Journal of Korean Medical Science
Vol. 10, No. 3, 155-163 June 1995

Effect of Growth Factors on the Expression of Proto-Oncogenes *c-fos* and *c-myc* in FRTL-5 Cell Line

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This study was performed to prove the hypothesis that oncogene expressions would have the same patterns with those of cellular growth to growth factors in FRTL-5 cells.
Ribonucleic acids of FRTL-5 were extracted at 15′, 30′, 60′ and 120′ after administration of growth factors to quiescent FRTL-5, and blotted to the nitrocellulose membrane. They were hybridized with radiolabelled c-fos, c-myc and β-actin probes. Hybridized dot blots were autoradiographed and the amount of radioactivity was measured by densitometry. Densitometric readings were used as the indices of oncogene expressions.
Expressions of c-fos and c-myc were more prominent in combined administrations of TSH (10 mU/ml) and IGF-I (100 ng/ml) or IgG of Graves′ disease (Graves′ IgG ; 1 mg/ml) and IGF-I than in combined administration of TSH and Graves′ IgG. IgG of primary myxedema suppressed oncogene expressions by TSH or Graves′ IgG, but not by IGF-I.
From the above results, it was suggested that expressions of c-fos and c-myc to growth factors would have similar patterns with those of cell growth to growth factors in FRTL-5, and the actions of TSH and Graves′ IgG would be manifested through same signal transduction system, but IGF-I would be manifested by its own.

Key Words : *Growth factors, c-fos, c-myc, Signal transduction system*

INTRODUCTION

The FRTL-5 cell strain(ATCC CRL # 8305) is a cloned normal thyroid cell of Fischer rat, which maintain the functional characteristics of iodide uptake, thyroglobulin synthesis, cyclic nucleotide metabolism over prolonged periods of culture, rapid growth with doubling time of approximately 36 hours, survival for prolonged periods in the absence of thyrotropin(thyroid stimulating hormone or TSH) and sensitivity to stimulators or inhibitors of thyroid function after withdrawal of TSH for long periods(Ambesi-Impiombato et al., 1989). FRTL-5 cells possess TSH-responsive adenylate cyclase and absolutely need TSH for their growth. These characteristics make the FRTL-5 strain of normal thyroid cells ideally suited to assays or analyses measuring thymidine incorporation, cAMP elevation and iodide uptake, to determine and quan-

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This study was supported by a 1990 research grant from Sammi Culture Foundation.

tify thyroid stimulatory and inhibitory factors.

It is well known that the responses of FRTL-5 cells to various growth factors are made through growth factor-receptor complex, followed by intracellular signal transduction pathways(Jin et al., 1986 ; Grollman et al., 1987 ; Marcocci et al., 1987a ; Brenner-Gati et al., 1988). TSH, which is the main growth factor of FRTL-5 cell, combines with its receptors on cell membrane. The TSH-receptor complex activates both the cAMP-dependent intracellular signal transduction system(adenylate cyclase system) and the cAMP-independent system(phosphoinositide turnover-protein kinase C-calcium system). Besides, there is a separate specific receptor for IGF-I(or somatostatin C) which activates the tyrosine kinase system. It has been known that each of these signal transduction systems takes an effect on cell growth.

The interactions between different signal transduction system were suggested by the fact that TSH and insulin(or IGF-I) have a synergistic effect on cell growth(Tramontano et al., 1988 ; Takahashi et al., 1991), or by the report on receptor cross-talk(Hidaka et al., 1993 ; Chung et al., 1993). In 1989, Cho et al performed an experiment to evaluate the effect of interactions of various growth factors and TSH receptor antibodies on the growth of cultured rat thyroid cells(FRTL-5 cells) and to verify their mechanisms of action. They measured ^{3}H-thymidine incorporation into FRTL-5 cells with various combinations of TSH, serum immunoglobulin G from patients with Graves' disease(Graves' IgG) and other growth stimulators or inhibitors. In this experiment, they postulated that there were at least two different pathways for the action of thyroid cell growth factors, one of which was the adenylate cylcase-cAMP pathway, and the other, the cAMP-independent pathway, and Graves' IgG or serum immunoglobulin G from patients with primary myxedema(myxedema IgG) had their effects of stimulation or inhibition on growth through the adenylate cyclase-cAMP pathway.

