Stimulation of c-myc protooncogene expression by transforming growth factor α in human ovarian cancer cells

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Abbreviations: TGF α , transforming growth factor α ; FBS, fetal bovine serum; OPT, phosphorothioate oligonucleotides

Abstract

To investigate whether transforming growth factor α (TGF α) treatment of human ovarian cancer cells was associated with the induction of c-myc protooncogene, the expression of this gene in NIH:OVCAR-3 cells was examined. TGF α induced increase in c-myc mRNA level, with a peak after 1 h of treatment; this stimulation was dose-dependent, with an optimal concentration of 5 ng/ml TGF α . Its primary action is probably at the transcription level since the half-life of c-myc mRNA measured in the presence of actinomycin D was not modified by TGF α treatment. In addition, TGF α stimulation of c-myc mRNA did not require protein synthesis since it was not suppressed by cycloheximide treatment. Antisense phosphorothioate oligonucleotide to c-myc specifically inhibited the TGF α -stimulated c-Myc protein expression and growth of NIH:OVCAR-3 cells. Our results indicate that induction of c-myc expression by TGF α plays an important role in the growth of NIH:OVCAR-3 cells.

Keywords: c-myc, ovarian cancer cells, TGF α

Introduction

Ovarian cancer is the most fatal gynecological malignancy, and although a great deal of effort has been devoted to it, prognosis has not been significantly improved. This is due in part to the occult nature of the malignancy as well as the fact that ovarian cancer's mechanism for growth regulation is as yet well known. Recent studies demonstrate that oncogenes, growth factors and their cognate receptors are involved in the genesis and proliferation of

neoplasms (Kommoss *et al.*, 1992; Berck and Martinez-Maza, 1994). The growth factors that may be involved in the pathogenesis of ovarian cancers, such as epidermal growth factor (EGF) or transforming growth factor α (TGF α), are being studied (Zhou and Leung, 1992), and a positive correlation between the TGF α and c-myc expression in ovarian cancer was reported (Bauknecht *et al.*, 1990). However, the relationship between these growth factors and cancer initiation, growth regulation of ovarian epithelial cells or growth of ovarian cancer cells remain largely unknown. TGF α , which has high structural homology to EGF, also binds to EGF receptor and exhibits EGF-like biological activities. That it functions as an auto-crine factor has been reported for a number of malignant cells in culture.

The expression of the c-myc protooncogene is closely correlated with cell proliferation and differentiation (Meichle *et al.*, 1992). An increase in the expression of c-myc gene by TGF α has been observed in a variety target tissues (Cutry *et al.*, 1989; Skouteris and McMenamin, 1992; Oliver *et al.*, 1995). Despite investigation in a variety of normal and neoplastic cells, the fundamental aspects of the kinetics and mechanisms underlying c-myc expression modulated by TGF α and the significance of its induction remain undetermined. The goal of this study was to investigate the role of c-myc expression and the mechanisms of the regulation by TGF α in ovarian cancer cells.

Materials and Methods

Cell culture

NIH:OVCAR-3 cells (ATCC, Rockville, MD) were grown as monolayer culture at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS).

For the experiments, cells were incubated for various periods in fresh medium containing 1% FBS and different concentrations of TGF α . In cycloheximide experiments, cells were treated with 10 μ g/ml cycloheximide and TGF α for 50 min. In c-myc mRNA stability analysis, cells were exposed to TGF α for 50 min, actinomycin D at a final concentration of 5 μ g/ml was then added and incubated for another 0-120 min. All cells were used within 10 passages from the original stock.

Cell growth rate

For plotting cell growth curves, cells were plated in 24-well tissue culture plates and incubated with 1% FBS in

the presence or absence of 5 ng/ml TGF α . After 2-8 days, the medium was removed, and the cells were detached with trypsin-EDTA. The viability of the cells was estimated by the trypan blue dye (0.4%) exclusion technique, and the number of cells was counted in a hemocytometer.

Extraction of RNA and Northern blot

Total RNA was extracted by using Ultraspec II (Biotecx) and quantified by absorption at 260 nm. For Northern blot studies, 20 μ g of total RNA was denatured in 50% formamide and fractionated in 1.0% agarose/formaldehyde gel. RNA was transferred to nylon membrane (Boehringer Mannheim) by a downward alkaline transfer method (Chomczynski, 1992). Membranes were hybridized in 0.25 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 20% SDS, 0.5% blocking solution (Boehringer mannheim) and digoxigenin (DIG)-labeled human *c-myc* probe at 68°C for 18 h. They were washed then three times in 20 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA and 10% SDS at 65°C. DIG-labeled hybridization signal was detected using DIG luminescent detection kit (Boehringer Mannheim) as manufacturer's protocol. Relative intensity of hybridization signals were quantified by laser densitometer (LKB). The human *c-myc* probe was obtained from ATCC, and a 1.4 kb *Clal/EcoRI* fragment, containing third exon of the human *c-myc* gene, was used and labeled with DIG-11-dUTP by using random primed DNA labeling kit (Boehringer Mannheim). Cyclophilin was used as a control.

