Korean J. Ginseng Sci.
Vol. 21, No. 3, 147~152(1997)

Identification of Nuclear Receptors by RT-PCR in F9 Cells Induced by Ginsenosides

Youl-Nam Lee[1], Shin-Il Kim[2], Ho-Young Lee[1], Seh-Yoon Yi[3], You Mie Lee[1], Chang Mo Kang[1] and Kyu-Won Kim[1]*

[1]*Department of Molecular Biology, Pusan National University, Pusan, 609-735*
[2]*Korea Ginseng and Tobacco Research Institute, Daejeon, 305-345*
[3]*Department of Chemistry, Ewha Womans University, Seoul, 120-750, Korea.*

(Received October 11, 1997)

Abstract : Ginsenosides Rh1 and Rh2 induced the differentiation of F9 teratocarcinoma stem cells. These agents are structurally similar to the steroid hormones, therefore, we speculated that the steroid receptor (s) or novel nuclear receptor (s) could be involved in the differentiation process induced by them. Based on this speculation, we tried to clone new nuclear receptors with reverse transcription-polymerase chain reaction (RT-PCR) method by isolating RNA from F9 teratocarcinoma cells induced by ginsenosides. By using RT-PCR with degenerated primers from highly conserved DNA binding domain of nuclear receptors, we identified several nuclear receptors. In northern blot analysis we found that these clones are transcriptionally regulated by ginsenoside Rh1 or Rh2 treatment. Further characterizations of these clones are needed to identify the mechanism of gene expression which has an important role in the differentiation of F9 cells induced by ginsenosides.

Key words : Ginsenosides, nuclear receptors, RT-PCR, F9 teratocarcinoma cell.

Introduction

Ginsenosides, ginseng saponins, are known as major effective compounds in Korean ginseng. Ginsenosides obtained from the root of *Panax ginseng* C.A. Meyer, have been reported to induce phenotypic reverse transformation in cultured Morris hepatoma cells.[1] Moreover, ginseng extract inhibited the incidence and the proliferation of tumors induced by some carcinogens, and the anticarcinogenic effect of ginseng was shown to be related to immune response.[2-4] It has been also reported that some ginseng saponins derived from different origins have various biological activities including anti-inflammatory activity,[5,6] and induction of gene expression such as tyrosine aminotransferase and human interleukin-1.[7] However, the detailed molecular action mechanism of ginsenosides is still unknown.

We previously identified that ginsenosides have the ability to induce the differentiation of F9 stem cells.[8] Among various purified ginsenoside derivatives, Rh1 and Rh2 caused the differentiation of F9 cells most effectively in terms of morphological change and marker gene expressions such as laminin B1 and type IV collagen. Since ginsenosides and steroid hormone are very similar in their gross molecular shapes, we speculated that steroid receptor (s) or novel nuclear receptor (s) could be involved in the differentiation process induced by them. According to the previous experiments such as gel shift assay, glucocorticoid receptor (GR) binding assay, and GRE-luciferase transactivation assay, we suggested that ginsenosides can interact with GR.[8] Therefore, it is necessary to characterize the nuclear receptor superfamily that is activated by ginsenosides in the differentiation process of F9 cells induced by ginsenosides.

Nuclear receptors are ligand-activated tran-

scription factors which regulate the expression of target genes by binding to specific *cis*-acting sequences.[9,10] This family includes nuclear receptors for several hydrophobic ligands such as steroids, vitamin D. thyroid hormones, retinoic acid and also for regulators of development, cell differentiation, and organ physiology. In addition to these receptors for known ligands, many of the new members have been described as orphan receptors which are putative receptors for ligands still to be identified.[9,11] Sequence analysis and functional studies revealed two important regions of these receptors that exhibit a high degree of amino acid residue conservation. The highest level of similarity among the receptors is found in a region that contains nine cysteine residues that bind zinc atoms to form two "zinc fingers", which interact with the cognate response elements of DNA.[12,13] We tried to use this conserved sequence of the zinc finger domain as a mean to find other members of the nuclear receptor superfamily in F9 cells induced by ginsenosides.