It has been reported that when growth factors were administered to the quiescent cells, expression of proto-oncogenes such as *c-fos* and *c-myc* increased early and immediately, in contrast to the increase in DNA synthesis which was the index for the cell growth and took several hours to days(Muller et al., 1984 ; Almendral et al., 1988). The function of *c-fos* and *c-myc* proto-oncogenes has resisted analysis. Recent results, however, have demonstrated that the protein products of these oncogenes could augment the transcription of other genes by binding to the genome, either directly to DNA or to DNA-associated proteins(Donner et al., 1982 ; Kelly et al., 1983 ; Muller et al., 1984 ; Zullo et al., 1985 ; Setoyama et al., 1986 ; Distal et al., 1987 ; Franza et al., 1988). Originally, oncogenes were considered to be "cancer"-causing genes with little inherent biological value. However, evidence has now occurred strongly suggesting that expression of cellular oncogenes in an orderly fashion is necessary for normal cell growth and differentiation(Newmark, 1987). When a cellular or viral oncogene is activated, the DNA message is transcribed into a protein product. The known activities of these translational products include protein kinase, guanosine triphosphate binding, DNA binding, and growth factor and growth-factor-receptor activities.

With these bases, we set the hypothesis that oncogene expressions would have the same stimulatory or inhibitory patterns with those of cellular growth to growth factors in FRTL-5 cell line. To prove this hypothesis, we observed the effect of growth factors and their interactions on the expression of *c-fos* and *c-myc* proto-oncogenes in cultured normal rat thyroid cell line, FRTL-5, by growth factors and combinations of them.

MATERIALS AND METHODS

Materials

FRTL-5 cell line was supplied by Dr. Kohn of the National Institute for Health in the U.S.A., and cultured as described by Ambesi-Impiombato et al.(1980).

TSH, IGF-I, Graves' IgG were used as the growth stimulatory factors and myxedema IgG was used as the growth inhibitory factors. Graves' IgG was extracted from Graves' disease patients with positive thyrotropin binding inhibitory immunoglobulin(TBII) activity, and myxedema IgG was extracted from primary myxedema patients with positive thyroid stimulating blocking antibody(TSBAb) activity. TSH was purchased from Sigma Chemical Company(Missouri, USA) and IGF-I was purchased from Boehringer Mannheim GmbH Biochemica(Mannheim, Germany).

The probes for *c-fos* and *c-myc* proto-oncogenes were purchased as 40-Mer oligonucleotides(Oncogene Science, Inc., Uniondale, NY, USA), and the probe for control *β-actin* was purchased as 2.0 kilobase-pair cDNA inserted into HFBCA46(American

Type-Culture Collection, Rockville, Maryland, USA). It was separated by restriction endonuclease Bam H1.

Methods

1) Culture of FRTL-5 cells

FRTL-5 cells were cultured in 6H media(Coons modified Ham F12 media containing 5% calf serum, TSH 10 mU/ml, bovine insulin 10 μg/ml, transferrin 5 μg/ml, hydrocortisone 10 nM, somatostatin 10 ng/ml, glycyl-L-hystidyl-L-lysine acetate 10 ng/ml), and subcultured with the ratio of 5-10 : 1 every 2 weeks. When the cells were confluent in the culture bottle, they were distracted from the bottom with Ca^{++} and Mg^{++} free Hank's balanced salt solution(HBSS) which contained 0.05% EDTA, 0.5 mg/ml trypsin(1 : 300), 20 U/ml collagenase and 2% chicken serum. After adding 6H media, they were centrifuged at 1000 rpm. The cells were suspended in 6H media and seeded on 100 mm plate. They were grown and refed every 3 or 4 days. After growing for 7 days, the 5H media(6H media without TSH) was added, and refed after 3 to 4 days. At this point when FRTL-5 cells were in quiescent status, various growth factors were given.

