Association of increased levels of TGF-β1 and p14ARF in prostate carcinoma cell lines overexpressing Egr-1

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Abstract. The present study examined the effect of the overexpression of early growth response gene (Egr-1) on transforming growth factor-β1 (TGF-β1) and p14ARF levels, in PC-3 and LNCaP prostate carcinoma cell lines. Amplification of EGR-1, TGF-β1 and p14ARF were observed in the two cell lines treated with different stimuli and resulted in a corresponding mRNA and protein expression. The downregulation of TGF-β1 and the attenuation of p14ARF expression by siRNA against Egr-1 predominantly suggested that TGF-β1 and p14ARF may be regulated by the transcription factor EGR-1. A marginal attenuation of cell growth in PC-3 and LNCaP prostate carcinoma cell lines overexpressing p14ARF was observed. Cells transfected with Egr-1 wild-type were able to grow and avoid cell cycle arrest and apoptosis in the presence or absence of p14ARF. In addition, EGR-1 stimulated the expression of TGF-β1 as well as the accumulation of the p14ARF proteins. The results suggested that TGF-β1 and p14ARF activities in the presence of EGR-1 overexpression can exist independently of the presence of cells carrying a mutant p53 (PC-3 cells) or cells carrying a wild-type p53 (LNCaP cells). Thus, the effect of EGR-1 on the growth of prostate carcinoma cells may occur through multiple mechanisms, but be independent of p53 expression control.

Introduction

EGR-1 belongs to the class of transcription factors known as immediate-early genes and is rapidly induced by growth factors to transduce the proliferative signal. Egr-1 induction by external stimuli is generally transient; however, it seems to be sustained in some prostate tumor cell lines and tumors, suggesting that Egr-1 stimulates tumor cell growth, which may be important as its expression level is enhanced with the degree of malignancy as measured by the Gleason tumor grade (1). This increase seems to be specific to prostate tumor cells as Egr-1 expression is low in mammary and lung tumors, as well as most normal tissues (2-6). By contrast, in breast, lung and brain tumor, Egr-1 expression is often not present or decreased and when re-expressed, results in growth inhibition (2,3,7,8). The induction of Egr-1 by external stimuli is generally transient but appears to be sustained in some prostate tumor cell lines and tumors, suggesting that Egr-1 stimulates tumor cell growth (9,10). Egr-1 is also involved in the regulation of p53 in human melanoma cells leading to apoptosis (11-13). The proapoptotic tumor-suppressor gene PTEN is also directly regulated by EGR-1 (14). Previously, it was demonstrated that the overexpression of Egr-1 in PC-3 and LNCaP prostate carcinoma cell lines increases cell growth and independent anchorage (15). Moreover, ablation of Egr-1 expression using small interfering RNA (siRNA) resulted in the induction of cell apoptosis and cell death (15-18).

Another important factor associated with EGR-1 regulation is the transforming growth factor β1 (TGF-β1) (19). It has been previously shown that the expression of EGR-1 in the HT1080 human fibrosarcoma cell line, increases the secretion of transforming growth factor-β1 (TGF-β1) in direct proportion to the amounts of EGR-1 expressed (20), and that EGR-1 protein specifically binds to two GC-rich EGR-1-binding sites in the TGF-β1 gene promoter and stimulates TGF-β1 promoter activity in HT1080 cells (20). TGF-β1 is a potent suppressive cytokine that inhibits immune system functions that otherwise may be effective in generating antitumor immunity. TGF-β is produced by many normal cells and is overexpressed in the tumor cells that comprise the most common forms of human cancer, including prostate carcinoma. TGF-β1 is induced by a variety of signals including those of oncopgenes and immediate-early genes, whereas the expression of TGF-β2 and TGF-β3 is considerably more developmentally and hormonally regulated (21). Thus, TGF-β1 influences various pathologies including fibrosis, parasite induction, autoimmune diseases and tumorigenesis (22-25). Another protein that is involved in the regulation of EGR-1 is p14ARF. This protein has emerged as an important tumor suppressor that can trigger the growth suppression and apoptosis of many cancer cells by a p53-dependent and -independent mechanism (26,27), and loss

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of p14ARF is likely to contribute to the failure of apoptosis in cancer. p14ARF is encoded within the same INK4a locus that encodes the p16 cyclin-dependent kinase inhibitor (27,28). Unlike p16, which inhibits cell cycle progression, p14ARF does not appear to have Cdk inhibitory activity. Instead, p14ARF stabilized p53 function through a complex formation with Mdm2, the principle mediator of p53 stability (29,30). A previous study demonstrated that EGR-1 binds to p14ARF which is associated with the tumor-suppressive effects of EGR-1 (31).