In this study, we examined a involvement of novel nuclear receptor (s) in the differentiation of F9 stem cells by RT-PCR analysis with degenerated primers from highly conserved DNA binding domain of the known nuclear receptors.

Materials and Methods

1. Materials

F9 cells were obtained from the American Type Culture Collection (ATCC CRL 1720). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). Tissue culture flasks were purchased from Nunc (Roskilde, Denmark), restriction endonucleases and ^{32}P-dCTP were from Amersham (Bucks, U.K.). Guanidinium thiocyanate and P60 gel were from Bethesda Research Laboratories and zetaprobe blotting membrane was from Bio-Rad (Zeta-Probe, Bio-Rad, CA, U.S.A.). Dibutyryl cAMP and other chemicals were also purchased from Sigma Chemical Co. (St. Louis, MD, U.S.A.).

2. Methods

(1) Cell Culture and differentiation of F9 teratocarcinoma stem cells

F9 stem cells were grown on gelatinized plastic flasks in DMEM, supplemented with 10% FBS, penicillin (100 U/m*l*) and streptomycin (100 μg/m*l*) in a humidified atmosphere of 5% CO_2 at 37°C. For the induction of differentiation, F9 stem cells were trypsinized and seeded onto gelatinized T75 tissue culture flasks containing 25 ml of culture medium and incubated for 24 hr prior to the addition of drugs. Cells used for differentiation experiments were between passages 1 to 7. When differentiation was induced by ginsenosides, F9 stem cells were treated with 50 μM ginsenosides, 500 μM dbcAMP and 250 μM theophylline. Ginsenosides were prepared as a 100 mM stock in ethanol, dbcAMP was prepared as 100 mM aqueous solution and theophyllin was a 100 mM stock in 0.1 N NaOH. Cell morphology was detected by phase-contrast microscopy for a judgement of the degree of the differentiation.

(2) Reverse transcription-polymerase chain reaction (RT-PCR)[14]

The conversion of RNA into cDNA was carried out at the conditons recommended by Perkin Elmer Cetus using random hexamers as a primer. Following completion of the reaction, 2 μ*l* of the cDNA solution were used in PCR reaction with degenerate primers SR-2 (sense), SR-3 (antisense) and SR-11 (sense) and SR-13 (anti- sense) [5'-GGCTGCAAIGBTTTCTTYA-3', 5-GCAITTSCKG-AASCGGCA-3',5'-TTGGATCCGAYDANDSNWS-NGGTNWBCAYTA-3', and 5'-AAGAATTCRCA-YT TNNNNWRNCKRCANGCDKGRCA-3', respectively] (Fig. 1) at a concentration of 50 pmol. After 40 cycles of amplification (30s at 94°C, 1 min at 50°C, 30s at 72°C), the products were analysed by electrophoresis in 2% agarose gels. Amplified DNA fragments of the expected size were recovered from the gel, and cloned with pGEM-T vector system (Promega, U.S.A.). Recombinant plasmids were transformed into the *E. coli* strain

DH5. White colonies were picked, and identified by restriction enzyme analysis. Correct clones were selected and sequenced by the dideoxy chain termination method 15 using sequenase version 2.0 (USB).