2) Administration of growth factors in quiescent FRTL-5 cells

In quiescent FRTL-5 cells, culture media was changed with 5H media, containing 0.01 mU/ml, 0.1 mU/ml, 1 mU/ml or 10 mU/ml of TSH, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml or 10 mg/ml of Graves' IgG or 1 ng/ml, 10 ng/ml or 100 ng/ml of IGF-I, respectively. Then RNA was extracted at 15, 30, 60 and 120 minutes after administration of growth factors, by acid phenol method. After getting the concentrations of each growth factor maximizing the enhancement of *c-fos* and *c-myc* proto-oncogene expressions(TSH 10 mU/ml, Graves' IgG 1 mg/ml and IGF-I 100 ng/ml), 5H containing TSH and IGF-I, TSH and Graves' IgG, IGF-I and Graves' IgG, TSH and myxedema IgG, Graves' IgG and myxedema IgG, TSH and IGF-I and myxedema IgG, or IGF-I and myxedema IgG, respectively, were administered to quiescent FRTL-5 cells. Then total RNA was extracted by the same method.

3) RNA extraction from cultured FRTL-5 cells

After irrigation of the culture dish with HBSS, guanidine thiocyanate was applied. When the cells were melted completely except for ribonucleic acids, they were transferred to tubes and 1.8 ml of 2M sodium acetate and 0.8 ml of phenol, 0.36 ml of CIA(49 : 1) were applied. After centrifuge, the supernatant were separated and 1.8 ml of 100% isopropanol were applied. It had been kept at −70℃ for over one hour. Then the precipitates by re-centrifuge were melted by applying 0.3 ml of guanidine thiocyanate, and after addition of 0.3 ml of 100% isopropanol, were kept in −70℃ for over 1 hour. RNA could be obtained by taking the precipitates after centrifuge at 1,500 rpm for 10 minutes. Concentration of extracted RNA was determined by measuring the optical density of RNA which was melted in distilled water, and purity was checked by comparing the O.D. at 260 nm with that at 280 nm. RNA was kept at −70℃ till use.

4) Isotope labelling of oncogene probes

40-Mer oligonucleotide probes for proto-oncogenes *c-fos* and *c-myc* were labelled by end-labelling method. A mixture of 0.2 μg of oligonucleotide probe, 1 μl of 10X buffer, 5 μl of [$\gamma^{32}P$] dATP, 1 μl of T_4 ligase and 1 μl of distilled water was incubated for 30 minutes at 37℃, and the enzyme reaction was stopped by adding 40 μl of 1 mM EDTA.

The labelling of *β-actin* cDNA of 2.0 kilobase-pair was done by nick translation method. 0.1 μg of *β-actin* was mixed with 3 μl solution each containing 1 μl of dATP, dGTP and dTTp, 2 μl of nick translation buffer, 2 μl of [$\alpha^{32}P$]dATP(3,000 Ci/mmol), autoclaved distilled water and 2 μl of a mixture of DNAse and DNA polymerase I. It was incubated for 35 minutes at 15℃ and the reaction was stopped by adding 2 μl of EDTA.

The radiolabelled probes for *c-fos*, *c-myc* and *β-actin* were refined by Sephadex G-50 spun column chromatography.

5) Dot blotting of extracted RNA and hybridization with radiolabelled oncogene probes

3 μg of each RNA was taken and mixed with 20 μl of 100% formamide, 7 μl of 37% formaldehyde and 2 μl of 20X SSC, and then incubated at 55℃ for 15 minutes. After application of suction on the dot blot apparatus to produce negative pressure inside of it, the RNA mixture was put into each dot well to be transferred onto nitrocellulose membrane. Each extracted RNA was applied to three dot wells to triplicate. The nitrocellulose membrane over which the RNA was transferred was dried up at room temperature and baked in a vacuum at 80℃ for 2 hours.

For hybridization, nitrocellulose membrane was incubated at 42℃ for one hour in prehybridization solution, which contained 50% formamide, 5X SSC, 2X Denhardt's solution, 0.5% SDS and denatured salmon sperm DNA 100 μg/ml. Then, radiolabelled oncogene probe was added to the solution, and hybridized at 42℃ for 18 hours. After hybridization, nitrocellulose membrane was washed with serially diluted mixture of SSC and SDS. Then the nitrocellulose membrane was wrapped with vinyl and autoradiographed in a cassette containing X-ray film. After overnight autoradiography, the X-ray film was developed. The measurements of the densities with a densitometer were used as the indices of mRNA expression of *c-fos*, *c-myc* and *β-actin*. The densitometric readings were subtraction of background density from densities of autoradiographed dot blots, which were described as arbitrary unit.

