Increased expression of p21<sup>Waf1/Cip1</sup> and JNK with costimulation of prostate cancer cell activation by an siRNA Egr-1 inhibitor

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Abstract. The p21<sup>Waf1/Cip1</sup> protein (hereafter, p21) and the c-Jun N-terminal kinase (JNK) are two well-characterized cell modulators that play a crucial role in cell differentiation, senescence and apoptosis. Here, we report that transcription of the p21<sup>Waf1/Cip1</sup> and JNK-1 genes is affected by inhibition of the early growth response-1 (Egr-1) in response to a small interfering RNA [siRNA]-Egr-1 in LNCaP and PC-3 prostate carcinoma cell lines. The expression levels of protein were determined by western blotting, and apoptosis was measured by propidium iodide staining and flow cytometric analysis. Inhibition of Egr-1, p21 and JNK-1 was carried out by siRNAs. LNCaP and PC-3 cells exhibited readily detectable Egr-1, JNK and p21, even in low serum medium without the addition of other exogenous agents. The expression of Egr-1, p21 and JNK was strongly increased after treatment of the cells with TPA, tumor necrosis factor-α (TNF-α) or arsenite. Suppression of Egr-1 expression by siRNA abrogated the ability of TPA to induce Egr-1 and JNK-1 activities, moderately increasing the p21 activity and abrogating the anti-apoptotic effect of Egr-1 observed in the prostate cancer cell lines. Moreover, blockade of p21 and JNK was unable to decrease the activity of Egr-1, while siRNA against p21 abrogated the pro-apoptotic effect of p21. The results demonstrated that Egr-1 acts as a key player in prostate tumor cell growth and survival, while p21 plays a key pro-apoptotic role in LNCaP and PC-3 prostate carcinoma cell lines.

Introduction

The p21<sup>Waf1/Cip1</sup> protein (p21) and the c-Jun N-terminal kinase (JNK) are two well-characterized cell modulators that play a crucial role in cell differentiation, senescence and apoptosis. p21 is a cyclin kinase inhibitor (CKI) that, among other things, directly inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4/6 complexes and functions as a regulator of cell cycle progression at the S phase (1,2). Results indicate that p21 expression is controlled by the tumor-suppressor protein p53 and that the expression of p21 is mainly dependent on two factors: i) the stimulus provided and ii) the type of the cell.

The JNK pathway is one of three principal mitogen-activated protein kinase (MAPK) pathways involved in signal transduction of extracellular receptor-mediated stimuli to the nucleus of the cell (3,4). The JNK pathway is rapidly and strongly activated by numerous pro-inflammatory cytokines and by many non-receptor-mediated events including DNA damaging agents such as UV light and numerous genotoxic chemotherapeutic agents (5). Once activated, JNK-1 can upregulate gene expression by phosphorylating the activating motif of transcription factors such as ATF-2 (6,7), c-Jun and Jun D (8) and the Ets domain of transcription factor Elk-1 and Sap-1 (9). JNK kinases are believed to participate in most aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis (10,11). Moreover, the role of JNK in apoptosis is unclear as these proteins have been assigned both pro- and anti-apoptotic properties (10-14).

In contrast, the early growth response protein 1 (Egr-1) is a C2H2-zinc finger-containing transcriptional regulator involved in the control of cell proliferation and apoptosis (15,16). Egr-1 is rapidly induced by growth factors to transduce the proliferative signal. 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 and could have an important function as measured by the Gleason grade of the tumor (17). In addition, overexpression of Egr-1 is correlated with the loss of its co-repressor NAB2 in primary prostate carcinoma (18,19). This disruption in the balance between Egr-1 and NAB2 expression results in a high Egr-1 transcriptional activity in prostate carcinoma cells (20). In contradiction, in breast, lung and brain tumors, Egr-1 expression is often absent or reduced and its re-expression results in growth suppression (18,21). Egr-1 also plays a role in tumor progression, through the hypoxic signal generated in growing tumors. Egr-1 is highly

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induced under these conditions and its activities stimulate angiogenesis (22,23). However, one of the major concerns about Egr-1 overexpression in carcinoma tumors is that it is associated with a loss in functionality of the prostate cells and/or the development of autonomous growth.