(3) RNA preparation and Northern blot analysis

Total cellular RNA was isolated according to the procedure of the acid-guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method.[16] Equal amounts (20 μg) of denatured RNA were fractionated on 1% agarose gels containing 1 M formaldehyde, and transferred to nylon membranes (Zeta-Probe, Bio-Rad, CA). After UV crosslinking at 0.12 J (UV Stratalinker 1800, Stratagene), blots were hybridized in a solution containing 50% deionized formamide, 0.25 M $NaHPO_4$, 7% SDS, and 1 mM EDTA at 42℃ in the presence of ^{32}P-labeled cDNA probes for 24 hr. The cloned RT-PCR products were used as DNA probes. They were labeled by the random primer labeling method.[17] Blots were washed 3 times for 5 min with 2×SSC and 0.1% SDS at RT, followed by two 15 min washes in 0.1×SSC and 0.1% SDS at 55℃. The filters were autoradiographed at -80℃ and the X-ray films were developed.

```
SR-2(sence)
5' -GGC TGC AAI GGT TTC TTC A- 3'
            C         T
            T
SR-3(antisence)
5' -GCA ITT GCG GAA GCG GCA-3'
      C T     C
SR-11(sence)
5' -TTG GAT CCG ATT ATT CTA CTG GTT ATC ATT A- 3'
                CA  CA GCT GC    C TC   C
                 G  AG   A   A    A G
                      G   G   G    G
SR-13(antisence)
5' -AAG AAT TCA CAT TTA AAA AAA CGA CAA GCA GGA CA- 3'
              G   C  T TTG TGG TG   G   G T G
                     G GGT   T      T   T
                     C CCC   C      C
```

Fig. 1. Degenerated PCR primers from the DNA binding domain of nuclear receptors.

Result and Discussion

In the previous report, we suggested that the induction of differentiation of ginsenosides might be mediated by the glucocorticoid receptor based on the experiments such as gel shift assay, GR binding assay, and GRE-luciferase transfection assay.[8] However, the relative weak binding affinities of ginsenosides to GR according to the result of GR-ligand binding assay suggest that ginsenosides may have a stronger binding affinity with unknown other nuclear receptor (s).

To identify a novel nuclear receptor which could act in the differentiation event induced by ginsenosides, RT-PCR was performed by using degenerated primers of the highly conserved DNA binding domain of the known nuclear receptor family. Reverse transcript cDNAs from RNA of F9 stem cells or ginsenoside-treated F9 cells were amplified by using degenerated primers. As the result, DNA fragment of about 130 and 200 base pairs (bp) were produced by using SR-2, 3 and SR-11, 13 primers, respectively (Fig. 2). The amplified cDNA was inserted into pGEM-

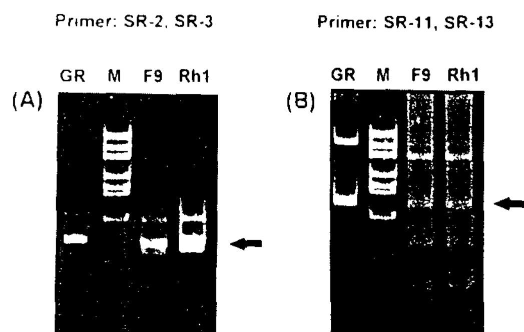

Fig. 2. Amplification of nuclear receptor cDNAs by RT-PCR with the degenerated primers in F9 cells treated witn ginsenosides. RT-PCR with primers SR-2,3 (A) and SR-11,13(B) was performed with RNA from F9 cells (F9) and F9 cell treated with Rh1 (Rh1). The amplified products were electrophoresed in 2% agarose gel. GR plasmid was used for a positive control of known nuclear receptors(GR). M means molecular weight marker, PUC19/Hae III(584, 458, 434, 298, 267/257, 174, 102, 80 bp, respectively).