The results showed that TGF-β1 and p14ARF activities in the presence of EGR-1 overexpression exist independently of the presence of cells carrying a mutant p53 (PC-3 cells) or cells carrying a wild-type p53 (LNCaP cells). The effect of EGR-1 on the growth of prostate cells may occur through multiple mechanisms, but be independent of p53 expression control.

Materials and methods

Cell lines and culture. Human prostate carcinoma cell lines PC-3 and LNCaP were a kind gift from Dr Dan Mercola (The SKCC; Sidney Kimmel Cancer Center, La Jolla, CA, USA). The cells were cultured in RPMI-1640 medium supplemented with 100 ml/l fetal bovine serum (FBS), 8x10^5 U/l penicillin and 0.1 g/l streptomycin in humidified incubator containing 50 ml l CO2 at 37°C.

Tris-borate-EDTA and acrylamide: bisacrylamide (29:1) were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Egr-1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Lipofectamine was purchased from Life Technologies, Inc. (Carlsbad, CA, USA) and TNF-α from Sigma Chemical Co. (St. Louis, MO, USA). Complete Mini-EDTA-Free Protease Inhibitor Cocktail Tablets and Annexin-V-Fluos were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Other reagents were purchased from Sigma Chemical Co. (Taufkirchen, Germany). Phorbol 12-myristate 13-acetate (TPA), TNF-α and TGF-β1 were purchased from Stratagene Inc. (La Jolla, CA, USA). Anti-Egr-1, anti-p14ARF, anti-TGF-β1, and β-actin antibodies were purchased from Santa Cruz Biotechnology.

siRNA preparation and transfection of small interfering RNA. The sequences designed used to specifically target human p14ARF and Egr-1 RNAs were: for p14ARF, 5'-GAACAUGGU GGCAGGUUCTT-3' and for Egr-1, 5'-AACGCAAGAGC AUACCAAGA-3'. The scrambled small interfering RNA (siRNA) oligonucleotides used as controls for all RNA interference experiments were: 5'-AAAGUGACGCGACGAA GTT-3' and 5'-CAAGAAAGCCAGUCCAGGT-3'. The cells were transfected with siRNA oligonucleotide duplexes and cultured in medium without antibiotics 24 h prior to transfection resulting in a confluence of the cell monolayer by 60-80%. Egr-1-siRNA or non-silencing siRNA (70 nmol) were mixed with Lipofectamine™ 2000 (Invitrogen-Life Technologies) according to the manufacturer's recommendations and added to the cells. After 6 h at 37°C, the medium was replaced and the cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (9,16,17).

3-(4,5-methylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. PC-3 and LNCaP cells (5x10^5) were plated in 96-well plates in RPMI-1640 containing 10% FBS at a final volume of 0.1 ml. The following day, the cells were treated with the pTER siRNA. MTT was added (20 ml/well of 5 g/l solution in PBS) after culture for 24, 48 and 72 h. Following incubation at 37°C for 4 h, the reaction was stopped by the addition of 100 ml DMSO. The reaction product was quantified by measuring the absorbance at 490 nm using an ELISA reader (WALLAC 1420 VICTOR 2; Wallac, Turku, Finland) and the Software HT-Soft (Perkin-Elmer). The samples were assayed repeatedly in 6-wells.