In this study, we showed that blocking of Egr-1 expression by a small interfering RNA (siRNA-Egr-1) increased the activity of p21\(^{Waf1/Cip1}\) and decreased the expression of JNK protein. In contrast, siRNA silencing of p21 or JNK-1 with siRNAs was unable to decrease the expression of Egr-1.

Materials and methods

The protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin and bestatin, were purchased from Roche (USA); T4 polynucleotide kinase and poly(dI-dC)2 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Tris-borate-EDTA buffer and acrylamide-bisacrylamide (29:1) were obtained from Bio-Rad (Richmond, CA, USA). Antibodies against JNK, p21, Egr-1 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MBP was purchased from Stratagene (La Jolla, CA, USA). Phorbol 12-myristate 13-acetate (TPA), tumor necrosis factor-α (TNF-α), arsenite and fetal bovine serum (FBS) were purchased from Sigma-Aldrich, Inc. (USA).

Cell lines and culture. Human prostate carcinoma cell lines LNCaP and PC-3 were a gift from Dr Dan Mercola (SKCC, La Jolla, CA, USA). The cells were cultured in RPMI-1640 medium supplemented with 100 ml/l FBS, 8x10\(^{-5}\) U/l penicillin and 0.1 g/l streptomycin in a humidified incubator containing 50 ml/l CO\(_2\) at 37°C (24). The antibodies used for western blotting included those against protein kinase JNK-1 and JNK-2, Egr-1 and p21. Western blotting was performed as previously described (12).

siRNA preparation and transfection of short interfering RNAs. The siRNAs for Egr-1 and JNK-1 were obtained as ready-annealed, purified duplex probes, and the scrambled control siRNAs were purchased from Shanghai Genechem Co. The siRNAs for Egr-1 were: sense, 5'-CAGCAGCAGCAGCGCAGCAGCAGTCT-3' and antisense, 5'-AACGTGCAGTCTGCTGCTGCTGCT-3'. The siRNA oligonucleotides for JNK-1 were: sense, 5'-AGGCCCAGTATAGTATGA-3' and antisense, 5'-TAC TATATATATTGCGCTT-3'. JNK-2 sense, 5'-CATGAT GTTATCATATCTT-3' and antisense 5'-ATAAGATATA GTAACACTATCT-3'. The p21\(^{Waf1/Cip1}\)-targeted siRNAs were obtained from Santa Cruz Biotechnology, Inc. Cells were treated in parallel with scrambled siRNA sense, 5'-AATTC TCCGACGGTCGAGCTG-3' and antisense, 5'-AACGTGACA CGTTGGGAGAATT-3'. The cells were cultivated in medium without antibiotics, and 24 h before transfection resulting in a confluence of the cell monolayer by 50-70%. Specific Egr-1, p21, JNK-1/2 siRNAs or non-silencing siRNA (70 nmol) were mixed with Lipofectamine\(^{\text{TM}}\) 2000 (Invitrogen) according to the manufacturer's recommendation and added to the cells. After 6 h at 37°C, the medium was replaced, and the cells were cultivated in RPMI-1640 supplemented with 10% heat-inactivated FBS.

Apoptotic cell death assay. The cells were treated with different siRNAs. At indicated time points, cells were collected and washed with phosphate-buffered saline (PBS). After fixation with 70% ethanol, cells were washed twice with PBS and stained with a solution containing 20 mg/ml propidium iodide (PI) and 50 mg/ml RNase A. Cells were incubated for 30 min at room temperature and the cell cycle profiles were determined by flow cytometry using a FACScan (Becton-Dickinson, San Jose, CA, USA).

