The effect of α -pinene from *Pinus densiflora* S. and a polysaccharide from *Angelica gigas* Nakai on differentiation and proliferation of human embryonic stem cells

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Abstract

The leukemia inhibitory factor (LIF), which is a very expensive reagent, can be used to efficiently control the differentiation of human embryonic stem (ES) cells at concentrations >1000 units/ml for 6–7 days. However, in supplement <500 units/ml, most ES cells differentiate within 3–4 days in *in vitro* cultures. α -Pinene from *Pinus densiflora* S. and a polysaccharide (MW 25 kDa) from *A. gigas* Nakai showed promising results as a substitute for LIF in cultivating ES cells. By adding both 0.5 (μ g/ml) of α -pinene and the polysaccharide, most of the ES cells could be maintained under undifferentiated conditions after adding only 100 units/ml of LIF. It was found that α -pinene can play a role in preventing the ES cells from differentiating and the polysaccharide can be used to grow the ES cells. The results suggest that human ES cells can be maintained under undifferentiated conditions by supplementing both plant extracts, which can result in a reduction in the amount of LIF needed.

Introduction

Recently, numerous reports on maintaining and/or differentiating embryonic stem (ES) cells derived from many sources such as humans, mice and rabbits, etc., have been released (Andrews 1981; Gluckman and Bulloch 1997; Fong et al. 1999; Shmablott et al. 2001). Among them, the results for human ES cells have attracted a great deal of attention because they can be widely used for transplantation medicine, human developmental biology and drug discovery, etc., even though work on human ES cells still has ethical problems

(Evans et al. 1981). However, knowledge of human ES cells is still primitive even though several great accomplishments regarding the establishment of human ES cells and transplantation of them in animal systems have been reported (Ham et al. 1996; Li et al. 1999). We are unsure of the mechanisms of the *in vitro* and *in vivo* differentiating embryonic cells and how they will be optimized for clinical treatments. It has been demonstrated that pluripotent human ES cells can proliferate on feeder cells while remaining karyotypically and phenotypically stable. These cells can eventually differentiate into a various types of cells *in vivo*

and/or *in vitro* by adding several growth factors such as the epidermal growth factor (EGF), the fibroblast growth factor (FGF) and the nerve growth factor (NGF), etc., (Becker 2002; Shamblott et al. 2001). Other polypeptide factors may also be involved in the differentiation and proliferation of committed embryonic progenitor cells (Thomson et al. 1995, 1998; Ling et al. 1997). However, this requires large quantities of undifferentiated ES cells to be maintained *in vitro*. Thus far, ES cells can grow only on feeder layer cells such as human and/or mouse primary fibroblast cells and also need cytokines such as the leukemia inhibitory factor (LIF) and the fibroblast growth factors (FGF) in the cultures in order to maintain them in the undifferentiated form (Wang et al. 1994; Marshall 1998; Schuldiner et al. 2000). Unfortunately, LIF is very expensive and may not be effective in controlling the differentiation of human ES cells growing on human fibroblast cells (Kim et al. 2000). This may be one of the main reasons that the research on ES cells cannot be accelerated due to the requirement of a labor intensive process with its associated high cost.

There is no report of an attempt to use plant extracts to control the proliferation and differentiation of ES cells even though they are inexpensive and safe resources for human clinical trials that the authors are aware of. *Angelica gigas* Nakai is a very well-known medicinal plant used for treating gynecological diseases, as well as has anti-tumor and anti-complementary activities, and immuno-stimulatory activation activity, etc., (Ahn et al. 1996; Kurtzberg, 1996). It contains many types of coumarin such as decursinol, decursin, nodakeentin, umbelliferon and beta-sis-tosterol, etc., which can control the blood circulation and other age-related diseases (Zhiming et al. 1995; Ahn et al. 1998). *Pinus densiflora* S. needles have also been used to treat blood circulation problems, anti-inflammation and aromatherapy for thousands of years (Kumazawa et al. 1982; Altman 1989; Zhiming et al. 1995). In particular, the essential oils from pine needles are used to control human nerve and mental problems (Kallós et al. 1998; Kim et al. 2001). Therefore, it is possible that the essential oils also play a role in controlling the growth of human ES cells in the early stage of the cell development. However, most work has been focused on screening and identifying the biological

activities of the herbs as sources of medicinal applications, which has not expanded the possibility of using its extracts as an biological modulator on the molecular and cell level. Therefore, this study explored the possibility of using new types of biologically active chemicals from *Pinus densiflora* S. needles and/or *A. gigas* Nakai for controlling the differentiation of human ES cells *in vitro* by substituting these materials for expensive mammalian-origin chemicals such as LIF.