Western immunoblot analysis. PC-3 and LNCaP prostate carcinoma cells lines (5x10^5) were seeded in 6-well plates. Forty-eight hours after transfection, the cells were collected and washed twice by cold PBS, and each well was treated with 50 ml lysis buffer (2 mmol/l Tris-HCl pH 7.4, 50 mmol/l NaCl, 25 mmol/l EDTA, 50 mmol/l NaF, 1.5 mmol/l NaVO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/l phenylmethylsulfonylfluoride, 10 mg/l pepstatin, 10 mg/l aprotinin and 5 mg/l leupeptin) (all from Sigma). Protein concentrations were determined using the Bradford protein assay. Equal amounts of protein (40 mg) were separated on a 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C; Amersham, Freiburg, Germany). The membranes were blocked in 5% non-fat dry milk in TBS for 1 h at room temperature and probed with rabbit anti-Egr-1 antibodies (1:500 dilution; Santa Cruz Biotechnology) overnight at 4°C. After being washed 3 times with TBS containing 0.1% Tween-20, the membranes were incubated with anti-rabbit IgG-horseradish-peroxidase (1:5,000; Santa Cruz Biotechnology), and developed by luminol-mediated chemiluminescence (Appylgen Technologies Inc., Beijing, China). To confirm equal protein loading, membranes were reprobed with a 1:1,000 dilution of an anti-actin antibody (Santa Cruz Biotechnology). Densitometric analyses were performed using Scion Image software. Intestinal alkaline phosphatase (IAP) activity was detected using an IAP kit from Sigma-Aldrich.

DNA fragmentation assay. The cells were plated in 96-well plates 24 h prior to treatment. Following treatment, DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions.

Flow cytometry. PC-3 and LNCaP prostate carcinoma (5x10^5) cell lines were seeded in triplicate in 6-well plates, and cultured in RPMI-1640 supplemented with 100 ml/l PBS. After transfection for 48 h, the cells were collected and washed with ice-cold PBS, and fixed in 70% ethanol overnight at 4°C. The fixed cells were pelleted, washed in PBS, resuspended in PBS containing 0.1 mg/ml of propidium iodide and analysed by flow cytometry.

Results

Expression of EGR-1, p14ARF and TGF-β in human prostate carcinoma cell lines overexpressing Egr-1. EGR-1 is an early response nuclear factor that is important in the regulation of
several genes (Fig. 1). To determine the effect of overexpressing Egr-1 in the expression of EGR-1, p14ARF and TGF-β1 in PC-3 (Fig. 1A) and LNCaP (Fig. 1B), prostate carcinoma cell lines, the cells were treated with FCS 0.5%, FCS 10%, TPA (30 nM) and TNF-α (20 ng/ml). The protein expression of EGR-1, p14ARF and TGF-β1 was assessed by western blot analysis (Fig. 1).

Knocking down Egr-1 expression by Egr-1-siRNA, strongly decreased the activity of TGF-β1 but only moderately the expression of p14ARF and was able to reverse the increasing effect of TPA (30 nM) only in PC-3 cells. PC-3 (Fig. 2A) and LNCaP (Fig. 2B) prostate carcinoma cells were transfected with a wild-type Egr-1-cDNA and the expression of EGR-1, TGF-β1 and p14ARF proteins was assessed by western blot analysis (Fig. 2A and B). We observed a strong induction of Egr-1 and p14ARF after the treatment of cells with TPA and TNF-α treatment (Fig. 2A and B, lanes 1 and 2). By contrast, TGF-β1 was significantly induced only after TNF-α treatment.

![Figure 1](image1.png)

**Figure 1.** Expression of EGR-1, p14ARF and TGF-β1 in (A) PC-3 and (B) LNCaP prostate carcinoma cell lines in response to several stimuli. (A) PC-3 and (B) LNCaP cells treated with FBS 0.5%, FBS 10%, TPA 30 nM and TNF-α 20 ng/ml. Untreated LNCaP and PC-3 cells were used as the control. β-actin was used as a loading control. One of three similar experiments is shown.

![Figure 2](image2.png)

**Figure 2.** Inhibition of EGR-1 (A and B) by siRNA against EGR-1 and inhibition of p14ARF (C and D) by siRNA against p14ARF. Western blot analysis shows the effect of blocking (A and B) EGR-1 and (C and D) p14ARF expression. Untreated PC-3 and LNCaP prostate carcinoma cell lines (lane 1), or treated with TPA (30 nM) (lane 2), a control siRNA (lane 3), a siRNA against EGR-1 or p14ARF (lane 4), and a combination of siRNA and TPA (lane 5). EGR-1, p14ARF and TGF-β1 were detected by enhanced chemiluminescence after film exposure of 10 min. β-actin was used as a loading control. One of three similar experiments is shown.
in PC-3 cells (Fig. 2A, lane 3). The expression of TGF-β1 in LNCaP cells was observed after treatment with TPA and TNF-α treatment (Fig. 2B, lanes 2, 3 and 5). A decrease in the cell protein expression in the PC-3 (Fig. 2A) and LNCaP (Fig. 2B) prostate carcinoma cells was observed following Egr-1 siRNA treatment (Fig. 2A and B, lane 4). Nevertheless, Egr-1-siRNA was unable to reverse the effect of TPA in the induction of TGF-β1 in LNCaP cells (Fig. 2B, lane 5).