T cloning vector and the positive clones were selected by the restriction enzyme analysis. The sequences of the clones were determined by Sanger's dideoxy method and then compared with those of the known nuclear receptors. As shown in Fig. 3, several clones having a very similar sequence homology were identified. One of the clones, c2-1 was highly homologous to embryonal LTR binding protein (ELP). ELP was initially identified as a repressor of transcription of the Moloney murine leukemia virus genome and it is exrpessed specifically in undifferentiated mouse embryonal carcinoma cells.[18] It was later found to be a mouse homologue of *Drosophila* FTZ-F1, which is also a member of the nuclear receptor superfamily and positively regulate transcription of the *fushi tarazu* gene.[19] The second clone, c2-2 showed a high degree of similarity to the Nur 77, which is also known as NGF1-B and N10.[20,21] It has been reported that Nur 77 encodes a member of nuclear receptor superfamily and can act as a potent transcription activator, which may function in mediating the cellular responses to growth factors by regulating the expression of specific genes.[22,23] The third clone, c3-4 showed a high degree of homology to glucocorticoid receptor (GR)[24] and it was regarded as a homologue of GR in F9 cells. As we have suggested, GR might act as a transcriptional regulator, which can control the expression of differentiation-specific genes of F9 cells. Sequence analysis of the c8-5 suggested that this clone is highly related to the peroxisome proliferator activated receptor (PPAR). PPAR is another member of the nuclear receptor superfamily and structurally related to the subfamily of receptors that includes the thyroid receptor (TR), retinoic acid receptor (RAR), and vitamin D3 receptor (VDR).[25] PPAR has been shown to be transcriptionally activated by treatment of cells with chemicals such as clofibric acid, nafenopin, and WY-14, 643 which induce peroxisome proliferation.[26,27] Due to this high degree of homology, we consider these clones to be related to the known nuclear receptors and hence further cloning for the full sequence of these clones would be very important to identify the interaction between ginsenosides and them. Nuclear hormone receptors have been implicated in a variety of functions during the development of vertebrates, such as morphogenesis, differentiation, and proliferation of cells. Elucidation of the functions of these receptors will help to explain the differential control of gene expression.

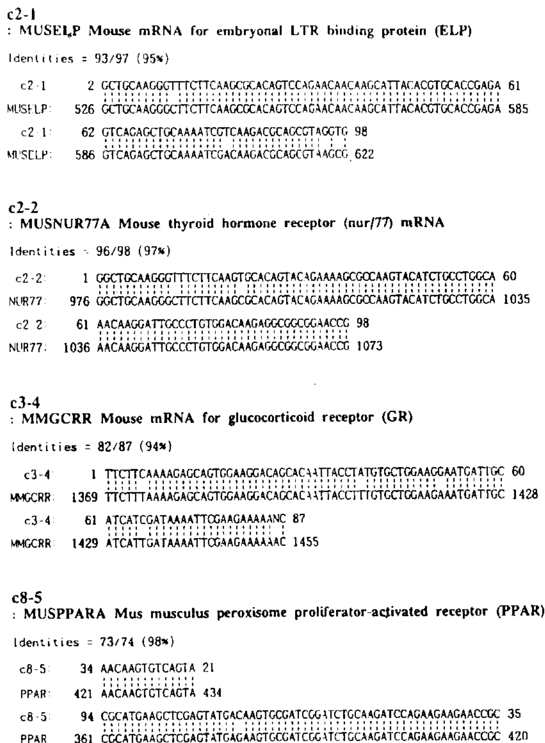

Fig. 3. Comparison of nucleotide sequence between the RT-PCR products and the nuclear receptor superfamily. The RT-PCR products were arbitrarily named as c2-1, c2-2, c3-4 and c8-5. The sequence homology search was performed by using the program of genebank. Identical sequences are marked with vertical lines.

To examine the expression of the clones during the differentiation of F9 cells induced by ginsenosides, northern blot hybridization was performed. Differentiation of F9 cells was induced into parietal endoderm-like cells by treatment of ginsenosides with dibutyryl cyclic AMP and theophylline (CT). The expression level of clone 2-1,