Phosphorothioate oligonucleotides (OPTs)

A 15-mer antisense OPT (5'-AACGTTGAAGGGCAT-3') complementary to the translation initiation region of *c-myc* mRNA was used. As a control, a sense OPT (5'-ATGCC CCTCAACGTT-3') were used in different experiments. OPTs were synthesized, purified and dried under vacuum by Bioneer, Inc., Korea. OPTs were resuspended in sterile PBS.

Western blot analysis of c-Myc protein

After a 4-day treatment with 5 μ M antisense *c-myc* or sense *c-myc* OPT, NIH:OVCAR-3 cells were stimulated with TGF α (5 ng/ml) for an additional 2 h, and then lysed directly in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2 mM phenylmethylsulfonylfluoride and 1 μ g/ml aprotinin). The samples were loaded onto 10% SDS-polyacrylamide gel, electrophoresed, and then transferred to a PVDF membrane (Amersham, UK). The membrane was incubated with anti-human c-Myc monoclonal anti-body (Oncoscience, Cambridge, MA) and then with horseradish peroxidase-conjugated goat anti-mouse antibody (Transduction Lab., Lexington, KY). Peroxidase activity was then detected following incubation with 4-chloro-1-naphthol, diaminobenzidine and hydrogen peroxide.

Effect of antisense c-myc DNA on cell growth

For the analysis of the effect on cell growth of antisense-*myc* OPT, cells were plated in 96-well plates. After pre-treated with serum-free medium for 48 h, cells were incubated in the fresh medium containing 1% FBS, 5 ng/ml TGF α , and 5 μ M of antisense-*myc* or sense-*myc* OPT for 4 days. Cell growth was assessed by XTT assay. At the end of incubation, cells were incubated with the XTT labeling mixture (Boehringer Mannheim) for 4 h and then the formazan dye formed is quantitated using an ELISA reader (Molecular Devices). Preliminary experiments were performed to establish that the absorption characteristics for NIH:OVCAR-3 cells would allow reproducible quantification of viable cell numbers within the range of cell density used in the proliferation experiments and that TGF α stimulation has no primary effect on the tetrazolium-reducing activity of NIH:OVCAR-3 cells.

Results

Effect of TGF α on cell growth

Cell growth was assessed by cell counting. As shown in Figure 1, the addition of 5 ng/ml TGF α caused a 1.8-fold increase in the growth of cells, compared to control cells.

c-myc mRNA induction by TGF α

Cells were treated with 5 ng/ml TGF α . *c-myc* mRNA levels were measured by Northern blotting at various times for up to 24 h. TGF α stimulated *c-myc* mRNA level with a peak (4.0 ± 0.5 fold) at 1 h after treatment (Figure 2). Cells were also treated for 1 h with various concentrations of TGF α and *c-myc* mRNA analyzed by Northern blot. The

Figure 1. Effect of TGF α on the growth of ovarian cancer NIH:OVCAR-3 cells. Cells were incubated in fresh 1% FBS media in the presence or absence of 5 ng/ml TGF α . The cells were harvested and cell number counted on the days indicated. Results are expressed as the mean values $\pm$ SD of three different experiments.

Figure 2. Time course of TGF α -stimulated *c-myc* mRNA expression. Cells were treated with 1% FBS media containing 5 ng/ml TGF α for the time designated. At the end of treatment, RNA were extracted and subjected to Northern blot analysis. *Upper panel*, Chemiluminescent hybridization signals of Northern blot. Cyclophilin mRNA in the samples was measured as a control. *Lower panel*, Quantification of the *c-myc* mRNA levels are normalized on the basis of the level observed before adding TGF α , considered as 1. Results are expressed as the mean values $\pm$ SD of four different experiments.

stimulation of *c-myc* was TGF α dose-dependent and the highest level being reached with the dose of 5 ng/ml TGF α (Figure 3).