To determine whether blocking the expression of p14arf by a siRNA against p14arf affected the expression of EGR-1, p14arf or TGF-β1, PC-3 (Fig. 2C) and LNCaP (Fig. 2D) cells were transfected with a vector carrying a p14arf cDNA. At 72 h after transfection, the cells were treated with TPA (30 mM), TNF-α (20 ng/ml) and siRNA against p14arf and cultured for an additional 12 h. At the indicated time point, the cells were harvested and analyzed for expression of EGR-1, p14ARF and TGF-β1. The protein expression was assessed by western blot analysis. As shown in Fig. 2C and D, p14arf-siRNA strongly decreased EGR-1 and p14ARF expression but was unable to reverse the effect of TPA.

Inhibition of Egr-1 but not p14arf increased apoptosis in PC-3 and LNCaP prostate carcinoma cell lines. We determined whether blocking Egr-1 or p14arf expression exerted an apoptotic effect on PC-3 or LNCaP cells treated with siRNA against the abovementioned proteins. To this effect, LNCaP cells were transfected with siRNA against Egr-1 (Fig. 3A) or against p14arf (Fig. 3B) and with a non-specific siRNA as the control. After culturing at the indicated times, the cells were harvested and analyzed for induction of apoptosis. Results of the flow cytometric analysis showed that knocking down Egr-1 using siRNA against Egr-1, significantly induced apoptosis in PC-3 and LNCaP cells (Fig. 3A and B). However, when PC-3 and LNCaP cells were treated with p14arf-siRNA and analyzed for induction of apoptosis, the effect was only moderate (Fig. 3C and D) and decreased after a 48-h transfection, suggesting a moderate role for p14arf inhibition in controlling apoptosis. However, unlike Egr-1-siRNA-treated PC-3 cells, the apoptotic activity decreased after 48 h as compared to that observed in Fig. 3A and B. The results suggested that the differential expression of EGR-1 and p53 was responsible for the differences in apoptotic response demonstrated in PC-3 and LNCaP cells (Fig. 3).

Egr-1 function in TGF-β1 induced proliferation and cell survival. Since EGR-1 is a potential tumor inducer for prostate cancer (1) and inhibition of Egr-1 by siRNA-Egr-1 decreases TGF-β1-mediated proliferation (6), we analysed the role of Egr-1 in TGF-β1-induced cell survival in PC-3 and LNCaP cells. Treatment with TGF-β1 (10 ng/ml) increased IAP activity (Fig. 4A and B), an inhibitor of apoptosis that plays a key role in preventing cell death by apoptosis. However, this increase was attenuated by the transfection of siRNA against Egr-1 (Egr-1-siRNA), suggesting a role for Egr-1 in TGF-β1-mediated cell viability.

The aforementioned results showed that inhibition of Egr-1 by siRNA enhanced TGF-β1-mediated cell viability in human prostate cancer cells (Fig. 4A and B). The effect
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of Egr-1 knockdown on TGF-β1-induced PC-3 and LNCaP cell survival was assessed. TGF-β1 induced obvious cell survival as shown by the decreased DNA fragmentation in the PC-3 and LNCaP cells (Fig. 4C and D) and this decrease was partially attenuated by the knockdown of Egr-1 using Egr-1-siRNA transfection. Inhibition of Egr-1 expression induced obvious cell death as shown by the increased DNA fragmentation (Fig. 4C and D), suggesting a survival role of Egr-1 in prostate cancer progression. Collectively, the results suggested that Egr-1 is involved in TGF-β1-mediated prostate cell death, survival and differentiation.