Western blot analysis of protein expression. Cells were chilled on ice and washed twice with ice-cold PBS (43 mM K\(_2\)HPO\(_4\), 9 mM Na\(_2\)HPO\(_4\), 120 mM NaCl; pH 7.4). They were solubilized on ice in lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM Na\(_2\)PO\(_4\), 1% (v/v) Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail (Sigma-Aldrich, Inc., St. Louis, MO, USA). Lysates were then clarified by centrifugation at 13,000 x g for 10 min at 4°C. The protein concentration was determined using the BCA\(^{\text{TM}}\) protein assay reagent (Pierce, Rockford, IL, USA). Cleared lysates were resuspended in sample buffer containing 70 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 1.5% (v/v) 2-mercaptoethanol. Samples were subjected to electrophoresis on a 12% acrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) using standard procedures. Membranes were blocked in saline buffer [25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% (w/v) Tween-20] containing 5% (w/v) non-fat milk for 2 h at 22°C before the addition of the antibodies for an overnight incubation at 41°C. Several washes were performed in saline buffer, and peroxidase-conjugated antibodies against mouse or rabbit immunoglobulins (Amersham Biosciences, Piscataway, NJ, USA) were added at a dilution of 1/6,000 for 45 min at 22°C. After washing, the membranes were soaked in western blotting luminol reagent (Santa Cruz Biotechnology, Inc.) followed by autoradiography. When appropriate, membranes were stripped using Restore\(^{TM}\) stripping buffer (Pierce) for 15 min at 22°C and reprobed with the indicated antibodies.

Antibodies. Antibodies to Egr-1 (sc-189), p21\(^{Waf1/Cip1}\) (sc-6264), JNK (FL) (sc-571), JNK-1 (C-17) (sc-474), and β-actin (sc-1616) were used at a concentration of 0.07 and 0.1 mg/ml, respectively (in a total volume of 12 ml). Antibodies to β-actin (clone AC-15) were from Sigma-Aldrich, Inc. and were used at a concentration of 0.22 mg/ml in a total volume of 12 ml.

Results

Egr-1, p21 and JNK are strongly induced by several different stimuli in LNCaP prostate cancer cells. In order to test the transforming role of Egr-1, JNK and p21\(^{Waf1/Cip1}\) in prostate carcinoma cell lines, we collected and examined two prostate cell lines. Both, LNCaP and PC-3 cell lines exhibited serum inducible growth (FBS 10%). Egr-1, p21 and total JNK activities were assessed under a similar condition, i.e. 48 h following plating in low serum followed by 3 h in complete serum-containing growth medium. Both cell lines exhibited readily detectable EGR-1, p21 and JNK activity, even in low serum (FBS 0.5%). However, all three proteins were strongly induced by FBS 10%,
TPA (30 nM), TNF-α (20 ng/ml) and arsenite (10 µM) (Fig. 1). β-actin was used as a loading control. The response of Egr-1 to all the stimuli was highly efficient and remained elevated when compared with the response of the other proteins.

Knockdown of Egr-1 expression by siRNA strongly decreases the activity of p21 and JNK and reverses the increasing effect of TPA (30 nM). LNCaP cells were transfected with a siRNA against Egr-1 or with a nonspecific siRNA (control). At 48 h after transfection, the cells were treated with TPA (30 mM) and cultured for an additional 12 h. At the indicated time point, the cells were harvested and analyzed for expression of EGR-1, p21 and JNK-1 proteins by western blot analysis (Fig. 2A). As shown in Fig. 2A, siRNA-Egr-1 strongly decreased p21 expression.
protein expression and reversed the effect of TPA. At the same time a high specificity to completely block the expression of EGR-1 was noted (Fig. 2A). To determine whether blocking the expression of p21 by an siRNA decreases the expression of EGR-1 and JNK-1, LNCaP cells were transfected with an siRNA against p21 or with a nonspecific siRNA (control). At 48 h after transfection, cell were treated with TPA (30 mM) and cultured for an additional 12 h. At the indicated time point, the cells were harvested and analyzed for expression of EGR-1, p21 and JNK-1 proteins by western blotting. As shown in Fig. 2B, p21-siRNA strongly decreased p21 protein expression but was unable to reverse the effect of TPA. Similar to the above experiments, we investigated the effect of the blockage of JNK-1 by an siRNA on the expression of EGR-1 and p21 proteins. As shown in Fig. 2C, siRNA-JNK-1 moderately decreased p21 protein expression but was unable to substantially decrease the expression of EGR-1.