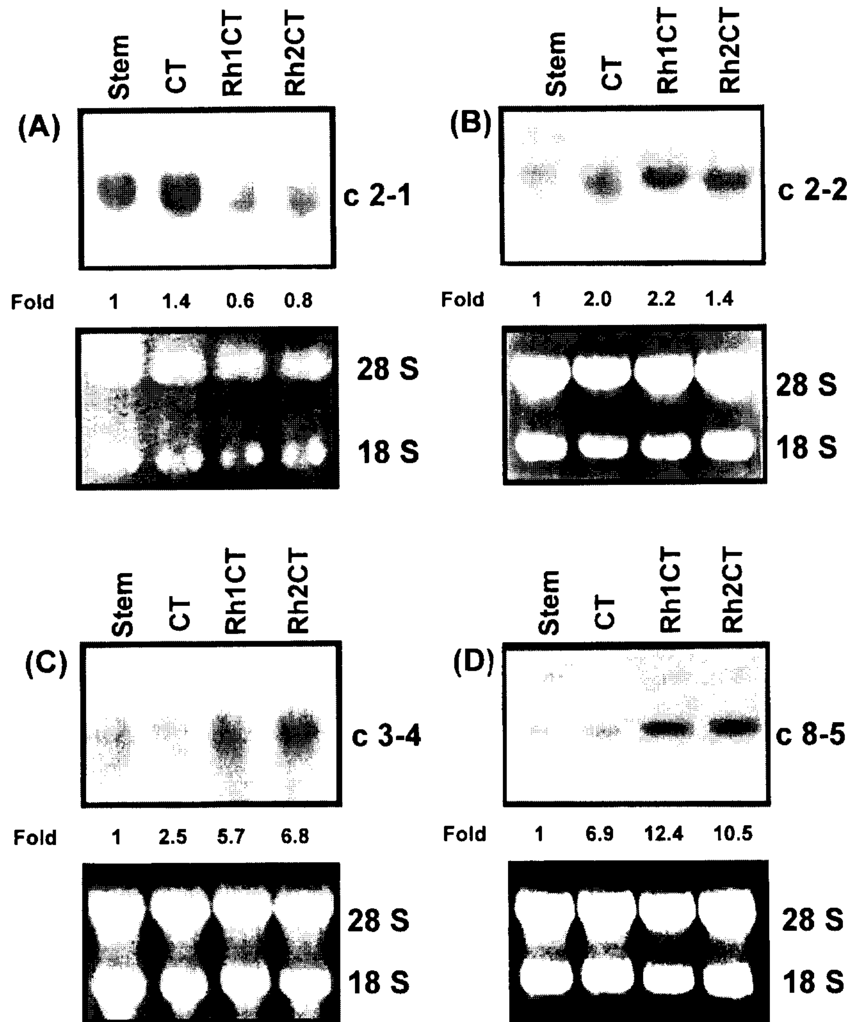

Fig. 4. Northern blot analysis of the newly identified RT-PCR clones in F9 cells. Total cellular RNAs from the F9 stem cells (Stem), F9 cells treated with 500 μM dbcAMP and 250 μM theophyline (CT), 50 μM Rh1 plus CT (Rh1CT), 50 μM Rh2 plus CT (Rh2CT) for 6 days were isolated, electrophoresed, and transferred onto nylon membranes. Then the RNAs were hybridized with ^{32}P-labeled probes of clone 2-1 (A), clone 2-2 (B), clone 3-4 (C), and clone 8-5 (D). Low panels, etidium bromide-stained RNA samples before transfer are shown and ribosomal RNA markers (28S and 18S) are indicated.

an analog of the known embryonal LTR binding protein (ELP) was decreased in F9 cells treated with ginsenosides Rh1 or Rh2, and this is consistent with the result of the previous report showing that ELP expression is specific in undifferentiated EC cells[19] (Fig. 4A). In contrast, the expression levels of clone 2-2, a homolog of Nur 77 and clone 3-4, a homolog of GR, clone 8-5, a homolog of peroxisome proliferator-activated receptor (PPAR) were increased in Rh1 or Rh2-treatd F9 cells (Fig. 4B, C, D).

These results suggest that ginsenosides may regulate the transcriptional level of these clones, which, in turn, may have a role in the differentiation process induced by ginsenosides. These clones may act as a monomer or oligomer with other nuclear receptor (s) or orphan receptor (s) in the regulation of gene expressions of

the differentiation of F9 cells. Further characterization of these nuclear receptor clones, that is, identification of true nuclear receptor (s) interacting with ginsenosides, or physical interactions of nuclear receptors will be needed for better understanding of the mechanism by which ginsenosides induce differentiation.

