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

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

Abstract

To investigate whether transforming growth factor α (TGFα) 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. TGFα 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 TGFα. 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 TGFα treatment. In addition, TGFα 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 TGFα-stimulated c-Myc protein expression and growth of NIH:OVCAR-3 cells. Our results indicate that induction of c-myc expression by TGFα plays an important role in the growth of NIH:OVCAR-3 cells.

Keywords: c-myc, ovarian cancer cells, TGFα

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 (TGFα), are being studied (Zhou and Leung, 1992), and a positive correlation between the TGFα 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. TGFα, which has high structural homology to EGF, also binds to EGF receptor and exhibits EGF-like biological activities. That it functions as an autocrine 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 TGFα 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 TGFα 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 TGFα 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 μg/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 TGFα. In cycloheximide experiments, cells were treated with 10 μg/ml cycloheximide and TGFα for 50 min. In c-myc mRNA stability analysis, cells were exposed to TGFα for 50 min, actinomycin D at a final concentration of 5 μg/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 TGFα. 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 μg 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 ClaI/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’-ATGCCCTCATTCC-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 μM antisense c-myc or sense c-myc OPT, NIH:OVCAR-3 cells were stimulated with TGFα (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 μg/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-naphtol, diaminobenzidine and hydrogen peroxide.

**Effect of antisense c-myc DNA on cell growth**
For the analysis of the effect on cell growth of antisense-c-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 TGFα, and 5 μM of antisense-c-myc or sense-c-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 TGFα stimulation has no primary effect on the tetrazolium-reducing activity of NIH:OVCAR-3 cells.

**Results**

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

**c-myc mRNA induction by TGFα**
Cells were treated with 5 ng/ml TGFα. c-myc mRNA levels were measured by Northern blotting at various times for up to 24 h. TGFα 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 TGFα and c-myc mRNA analyzed by Northern blot. The

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**Figure 1.** Effect of TGFα 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 TGFα. The cells were harvested and cell number counted on the days indicated. Results are expressed as the mean values ± SD of three different experiments.
Stimulation of c-myc expression by TGFα

The stimulation of c-myc expression by TGFα was dose-dependent and the highest level was reached with the dose of 5 ng/ml TGFα (Figure 3).

Effect of cycloheximide on induction of c-myc mRNA by TGFα

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 TGFα was tested to determine whether new protein synthesis is required. Cells were treated with vehicle control, 5 ng/ml TGFα, or 10 μg/ml cycloheximide alone or with 5 ng/ml TGFα plus 10 μg/ml cycloheximide. Total cellular RNA was isolated after 50 min of treatment and subjected to Northern blot. When cycloheximide was added together with TGFα, the transcript level increased 490 ± 24% of control (Figure 4). This increase was higher than that observed with TGFα alone. Therefore, cycloheximide does not prevent TGFα 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 al., 1993; Yokota et al., 1995). These results show TGFα 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 TGFα has a stabilizing action on c-myc mRNA, the half-life of this transcript was studied in the presence or absence of TGFα. Cells were exposed to 5 ng/ml TGFα for 50 min and then actinomycin D at a concentration of 5 μg/ml was added. The degradation of existing c-myc mRNA was monitored at various time points.
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 TGFα (Figure 5). The results suggest that TGFα 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 TGFα, and 5 μM antisense-c-myc or sense-c-myc OPT for 4 days, antisense-c-myc OPT inhibited TGFα-stimulated c-Myc protein expression and cell growth. The sense-c-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 TGFα-induced cell growth (Figure 6 and 7).

Discussion
In the present investigation, treatment of NIH:OVCAR-3 cells with 5 ng/ml TGFα 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 TGFα. 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 TGFα.

We have found that TGFα 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 TGFα-stimulated c-Myc protein expression and cell proliferation. These findings suggest that c-Myc protein plays important roles in the mechanism of TGFα-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 TGFα-stimulated cells.

Detailed time-course studies showed that TGFα treatment was apparently associated with biphasic induction of c-myc mRNA. The biphasic pattern of c-myc mRNA stimulation by TGFα can be due to a combination of several regulatory mechanisms that are known to modul-
activate 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 TGFα does not require protein synthesis, since it is not inhibited by cycloheximide. The half-life of c-myc mRNA was not modified by TGFα. It therefore seems that the TGFα 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 TGFα as demonstrated in this study.

The signal transduction pathway of TGFα in stimulation of c-myc expression have been examined in other tissues. In rat hepatocyte cultures, TGFα increased c-myc expression and this effect was overcome by the presence of an antibody against TGFα in the hepatocyte culture medium (Skouteris and McMenamin, 1992). A clear link emerged between c-myc activation and coupling of TGFα with its receptor. Furthermore, indomethacin inhibited the TGFα-induced hepatocyte DNA synthesis, progaptaglin production and at the same time TGFα-induced c-myc expression. Prostaglandins added to hepatocyte cultures supplemented with indomethacin and TGFα restored the ability of TGFα to stimulate DNA synthesis and to increase c-myc expression. Thus prostaglandins mediate the transduction of TGFα-generated signals. These data suggest that TGFα 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) TGFα 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 TGFα in NIH:OVCAR-3 ovarian cancer cells. In these cells, therefore, the growth-promoting effect of TGFα may, at least partly, be mediated through c-myc stimulation. As a consequence, any abnormality in this process could result in cell growth disregulation. Further studies are underway to understand the exact mechanisms of TGFα action, at the transcriptional and/or posttranscriptional levels.

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