RESULTS

1. Expression of *c-fos* and *c-myc* in quiescent FRTL-5 cells

Expression of *c-fos* and *c-myc* in quiescent FRTL-5 cells was measured to find the baseline expression of *c-fos* and *c-myc*. There was nearly nill expression of *c-fos* and *c-myc*, so that the baseline expression of *c-fos* and *c-myc* was set to density of 0.

2. Expression of *c-fos* and *c-myc* with time sequence by growth factors

After administration of growth factors, the expression of *c-fos* reached its maximum within 30 minutes and decreased gradually with time. But the expression of *c-myc* reached its maximum in about 60 minutes, and showed a decreasing trend after then (Fig. 1~5).

3. Expression of proto-oncogenes *c-fos* and *c-myc* by the different concentrations of TSH, IGF-I and Graves' IgG

1) Expression by different concentrations of TSH

Expression of *c-myc* increased gradually, as the concentration of TSH increased gradually(0.01 mU/ml, 0.1 mU/ml, 1 mU/ml and 10 mU/ml). The expression of proto-oncogenes *c-fos* and *c-myc* by TSH were more prominent than by those of IGF-I or Graves' IgG(Fig. 1).

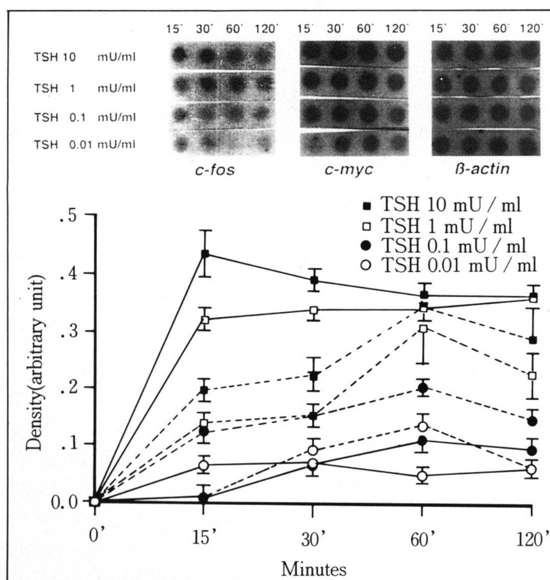

Fig. 1. Changes(mean±SEM) of *c-fos* and *c-myc* expression with time sequence by different TSH concentrations. Densities are densitometric readings of autoradiographed X-ray film from dot blots hybridized with radiolabelled probes. Solid lines for *c-fos*; dotted lines for *c-myc*.

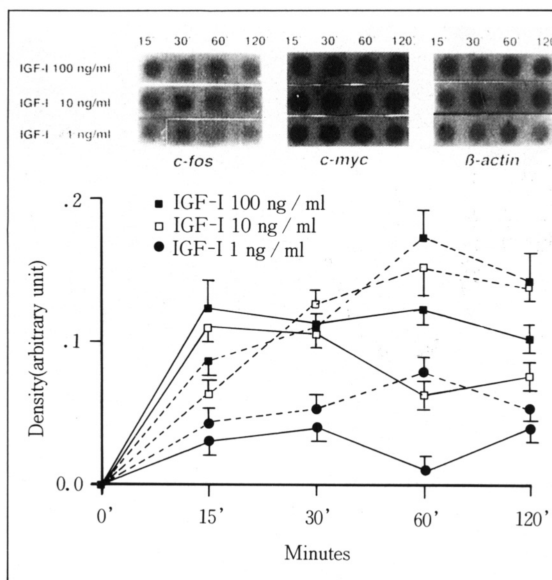

Fig. 2. Changes(mean±SEM) of *c-fos* and *c-myc* expression with time sequence by different IGF-I concentrations. Densities are densitometric readings of autoradiographed X-ray film from dot blots hybridized with radiolabelled probes. Solid lines for *c-fos*; dotted lines for *c-myc*.

2) Expression by different concentrations of IGF-I

The expression of *c-fos* increased gradually as the concentration of IGF-I increased(1 ng/ml, 10 ng/ml, 100 ng/ml). The expression of *c-myc* was more prominent at the concentrations of IGF-I of 10 ng/ml and 100 ng/ml than at 1 ng/ml, but there was no difference between the expressions of *c-myc* at the two concentrations(Fig. 2).