Knockdown of Egr-1 or JNK-1 but not p21 increased apoptosis in LNCaP and PC-3 cells treated with siRNAs against these proteins. Next, we sought to determine whether blocking EGR-1, p21 or JNK-1 has an apoptotic effect in LNCaP or PC-3 cells treated with siRNAs against these proteins. To this effect, LNCaP and PC-3 cells were transfected with an siRNA against Egr-1 (Fig. 3A), against p21 (Fig. 3B) or against JNK-1 (Fig. 3C) and with a nonspecific siRNA as control. After the indicated times of the culture, LNCaP cells were harvested and analyzed for induction of apoptosis. As shown by flow cytometric analysis, knockdown of Egr-1 using siRNA significantly induced apoptosis in LNCaP cells (Fig. 3A). However, when LNCaP cells were transfected with p21-siRNA and analyzed for induction of apoptosis, the effect was only marginal (Fig. 3B) compared with the effect induced by siRNA-Egr-1 (Fig. 3A), and was decreased after 72 h transfection (data not shown). We then proceeded to analyze the
effects of blocking JNK expression on the apoptosis process. As shown in Fig. 3C, blocking JNK-1, moderately induced apoptosis after 48 h transfection, compared with untreated and control cells. The results suggest that the differential expression of Egr-1 was responsible for the differences in the apoptotic response demonstrated in the LNCaP and PC-3 cell lines.

**Discussion**

**Egr-1** is a member of the immediate early gene family and encodes a nuclear phosphoprotein involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors and stress stimuli (15,16,18,25). However, in other circumstances, **Egr-1** is induced very early in the apoptotic process (26), where it mediates the activation of downstream regulatory genes (27,28). It has been previously demonstrated that Egr-1 is required for tumor formation by a variety of human cancers (15,16,21,25). As we know, degradation of mRNA mediated by siRNA is a powerful means of specifically knocking down the expression of a target gene (29,30). We previously used small interference RNAs to treat cells expressing the nuclear factor Egr-1 (12,14,20,24,31).

In this study, we showed that blocking Egr-1 by specific siRNA, strongly decreased the activity and expression of p21 and JNK proteins. Similar to Egr-1, both LNCaP (wild-type p53) and PC-3 (p53-deficient) were able to strongly express JNK and p21 upon exposure to several stimuli, suggesting that the expression of these protein may be initiated through intracellular signaling through the stress-activated kinases or other pathways (32,33). As expected, the response of PC-3 and LNCaP cells to the different stimuli was followed by high expression of EGR-1 and JNK, while the expression of p21 was only moderate, suggesting that the activation of p21 requires the participation of several factors (32,33). We simultaneously examined the effect of siRNAs on the protein expression of Egr-1, p21 and JNK. We found that the expression of all three proteins was decreased when cells were treated with specific Egr-1-siRNA or p21-siRNA or with JNK-1-siRNA. Both p21-siRNA and JNK-1-siRNA were unable to decrease the expression of EGR-1 in the PC-3 and LNCaP cells, suggesting a regulatory role of Egr-1 in controlling the expression of these proteins (34). As we know, JNK activation participates in DNA repair pathways that induce cell cycle arrest and has been implicated in the regulation of the cell cycle regulators p21Waf1/Cip1 and cyclin D1. Studies have shown that other MAPK pathways participate in cell cycle control (32,33,35). ERK induces G1/S transition while p38 inhibits G2/M transition (32,33). Other studies have shown that JNK is required for efficient induction of apoptosis in response to ionizing radiation (36). Based on these findings, it is reasonable to hypothesize that activation of JNK-1 induces apoptosis and cell cycle arrest (37). Although Egr-1, but not JNK-1 (38) activation, is associated with the induction of both cyclin D1 and p21, several studies have shown that the cellular decision to induce p21 in the G1 phase of the cell cycle could be dictated by the magnitude of the ERK1/2 signal (39). In this respect it is interesting to note that in both cell lines we found a moderate expression of p21 expression associated with reduced cell apoptosis when compared with the scramble-transfected cells. Because we were able to show that Egr-1 inhibition strongly blocks JNK-1 and moderately blocks p21, it is tempting to speculate that the strong inactivation of Egr-1 by an siRNA in these cells may lead to a strong and persistent p21Waf1/Cip1 expression favoring apoptosis.

In conclusion, we showed that inhibition of Egr-1 activity by Egr-1-siRNA was associated with a marked reduction in p21 and JNK activities. However, siRNAs against p21 and JNK-1 were unable to decrease the activity of Egr-1. We found that siRNA-mediated Egr-1 inhibition also resulted in the cell death of >60% cancer cells at 48 h, when compared with this percentage in the cells treated with either p21-siRNA or JNK-1-siRNA.

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**References**