Materials and methods

Sample preparation

The oils from the needles of *Pinus densiflora* S. were isolated by steam distillation followed by diethyl ether extraction of the distillate (Whish 1996). The composition of the oil was analyzed and isolated by gas chromatography-mass spectrometer (GC-MS, HP 5890, New Brunswick, USA) equipped with a flame ionization detector with a 2.0×0.25 mm capillary column coated with SGE BP-5. A major component in the oils was determined to be α -pinene (extraction yield 12.56%, v/w), and α -pinene (Sigma, St. Louis, USA) was used as a single component in the following experiments. The root of *A. gigas* Nakai produced in Pyungchang, Kangwondo, Korea, 2001 was dried in the shade, and extracted by adding 5 times the volume of a mixed solvent, water:ethanol (1:1 v/v) at 80 °C for 10 h. 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 h. The precipitate was then dissolved in water and separated by a DEAE cellulose column at a rate of 5 ml/min. The solution was centrifuged at 5000 rpm for 25 min, then washed with a Tris buffer (pH 7.0) and dissolved in 15% NaCl. The solution was then dialyzed and freeze-dried. The sample was stored at -4 °C prior to use. The molecular weight of the sample was estimated to be 25 kDa by gel permeation HPLC (Waters, St. Louis, USA). Detailed operation conditions were as follows: column, PL-GF (18 mm, 7×300 mm, Polymer Lab. USA); mobile phase, water; flow rate, 1 ml/min; detector, RI detector; standard, standard polymers (10, 50, 100, 500 kDa). It was

found that the polysaccharide was composed of fructose, galactose and glucose (1.4:1.5:2, w/w) including uronic acid.

Maintenance of ES cell cultures

The human ES cells were generously donated by Dr Pera (Monash University, Australia) and maintained in a 75 T-flask coated with a mouse fibroblast feeder layer (Schuldiner et al. 2000). The human ES cells initially were cultured and subsequently passaged on a mouse fibroblast feeder layer that was mitotically inactivated by adding 0.5 mg/l of mytomicin C. The cells were grown in DMEM (GIBCO) supplemented with 20% fetal bovine serum (GIBCO) and 100 µg/ml of streptomycin (GIBCO/BRL). Initially 1,000 units/ml of the human recombinant leukemia inhibitory factor (LIF, Genzyme, New Brunswick, USA) was added to limit the differentiation of the ES cells. The cultures were grown in 5% CO₂ incubator at 37 °C and were routinely passaged every 7 days after disaggregation with 0.05% trypsin at 37 °C for 5–10 min. In order to test the effect of the samples on differentiation, LIF or various concentrations of the plant extract samples were added to the culture medium and monitored by an inverted microscope when the undifferentiated ES cells were first inoculated on the feeder layers.

Measurement of differentiation of ES cells

In order to identify the change in the morphology of the ES cells, the propagated ES cells grown on the mouse feeder layer were observed under an inverted microscope. This is considered to be the clearest evidence showing whether or not the ES cells have differentiated since the morphology of undifferentiated ES cells changes when the cell begins to differentiate during proliferation (Rose et al. 1994). The ratio of differentiated to undifferentiated cells was estimated by calculating the total number of cells and the number of differentiated cells in same spot of a 75 T-flask, observing the changes in their morphology under a microscope, which can be easily identified by alkaline phosphatase. The alkaline phosphatase (AP) activity, which can be characterized as undifferentiated ES cells, was measured as follows:

The cells were fixed with a 66% acetone/3% formaldehyde solution at 4 °C for 30 min., and then stained with naphthol/FRV-alkaline AP substrate (Sigma, USA) (Hong et al. 1999).

The telomerase activity, which is often used to estimate the degree of cell differentiation (Harley et al. 1990; Kim et al. 1994) was measured by the telomerase repeat amplification protocol (TRAP) as follows (Harley and Villeponteau 1995): The DNA was isolated when the cells reached confluence and was digested with restriction enzymes *MspI* and *RsaI*. The fraction was then loaded onto 0.7% agarose gel electrophoresis. The DNA was depurinated by soaking the gels in 0.1 M sodium citrate (pH 3.0) for 2 h, transferred to Nytran and hybridized in 6× SSC at 37 °C with end-labeled (TTAGGG)₃. The filters were washed in 3× SSC at 42 °C for the (TTAGGG)₃ probe in a PCR (Bio-Rad, USA). The positive control was a cell extract from the telomerase expressing tumor cell line HL299 cells. The length of the telomere in the cells was measured by measuring the genomic DNA with a Southern blot. The oligonucleotide probe was (TTAGGG)₃ (Harley et al. 1995; Reubinoff et al. 2000).