Effect of cycloheximide on induction of *c-myc* mRNA by TGF α

Modulation of the levels of mRNA species by a particular agent can occur in the absence of protein synthesis if this is a primary response to the agent. Therefore, the effect of the protein synthesis inhibitor, cycloheximide, on the stimulation of *c-myc* by TGF α was tested to determine whether new protein synthesis is required. Cells were treated with vehicle control, 5 ng/ml TGF α , or 10 μ g/ml cycloheximide alone or with 5 ng/ml TGF α plus 10 μ g/ml cycloheximide. Total cellular RNA was isolated after 50 min of treatment and subjected to Northern blot. When cycloheximide was added together with TGF α , the transcript level increased $490 \pm 24\%$ of control (Figure 4). This increase was higher than that observed with TGF α alone. Therefore, cycloheximide does not prevent TGF α from stimulating *c-myc* expression. These results suggest additive effects since cycloheximide alone induced a 90% increase in the *c-myc* mRNA level. This cycloheximide action has been observed in other model systems and is primarily due to prolongation of mRNA half-life resulting from the inactivation of a labile regulatory protein, known to destabilize *c-myc* mRNA (Kelly *et al.*, 1983; Miano *et*

Figure 3. Dose response of TGF α -stimulated *c-myc* mRNA expression. Cells were treated with 1% FBS and various concentrations of TGF α and RNA was extracted 1 h later for Northern blot analysis. Quantification of the *c-myc* mRNA levels are normalized on the basis of the level observed before adding TGF α , considered as 1. Results are expressed as the mean values $\pm$ SD of three different experiments.

Figure 4. Effect of cycloheximide on *c-myc* mRNA expression. Cells were treated with control vehicle, TGF α (5 ng/ml), cycloheximide (10 μ g/ml), or combination of TGF α and cycloheximide for 50 min. RNA was extracted and subjected to Northern blot analysis. Quantification of the *c-myc* mRNA levels are normalized on the basis of the level observed in control vehicle only, considered as 1. Results are expressed as the mean values $\pm$ SD of three different experiments. C, control vehicle (1% FBS only); T, TGF α ; CHX, cycloheximide.

al., 1993; Yokota *et al.*, 1995). These results show TGF α increased *c-myc* mRNA level in NIH:OVCAR-3 cells to the same extent with or without cycloheximide, suggesting that new protein synthesis was not required for this stimulation.

c-myc mRNA stability analysis

In order to verify TGF α has a stabilizing action on *c-myc* mRNA, the half-life of this transcript was studied in the presence or absence of TGF α . Cells were exposed to 5 ng/ml TGF α for 50 min and then actinomycin D at a concentration of 5 μ g/ml was added. The degradation of existing *c-myc* mRNA was monitored at various time

Figure 5. Stability of *c-myc* mRNA. Cells were cultured in the presence or absence of 5 ng/ml TGF α for 50 min. Cells were then treated with 5 μ g/ml of actinomycin D for the time indicated. RNA was extracted and subjected to Northern blot analysis. Results are expressed as the mean values $\pm$ SD of three different experiments.

intervals for a period of 120 min. The half-life of *c-myc* mRNA of NIH:OVCAR-3 cells was estimated to be 64 ± 5 min in the presence or absence of TGF α (Figure 5). The results suggest that TGF α has no effect on the *c-myc* mRNA stability in NIH:OVCAR-3 cells.

Effect of antisense *c-myc* oligonucleotide on cell growth

In order to study the effect of antisense inhibition of *c-myc*, *c-Myc* protein expression and cell growth of NIH:OVCAR-3 cells were assessed by Western blot and XTT assay, respectively. When cells were incubated in medium containing 1% FBS, 5 ng/ml TGF α , and 5 μ M antisense-*myc* or sense-*myc* OPT for 4 days, antisense-*myc* OPT inhibited TGF α -stimulated *c-Myc* protein expression and cell growth. The sense-*myc* OPT had a negligible effect on *c-Myc* protein expression and cell growth at the same concentration. These findings suggest that *c-Myc* protein plays important roles in the mechanism of TGF α -induced cell growth (Figure 6 and 7).

Discussion

In the present investigation, treatment of NIH:OVCAR-3 cells with 5 ng/ml TGF α resulted in a 1.8-fold increase in cell growth, suggesting a fully functional EGF receptor system. *c-myc* protooncogene is involved in regulating cell proliferation (Kelly *et al.*, 1983; Kaczmarek *et al.*, 1985; Studzinski *et al.*, 1986), and seems to be one of the key genes required for a cell to progress through the cell cycle (Shichiri *et al.*, 1993). Its expression is activated by several mitogenic factors including TGF α . In this study, we have studied the mRNA expression of *c-myc* in human ovarian cancer cells as an indicator of possible protooncogene mediation of the signals transmitted by

Figure 6. Effect of antisense *c-myc* OPT on *c-Myc* protein expression. Cells were incubated in fresh medium containing 1% FBS and 5 ng/ml TGF α in the presence or absence of 5 μ M OPTs. *c-Myc* protein expression was assessed by Western blot analysis. C, 1% FBS + TGF α , no OPT; AS, antisense *c-myc* OPT-treated; S, sense *c-myc* OPT-treated.