3) Expression by different concentrations of Graves' IgG

The expression of *c-myc* increased, as the concentration of Graves' IgG gradually increased(0.01 mg/ml, 0.1 mg/ml, 1 mg/ml), but it decreased slightly, as the concentration of Graves' IgG increased to 10 mg/ml(Fig. 3).

4. The expression of proto-oncogenes *c-fos* and *c-myc* by the interaction of TSH, IGF-I and Graves' IgG

The expression of proto-oncogenes *c-fos* and *c-myc* was more prominent in cases of combined administration of TSH(10 mU/ml) and IGF-I(100 ng/

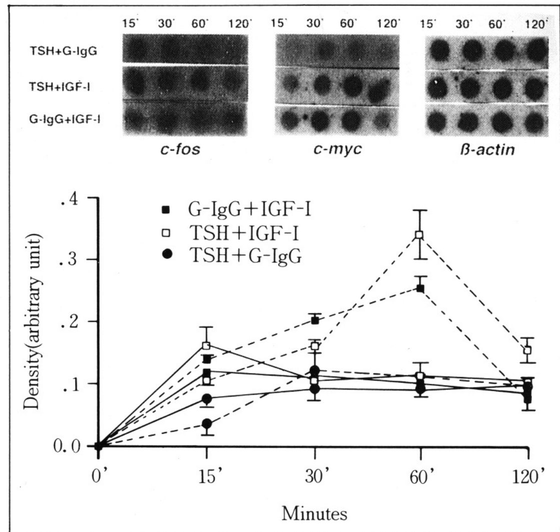

Fig. 4. Changes(mean±SEM) of *c-fos* and *c-myc* expression with time sequence by mixture of TSH(10 mU/ml), IGF-I(100 ng/ml) and IgG from patients with Graves' disease(1 mg/ml). Densities are densitometric readings of autoradiographed X-ray film from dot blots hybridized with radiolabelled probes. Solid lines for *c-fos*; dotted lines for *c-myc*.

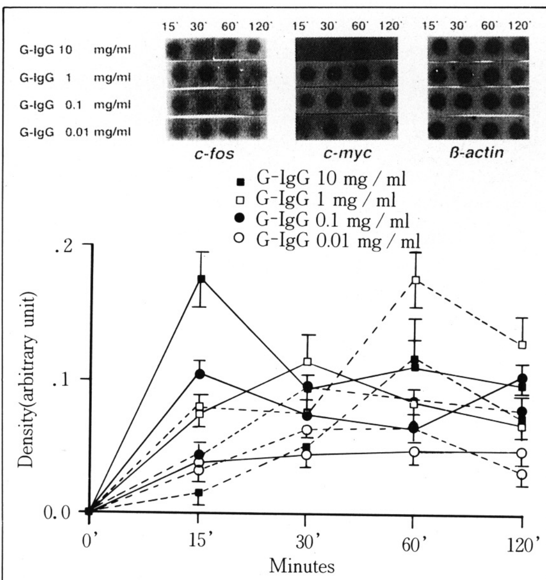

Fig. 3. Changes(mean±SEM) of *c-fos* and *c-myc* expression with time sequence by different concentrations of IgG from patients with Graves' disease. Densities are densitometric readings of autoradiographed X-ray film from dot blots hybridized with radiolabelled probes. Solid lines for *c-fos*; dotted lines for *c-myc*.

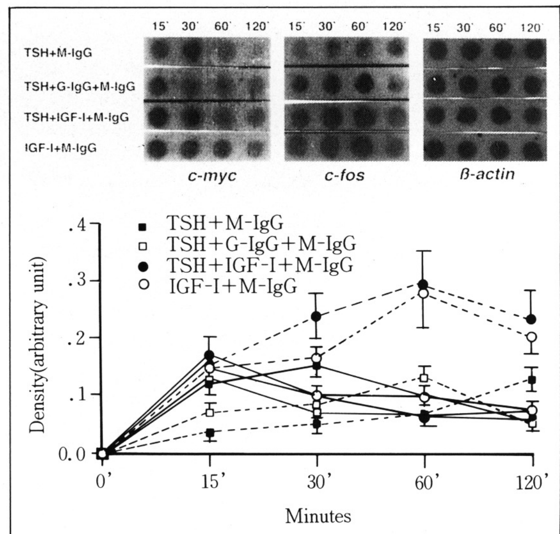

Fig. 5. Changes(mean±SEM) of *c-fos* and *c-myc* expression with time sequence by addition of IgG from patients with primary myxedema(1mg/ml) to the mixture of TSH(10 mU/ml), IGF-I(100ng/ml) and IgG from patients with Graves' disease(1 mg/ml). Densities are densitometric readings of autoradiographed X-ray film from dot blots hybridized with radiolabelled probes. Solid lines for *c-fos*; dotted lines for *c-myc*.

ml), and Graves' IgG(1 mg/ml) and IGF-I than in case of TSH and Graves' IgG(Fig. 4).

