel electrophoresis. DNA was depurinated by soaling the gels in 0.1M sodium citrate (pH 3.0) for two hours, thransferred to Nytran and hybridized in 6xSSC at 37 ′ C with end-labelled $(TTAGGG)_3$. Filters were washed in 3xSSC at 42 ′ C for the $(TTAGGG)_3$ probe in a PCR (BioRad, USA). Positive control was a cell extract from the telomerase expressing tumor cell line H1299 cells. The ratio of telomerase expressed in treated or untreated ES cells read through a densitometer (BioRad) to the telomerase activity in positive control was expressed as a percentage in the Figs.

RESULTS AND DISCUSSION

Fig. 1 shows the morphology of ES cells embedded on feeder layers. Picture (A) must be mostly undifferentiated ES cells only for one day cultivation after being innoculated, which seems to be similar to other reported morphology of human ES cells stained with AP (Hogan et al., 1994). After six days cultivation without adding any undifferentiated agents, the morphology of the cells definitely changed to other forms (B), in case to neuronal cells. Therefore, it is obvious that human ES cells were automatically differentiated to other many types of cells during proliferation. Fig. 2 is to illustrate the effect of LIF (1000 unit/ml), which is most used for limiting differentiation of ES cells and the sample from *A. gigas* (0.8 ug/ml) on controlling the differentiation of them during the cultivation. The cell growth was fairly well increased in adding the extracts, compared to the control. It may be caused by several unknown components in *A. gagis* Nakai which can enhance the cell growth (Ham et al., 1996). While the growth of ES cells seemed to be inhibited in adding LIF even it controlled the differentiation. It is also interesting that the purified extract accelerated the cell growth when the cell reached to exponential growth phase three to four times, compared to the control. Therefore, it is obvious that the extract from *A. gigas* did not negatively affect the cell growth but LIF, even enhanced the cell growth.



(A)



(B)

Fig. 1. Pictures of undifferentiated (A) and differentiated (B) ES cells grown on mouse feeder layer (400 times magnification). A mans are the matured ES cells.

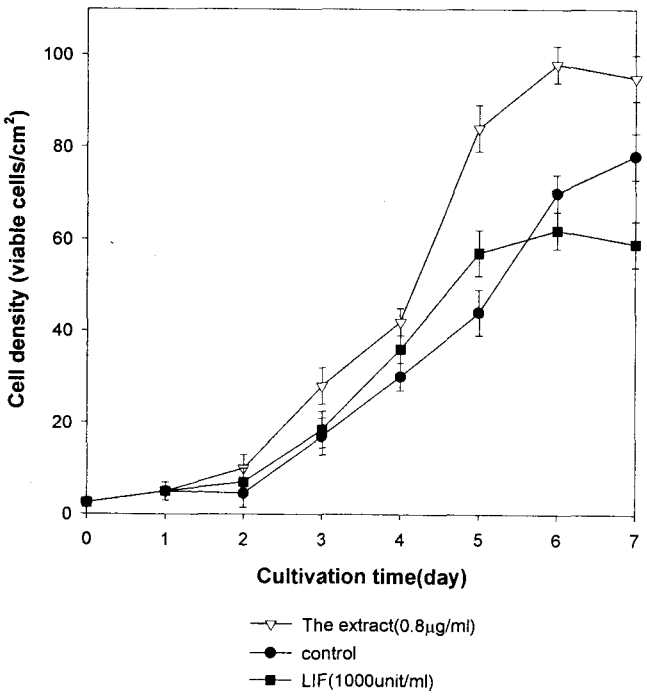


Fig. 2. The growth of human ES cells by adding LIF and the extract from *A. gigas* Nakai.

Fig. 3 compares the effect of purified extract and LIF on limiting the differentiation of ES cells according to the cultivation time. Without adding the agents (control), more than 50% of the cells was differentiated into neuronal cells in this case; however, only 15% of the cells turned into differentiated cells in adding 0.8 ug/ml of the extract. In adding 1000 unit/ml of LIF, ca. 80 % of the cells remained in undifferentiated at 6th day of the cultivation even though the cell growth was slightly inhibited (Fig. 2). It was also found that both LIF and the extract maintained the cells as undifferentiated morphology even the cell reached to stationary phase, while the cells was continuously differentiated without any supplementation. It tells that ES cells definitely need the agent controlling the differentiation for long-term cultivation. The purified extract from *A. gigas* Nakai meets the demand that limits the differentiation without affecting the cell growth while commercially available LIF does not. Then, Fig. 4 is to show the effect of the extract as a function of cell growth and undifferentiation ratio according to addition concentrations. It was found that an optimal supplementation of the extract was 0.8 (ug/ml) to maintain the highest undifferentiated cells. In adding this concentration, maximum cell density was also maintained. At the above 0.8 ug/ml both cell growth