Figure 7. Effect of antisense-*myc* OPT on cell growth. Cells were incubated in fresh medium containing 1% FBS and 5 ng/ml TGF α in the presence or absence of 5 μ M OPTs. Cell growth was assessed by XTT assay. Results are expressed as the mean values $\pm$ SD of four wells of three independent experiments. F, 1% FBS, unstimulated; C, 1% FBS + TGF α , no OPT; AS, antisense *c-myc* OPT-treated; S, sense *c-myc* OPT-treated.

TGF α .

We have found that TGF α increased the *c-myc* mRNA expression and the proliferation of human ovarian cancer NIH:OVCAR-3 cells. Moreover, antisense *c-myc* oligonucleotide treatment resulted in the inhibition of TGF α -stimulated *c-Myc* protein expression and cell proliferation. These findings suggest that *c-Myc* protein plays important roles in the mechanism of TGF α -induced cell growth. As a cell cycle regulator (Studzinski *et al.*, 1986), the *c-Myc* protein has been shown to be a target of growth factor (Luscher *et al.*, 1989); it can also act as a transcription factor (Schweinfest *et al.*, 1988; Penn *et al.*, 1990). In this sense, the *c-Myc* protein may play a role in regulating the expression of certain growth-related genes in TGF α -stimulated cells.

Detailed time-course studies showed that TGF α treatment was apparently associated with biphasic induction of *c-myc* mRNA. The biphasic pattern of *c-myc* mRNA stimulation by TGF α can be due to a combination of several regulatory mechanisms that are known to modul-

ate the expression of this protooncogene, both at the transcriptional and post-transcriptional levels (Spencer and Groudine, 1991; Marcu *et al.*, 1992). A negative autoregulatory loop may contribute such a combination (Grignani *et al.*, 1990).

This stimulation of *c-myc* mRNA expression by TGF α does not require protein synthesis, since it is not inhibited by cycloheximide. The half-life of *c-myc* mRNA was not modified by TGF α . It therefore seems that the TGF α action is not due to a stabilization of *c-myc* mRNA. The stability of RNA was known to be regulated by polyadenylated RNA species (Swartwout and Kinniburgh, 1989), or by A+U rich element RNA binding factor (Brewer, 1991); apparently it is not altered by TGF α as demonstrated in this study.

The signal transduction pathway of TGF α in stimulation of *c-myc* expression have been examined in other tissues. In rat hepatocyte cultures, TGF α increased *c-myc* expression and this effect was overcome by the presence of an antibody against TGF α in the hepatocyte culture medium (Skouteris and McMenamin, 1992). A clear link emerged between *c-myc* activation and coupling of TGF α with its receptor. Furthermore, indomethacin inhibited the TGF α -induced hepatocyte DNA synthesis, prostaglandin production and at the same time TGF α -induced *c-myc* expression. Prostaglandins added to hepatocyte cultures supplemented with indomethacin and TGF α restored the ability of TGF α to stimulate DNA synthesis and to increase *c-myc* expression. Thus prostaglandins mediate the transduction of TGF α -generated signals. These data suggest that TGF α induces hepatocyte proliferation and *c-myc* expression either through coupling with its receptor or through activation of arachidonic acid metabolism (Skouteris and McMenamin, 1992). On the other hand, in rat small intestinal epithelial cells (IEC-6 cells) TGF α stimulation of IEC-6 cells increases the activities of 42- and 44-kDa isoforms of mitogen-activated protein kinase (MAPK). And the increase in activities of these enzymes leads to an increase in the synthesis of c-Fos and c-Myc protein levels as well as mRNA levels, suggesting a link to nuclear events (Oliver *et al.*, 1995).

In conclusion, *c-myc* protooncogene is expressed and stimulated by TGF α in NIH:OVCAR-3 ovarian cancer cells. In these cells, therefore, the growth-promoting effect of TGF α may, at least partly, be mediated through *c-myc* stimulation. As a consequence, any abnormality in this process could result in cell growth dysregulation. Further studies are underway to understand the exact mechanisms of TGF α action, at the transcriptional and/or posttranscriptional levels.

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