Acknowledgments

This work was supported by the research grant (1995) from the Society for Korea Ginseng and Korea Tobacco and Ginseng Corp.

References

1. Odashima, S., Nakayabu, Y. Honjo, N., Abe, H., and Arichi, S. : *Eur. J. Cancer.* **15** 885-892 (1979).
2. Yun, T. K., Yun, Y. S., and Han, I. W. : *Cancer Detect. Prev.* **6** 515-525 (1983).
3. Yun, Y. S., Jo, S. K., Moon, H. S., Kim, Y. J., Oh, Y. R., and Yun, T. K. : *Cancer Detect, Prev.*, **1**, 301-309 (1987).
4. Yun, T. K., and Choi S. Y. : *Int. J. Epidemiology* **19**, 871-876 (1993).
5. Okita, K., Li, Q., Murakamio, T., and Takahashi M. : *Eur. J. Cancer Prev.* **2**, 169-175 (1993).
6. Sharma, R. K., and Kalra, J. : *Biochemistry* **32**, 4975-4978 (1993).
7. Tian, Z. G., and Yang, G. Z. : *Chung Kuo Yao Li Hsueh Pao* **14**, 159-161 (1993).
8. Lee, Y. N., Lee, H. Y., Chung, H. Y., Kim, S. I., Lee, S. K., Park, B. C., and Kim, K. W. : *European J. Cancer.* **32**, 1420-1428 (1996).
9. Evans, R. M. : *Science* **240**, 889-895 (1988).
10. Beato, M. : *Cell.* **56**, 335-344 (1989).
11. Moore, D. D. : *New Biol.* **2**, 100-105 (1990).
12. Miller, J., McLachlan, A. D., and Klug, A. : *EMBO J.* **4**, 1609-1614 (1985).
13. Evans, R. M., and Hollenberg, S. M. : *Cell* **52**, 1-3 (1988).
14. Becker-Andre, M., Andre, E., and DeLaarter, J. F. : *Biophys. Res. Commun.* **194**, 1371-1379 (1993).
15. Sanger, F., Niklen, S., and Coulson, A. R. : *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467 (1977).
16. Chomczynski, P., and Sacchi, N. : *Anal. Biochem.* **162**, 156-159 (1987).
17. Feinberg, A. P., and Vogelstein, B. A. : *Anal. Biochem.* **137**, 255-267 (1984).
18. Tsukiyama, T., Niwa O., and Yokoro, K. : *Mol. Cell. Biol.* **9**, 4670-4676 (1989).
19. Tsukiyama, T., Ueda, H., Hirose, S., and Niwa O. : *Mol. Cell. Biol.* **12**, 1286-1291 (1992).
20. Milbrandt, J. : *Neuron* **1**, 183-188 (1988).
21. Ryseck, R. P., MacDonald Bravo, H., Mattei, M. G., Ruppert, S., and Bravo, R. : *EMBO J.* **8**, 3327-3335 (1989).
22. Davis, I. J., Hazel T. G., and Lau. L. F. : *Mol. Endocrinol.* **5**, 854-859 (1991).
23. Wilson, T. E., Fahrner, T. J., Johnston, M., and Milbrandt J. : *Science* **252**, 1296-1300 (1991).
24. Stanley, M. H., Cary, W., Estelita, S. O., Gail, C., Anthony, O., Roger, L., Thompson, E. B., Michael, G. R., and Ronald, M. E. : *Nature.* **318**, 635-641 (1985).
25. Laudet, V., Hanni, C., Coll, J., Catzeflis, F., and Stehelin, D. : *EMBO J.* **11**, 1003-1013 (1992).
26. Issemann, I., and Green, S. : *Nature* **347**, 645-650 (1990).
27. Keller, H., Dreyer, C., Medin J., Mahfoudi, A., Ozato, K., and Wahli, W. : *Proc. Natl.. Acad. Sci. USA* **90**, 2160-2164 (1993).