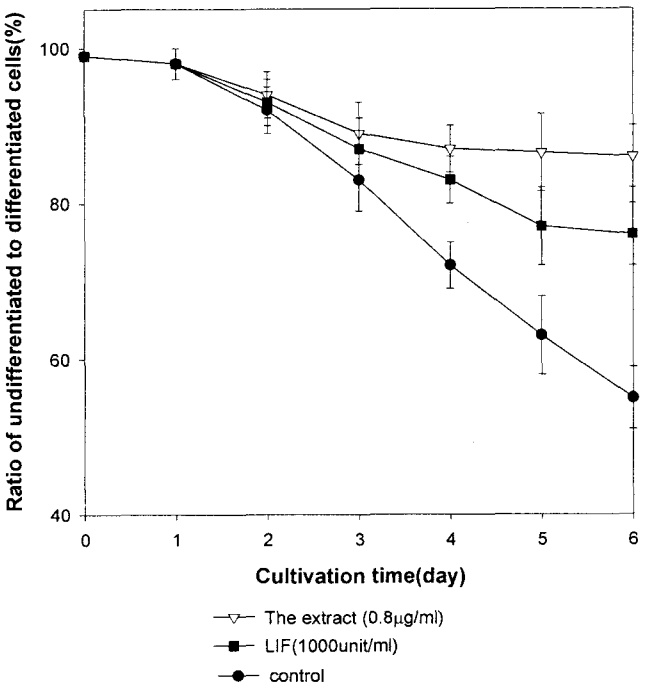


Fig. 3. Changes of the ratio of undifferentiated ES cells by adding two different samples; LIF and the extract from *A. gigas* Nakai.

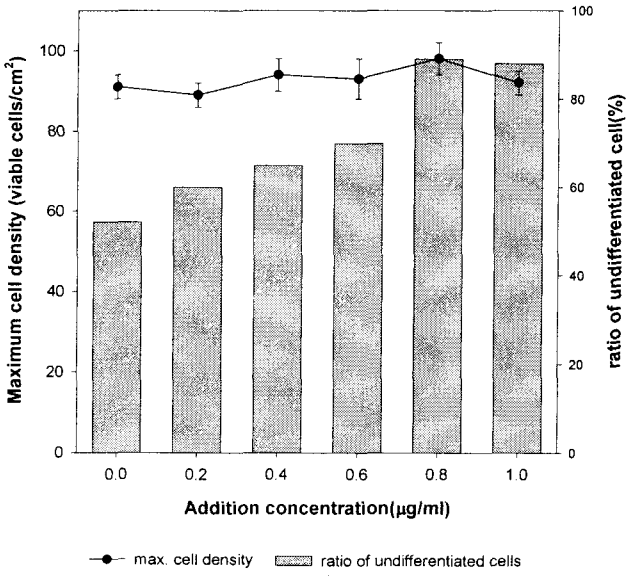


Fig. 4. The effect of the extracts on growth and differntiation of human ES cells at exponential growth phase.

and undifferentiation ratio were decreased while the differentiation ratio was gradually increased as the supplementation concentration was increased. However, the maximum cell density did not seem to be correlated to the supplementation. It implies that the purified extract could more directly affect on controlling the differentiation rather than cell growth even the extract could positively play a role in enhancing the cell growth.

Fig. 5 compares the effect of the samples on telomerase activity .which is most evidence for the cell to be differentiated or not in molecular level within the cells (Allsopp et al., 1992). The telomerase activity was also increased as the supplementation was increased. It was interesting that the telomerase activity was continuously increased as the supplementation was increased, compared to that the undifferentation ratio was not increased at 0.8 (ug/ml) (Fig. 4). It may tell that the purified extract first attack the cell membrane because of relatively high molecular weight and control signal system on the cell membrane, then trigger the gene expression of telomere for controlling the differentiation. The telomerase activity in adding the extract was enhanced about three times higher than that for the control, and two times in adding LIF. It was confirmed that both LIF and the extract did not influence the maximum cell growth. It can also be supported the result that relatively high cell density was maintained without the supplementation when the telomerase activity was severly dropped.

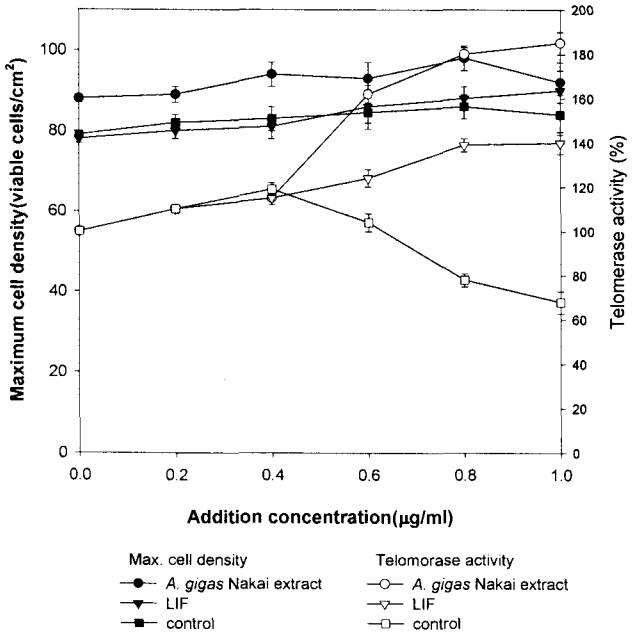


Fig. 5. The effect of the extracts on growth of human ES cells and telomerase activity.

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