5. The expression of proto-oncogenes *c-fos* and *c-myc* by the interaction of growth factors such as TSH, IGF-I and Graves' IgG, and myxedema IgG

Myxedema IgG(1 mg/ml) suppressed the expression of proto-oncogene *c-myc* provoked by TSH(10 mU/ml) and Graves' IgG(1 mg/ml), but not by IGF-I(100 ng/ml) or combined administration of TSH and IGF-I. The expression of proto-oncogene *c-fos* has shown similar patterns, although not so prominent as that of *c-myc*(Fig. 5).

DISCUSSION

Cell growth regulation appears to be mediated not only by classic endocrine mechanisms in which cell function is controlled by hormones derived from a distant source, but also by cellular factors derived from the same cells(Melmed, 1988). These cellular factors act as growth factors or growth inhibitory factors, and recently the signal transduction system by which these cellular factors acted in normal cells have been slowly uncovered. After it was suggested that the signal transduction system was involved in normal cellular growth and differentiation, many efforts have been made to prove the hypothesis that the abnormalities in this system would induce cancer.

The synthesis of new mRNA is an absolute requirement for quiescent cells to respond to mitogenic factors indicating that transcriptional activation of genes is part of the cellular reaction. The genes induced by growth factors have been pursued by looking for changes in the levels of mRNA of a known gene after stimulation of quiescent cells with a purified growth factor or serum. This strategy quickly led to the identification of *c-fos* and *c-myc* targets for a variety of growth factors such as PDGF (platelet derived growth factor), EGF(epidermal growth factor), serum and NGF(nerve growth factor) (Kelly et al., 1983 ; Cochran et al., 1984 ; Greenberg and Ziff, 1984 ; Kruijer et al., 1983, 1984).

Oncogenes exist as part of normal mammalian cells as well as retroviruses. These cellular genes are called cellular oncogenes or proto-oncogenes(Bishop, 1983 ; Melmed, 1988 ; Sibbitt Jr, 1988). These proto-oncogenes are known to be necessary for normal cell growth and differentiation(Bishop, 1983 ; Sibbitt Jr, 1988), and expressed rapidly by application of growth factors to quiescent cells(Dere et al., 1985 ; Colleta et al., 1986 ; Tramontano et al., 1986 ; Heldin & Westermark, 1988). The peptides encoded in the cellular oncogenes are thought to have potent regulatory activities involving membrane function, receptor activity, enzyme activation(Sibbitt Jr, 1988), and expression of genes in the chromosome(Hunter, 1984), or have the role of autocrine growth factors controlling cellular growth(Sporn & Todaro, 1980 ; Sporn & Roberts, 1985 ; Weinberg, 1985). Generally, the translational products of oncogenes are relatively small peptides that have direct or indirect effects on cell replication. The known activities of these tranlational products include protein kinase, guanosine triphosphate binding, DNA binding, and growth factors and growth-factor-receptor activities(Sibbitt Jr, 1988). A certain number of oncogene translational products have no known function. It has been suggested that when these oncogenes have changes in their functions or damages such as point mutation, translocation, loss of regulator sequence or amplication of certain sequences, malignant transformation of normal cells would occur(Slamon et al., 1984 ; Kelly, 1986 ; Bishop, 1987 ; Sibbitt Jr, 1988).