Results and discussion

Figure 1 shows the change in the morphology of the ES cells embedded on the feeder layers grown with or without LIF in 10% FBS enriched medium. In Figure 1, (a) and (c) show the morphology of the ES cells when the cells were grown with 1000 units/ml of LIF (a) and without (c), respectively, for 3 days after the inoculation. In (a) the cells appear like the undifferentiated ES cells, which are similar to the reported morphology of human ES cells (Shamblott et al. 2001). However, the morphology of (c) has already begun to change to other forms after only 3 days of cultivation. Some of the ES cells in (b) had changed to other forms, for this case, possibly to neuronal cells after 6 days of cultivation. The cells in (d), which were grown without adding LIF for 6 days, had completely differentiated to other forms, but did not propagate. It is obvious that LIF plays a role in delaying and/or limiting the differentiation of ES cells on the fibroblast feeder layers for approximately 6–7 days. It was also confirmed that the human ES cells could not be maintained

Figure 1. Comparison of the morphology of the human ES cells after adding 1000 units/ml of LIF (a, b) or not (c, d) after the 3rd and 6th days of cultivation.

in the undifferentiated form without LIF, which means that the ES cells require undifferentiating agents in the culture medium. Figure 2 also confirms the effect of LIFs on controlling the differentiation of ES cells by AP staining (Hong et al. 1999). In (a), the cells grown for 3 days in the supplementing 1000 units/ml of LIF is shown, which were then stained with AP, and (b) is not supplemented with LIF. The cells cultivated in the presence of LIF (Figure 2a) had a strong AP activity, showing that the AP stained most parts. However, in (b), only small parts within the cell were stained by AP, which means that the ES cells had already differentiated to other cells in the medium without the added LIF for 3 days. This

result is well matched to the morphological observations as seen in Figure 1.

The effect of LIF concentration on the differentiation of ES cells is shown in Figure 3. In Figure 3, (a) is the result of supplementing half of the conventional concentration of LIF (500 units/ml) after 4 days cultivation, and (b) is for the case of adding only one tenth of the original concentration (100 units/ml). After adding 500 units/ml of LIF, many of the cells had differentiated within 4 days of cultivation, which is in contrast to those maintained in the 1000 units/ml. After adding 100 units/ml, the cells could not be maintained in the undifferentiated form even for 3–4 days. This shows that the minimum concentration of the

Figure 2. Comparison of the stained cells by alkaline phosphatase after adding 1000 units/ml of LIF (a) or not (b) on the 3rd day of cultivation.

Figure 3. The effect of LIF on the differentiation of the ES cells on the 4th day of cultivation: (a) 1000 units/ml of LIF; (b) 100 units/ml of LIF.

supplementing LIF is 1000 units/ml for the *in vitro* cultivation of human ES cells.

Figure 4 shows the effect of the *Pinus densiflora* S. or *A. gigas* Nakai extracts on controlling the differentiation of ES cells after adding 100 units/ml of LIF and 10% FBS enrichment. The ES cells had completely differentiated within 3 days after adding 1.0 μ g/ml of the samples without LIF (data not shown). In Figure 4, (a) and (b) show the change in the ES cells after adding α -pinene from the needles of *Pinus densiflora* S. for 3 and 6 days cultivation, respectively. Figure 4 (c) and (d) show the results of adding the polysaccharides from *A. gigas* Nakai. It is apparent that α -pinene has the effect of limiting

differentiation of the ES cells since most of the cells had not differentiated even after 6 days of cultivation, compared to (b) in Figure 1 (1000 units/ml of LIF). However, the propagation of the cells appeared to be inhibited due to the addition of α -pinene. On the other hand, better propagation of the ES cells was achieved by adding polysaccharides based on a larger number of ES cells even though a relatively higher differentiation rate was observed, compared to the morphology in Figures 1 and 3. This suggests that a polysaccharide from *A. gigas* could not control the differentiation but improved the growth of the ES cells. α -pinene has the potential of substituting for LIF for the control of ES cell

Figure 4. The effect of α -pinene (a, b; 1.0 μ g/ml) and polysaccharides from *A. gigas* Nakai (c, d; 1.0 μ g/ml) on the differentiating human ES cells after the 3rd and the 7th days of cultivation.

differentiation by possibly limiting the propagation of the ES cells.