The effect of p14arf knockdown on TGF-β1-induced cell survival and proliferation was assessed. TGF-β1 induced obvious cell survival as shown by the decreased DNA fragmentation in the PC-3 and LNCaP cells (Fig. 5C and D) and this decrease was not attenuated by the transfection of siRNA against p14arf (p14arf-siRNA) suggesting a key role of TGF-β1-mediated proliferation in allowing cell viability in human prostate cancer cells (Fig. 5A and B).

Discussion

In previous studies, we demonstrated decreased prostate carcinoma cell survival and independent anchorage by Egr-1-siRNA (15,16) and defined a novel feedback regulation of EGR-1 through the regulation of NF-κB and AP-1 (10). In the present study, we delineated the signaling pathway involved in this regulation. The results of the present study show that TGF-β1-treated cells increased EGR-1 expression and
decreased the p14ARF suppressor effect in PC-3 and LNCaP prostate carcinoma cell lines. This TGF-β1-mediated EGR-1 induction and further enhancement in the increase of EGR-1 expression was independent of the presence or absence of the tumor suppressor protein p14ARF. Treatment of the PC-3 and LNCaP prostate carcinoma cell lines with TPA (30 nM) or TNF-α (20 ng/ml) activated EGR-1, JNK-1 and JNK-3 (32) and attenuated cell apoptosis (32). Findings of a recent study have shown that the treatment of PC-3 and LNCaP cells with Egr-1-siRNA induced cell survival, which was associated with the activation p21Waf1/Cip1 protein and with a decreased JNK expression (33). By contrast, inhibition of JNK by a siRNA, did not attenuate EGR-1 expression (18). Our observation that the blockade of EGR-1 by a genetic mechanism (transfection of Egr-1-siRNA) attenuated TGF-β1 induction, while it did not affect p14ARF induction, suggests a functional role for EGR-1 activation in TGF-β1 induction in PC-3 and LNCaP prostate carcinoma cell lines. The induction of proliferation by TGF-β1 via the induction of EGR-1 may play a role in the autonomy of prostate carcinoma cell growth and, thus, in the pathogenesis of prostate cancer.

EGR-1 expression in certain cells, such as breast and lung carcinoma cells, as well as most normal tissues, is low (10). In addition, EGR-1 overexpression is correlated with loss of its co-repressor NAB2 in primary prostate carcinoma (6-9). This disruption of the balance between EGR-1 and NAB2 expression results in high EGR-1 transcriptional activity in prostate carcinoma cells (6).

Crosstalk between EGR-1 and TGF-β1 pathway has been previously reported (34). The levels of EGR-1 protein and mRNA were rapidly and transiently upregulated by TGF-β1 in vitro, and EGR-1 promoter activity was enhanced. Liu et al (25) observed that the EGR-1 gene product directly controls TGF-β1 gene expression. Another study showed that TGF-β1 treatment also activated the ERK1/2 signaling pathway, causing an increase in EGR-1 (35). However, the tumor-suppressor p14ARF protein showed antagonist activity to the effect induced by EGR-1 in PC-3 and LNCaP cells. Previously, Egr-1 and PTEN were identified as mediators of p14ARF function (36). Egr-1-dependent expression of PTEN is controlled by p14ARF through the ARF-mediated sumoylation of Egr-1. This requires the phosphorylation of
Egr-1 by the protein kinase AKT, which promotes the association of Egr-1 with p14ARF fibroblasts or ARF-/ARF (36). p14ARF has been identified as a potent tumor suppressor in vitro and in vivo (37,38). p14ARF has been shown to protect against uncontrolled growth and tumorigenesis induced by hyper-proliferative stimuli (39). In agreement with these findings, results of this study have shown that inhibition of p14ARF attenuated cell apoptosis, leading to the enhancement of EGR-1 induction. p14ARF was found to be primarily dependent on the presence of functional p53. However, p14ARF is involved in p53-independent mechanisms of cell cycle regulation and apoptosis induction, respectively. Failure of apoptosis is central to the development of cancer, and in many types of cancer, this failure results from loss of p53, a key mediator of apoptosis (40). Consequently, the tumor-suppressor p14ARF appears to be a possible target of the EGR-1 pathway.

In summary, the present study provides important insights regarding the signaling mechanisms regulating EGR-1 expression and function in prostate carcinoma cell lines. The importance of the antagonistic crosstalk between EGR-1 and p14ARF in prostate carcinoma cell lines deserves further investigation as a novel approach to cancer therapy.

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