From the fact that TSH and cAMP or its analogues which mediate TSH action stimulates expression of proto-oncogenes *c-ras*, *c-myc* or *c-fos* before the cellular growth in quiescent FRTL-5 cells, increase in expression of oncogenes was thought to be an early and immediate response to growth factors(Dere et al., 1985 ; Colletta et al., 1986 ; Reuse et al., 1986 ; Tramontano et al., 1986). Colleta et al.(1986) and Ran et al.(1986) reported that administration of growth factors, which is mediated by cAMP for cell growth, into quiescent BALB/c 3T3(A31) fibroblast cell line or normal rat thyroid cell line, induced expression of *c-fos* in 5 minutes, and then it disappeared in two hours, but *c-myc* was expressed maximally in one hour and disappeared over a longer time duration. From the rapid and massive response of these proto-oncogenes to growth factors, they postulated that proto-oncogenes, such as *c-fos* or *c-myc*, would be related to normal cell growth. And also they suggested that TSH would not only have an effect on the thyroid hormone synthesis, but strongly stimulate proto-oncogene expression by the mediation of cAMP as a second messenger, and, in turn, these oncogene products stimulate cellular proliferation.

Our results have shown that growth factors stimu-

lated expression of proto-oncogenes *c-fos* and *c-myc* earlier and more immediately than that of DNA synthesis which was shown in previous reports. The expression of *c-fos* reached its maximum in 15 to 30 minutes after administration of growth factors, and there was no instance in which it reached its maximum in over 30 minutes. But expression of proto-oncogene *c-myc* reached its maximum in over 60 minutes, and gradually decreased thereafter, which was also more immediate and earlier than DNA synthesis after administration of growth factors that appeared in 24 to 48 hours.

But TSH was known to stimulate phosphatidylinositol 4,5-biphosphate(PIP_2) as well as cAMP signal transduction in rat FRTL-5 thyroid cells, from the fact that there was a single TSH receptor gene(Akamizu et al., 1990 ; Rousseau-Merck et al., 1990 ; Ikuyama et al., 1992), transfected TSH receptor cDNA confers both adenylate cyclase and PIP_2 responses(Van Sande et al., 1990 ; Kosugi et al., 1992 ; Chung et al., 1993), and mutation of alanine-623 in the TSH receptor eliminates the TSH-induced inositol phosphate, but not the cAMP, signal(Kosugi et al., 1992). In studies using FRTL-5 cell line, it has been previously described that regulation of iodide uptake, thyroid peroxidase, and thyroglobulin synthesis were made via the TSH-induced cAMP signal(Ekholm et al., 1989), and that TSH-induced PIP_2 cascade was involved in iodide efflux(Corda et al., 1985 ; Marcocci et al., 1987b ; Ekholm et al., 1989), hydrogen peroxide generation(Bjorkman and Ekholm, 1992), and iodination(Marcocci et al., 1987b).

Increase in ^{3}H-thymidine incorporation into FRTL-5 cells could be induced by cAMP elevation by activation of adenylate cyclase when TSH was administered. Increase in ^{3}H-thymidine incorporation by activation of adenylate cyclase could also be possible by cAMP and cAMP analogues, and Graves' IgG(Cho et al., 1989a ; Wilders-Truscnig et al., 1990). IGF-I also increased ^{3}H-thymidine incorporation when administered to quiescent FRTL-5 cells. Its action was synergistic with TSH, and as its action was not suppressed by myxedema IgG which blocked the action of cAMP-dependent growth factors, it was thought to act through a different signal transduction system from activation of adenylate cyclase, which has been reported to enhance the expression of *c-myc* and *c-fos*(Tramontano et al., 1986 ; Santisteban et al., 1987 ; Cho et al., 1989b). As our results have shown that myxedema IgG, which has been known to act on the same receptor for TSH, suppressed expression of proto-oncogenes *c-fos* and *c-myc* by Graves' IgG which also has known to act on the same receptor sites for TSH, but not the expression by IGF-I, it can be suggested that oncogene expression by growth factors has similar patterns with ^{3}H-thymidine incorporation which is an index of DNA synthesis, and thereby precedes cellular growth and proliferation.

This study is an experiment on the effect of growth factors and their interactions on the expression of *c-fos* and *c-myc* proto-oncogenes in cultured normal rat thyroid cell line(FRTL-5), and we hope that it will be of help in the search for the mechanism of action of the growth factors, cell growth and differentiation, and, in advance, the signal transduction system related to the carcinogenesis.

At this time, as the function of *c-fos* and *c-myc* is only known to be related to the cell growth and differentiation through binding to the intracellular DNA or DNA associated proteins, further study on this issue would be necessary to clarify the function of oncogenes in cancer cells as well as in normal cells.

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