It is essential to seek the possibility of simultaneously adding both the samples in cultivating ES cells since it was found that α -pinene and polysaccharides, respectively, have different effects on the ES cells (Figure 4). Figure 5 illustrates the morphology of human ES cells for 3 and 6 days cultivation after adding 0.5 ($\mu\text{g/ml}$) of α -pinene and 0.5 ($\mu\text{g/ml}$) of polysaccharide from *A. gigas* Nakai as well as 100 units/ml of LIF. The FBS concentration was also reduced to 2% from 10% because the polysaccharides improved the growth of the ES cells. The morphology of (b) was not much different from (b) in Figure 1, which was taken by adding 1000 units/ml of LIF and 10% FBS after 6 days cultivation. However, below 0.5 ($\mu\text{g/ml}$) of the supplementation of each sample, a relatively higher differentiation and a severe inhibition of ES cell proliferation were observed (data not shown here). This means that

Figure 5. The morphology of the human ES cells after adding 0.5 $\mu\text{g/ml}$ α -pinene and 0.5 $\mu\text{g/ml}$ of the polysaccharides from *A. gigas* Nakai enriched with 2% FBS and 100 units/ml of LIF. Arrows show the undifferentiated human ES cells.

there must be a minimal supplementation concentration for the substitution of LIF and FBS for the ES cell cultures, for this case, determined at about 0.5 ($\mu\text{g/ml}$).

The effect of the extracts in controlling differentiation was more closely examined by measuring the telomerase activity by adding the extracts into the ES cells because the telomerase activity is the best evidence for cell differentiation at the molecular level within the cells (Harley et al. 1992). Figure 6 compares the telomerase activity from each cell when LIF or plant extracts were added to the medium. Lane A shows the results of estimating the telomerase activity in the presence of 1000 units/ml of LIF on the 3rd day of cultivation. The enzyme shows the strongest activity among other cells, which is similar to the result shown in Figures 1 and 2. Lane B shows the activity after adding both 0.5 ($\mu\text{g/ml}$) of α -pinene and 0.5 ($\mu\text{g/ml}$) of polysaccharides along with 100 units/ml of LIF, as used in Figure 5 (A or B2). The enzyme activity (B) was similar to that observed in Lane A. This also demonstrates that the addition of the plant extracts definitely limited the differentiation of the ES cells in the presence of one tenth of LIF as LIF does. Lanes C, D and E show the telomerase activity after adding only α -pinene or polysaccharides or 100 units/ml of LIF, respectively. The activity became weaker after adding only 100 units/ml. This also provides evidence that the plant extract could play a role in controlling the differentiation of ES cells and possibly substitute an expensive mammalian-origin LIF in some ways. The last lane F shows the result of the telomerase activity without adding any of the undifferentiating chemicals. The lane shows that most of the enzyme activity appears to be lost. Table 1 also confirms the effect of the

Figure 6. *In vitro* reconstitution of hTRP as a telomerase activity in human ES cells cultivated for 3 days. A, 1000 units/ml, LIF; B, 0.5 $\mu\text{g/ml}$ α -pinene + 0.5 $\mu\text{g/ml}$ polysaccharides; + 100 units/ml, LIF; C, 1.0 $\mu\text{g/ml}$ α -pinene; D, 1.0 $\mu\text{g/ml}$ polysaccharides; E, 100 units/ml LIF; F, none (control).

Table 1. Effect of the plant extracts on the telomere length in the grown human ES cells (derived from Fig. 6)

Treatment	Mean telomere length (kb $\pm$ sd (n)*)
LIF (1000 units/ml)	9.8 $\pm$ 0.4 (5)
LIF (100 units/ml)	6.3 $\pm$ 0.4 (4)
α -pinene (0.5 μ g/ml) and polysaccharide (0.5 μ g/ml), LIF (100/units/ml)	9.4 $\pm$ 0.5 (5)
α -pinene (0.5 μ g/ml)	7.1 $\pm$ 0.3 (4)
Polysaccharide (0.5 μ g/ml)	6.0 $\pm$ 0.5 (5)
None	5.1 $\pm$ 0.5 (4)

* n is the number of repetitions. SD is the standard deviation.

plant extracts on limiting the differentiation of the ES cells by measuring the telomere length for each condition tested (extracted from the experiments in Figure 6). As expected from the data shown in Figure 6, the length of the telomere was the longest after adding 1000 units/ml of LIF (9.8 kb), which was followed by the case of adding both extracts with 100 units/ml of LIF (9.4 kb). After adding only 0.5 (μ g/ml) of α -pinene, the length dropped to 7.1 kb. It further dropped to 6.0 kb when only adding the polysaccharides. This might suggest that α -pinene plays a large role in controlling the differentiation, and the polysaccharides from *A. gigas* Nakai play an important role in controlling the proliferation of cell growth. This is because the length of the telomere in adding only 100 units/ml of LIF was slightly longer than that after adding only the polysaccharides. Without adding any supplement, the length of the telomeres was dramatically reduced to 5.1 kb, which is lower than for those of other cell lines (range of 6.5–8.5 kb) (Hogan et al. 1994). This also demonstrates that α -pinene has the effect of delaying or limiting the differentiation of the ES cells. These results will eventually lead, to a reduced use of LIF in culture medium and hence to a reduction of the costs of mass production of undifferentiated ES cells. Further studies should be carried out for studying the detailed mechanism of α -pinene in ES cells on a molecular and cellular level.

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