C-Jun NH$_2$-terminal Kinase Mediates Proliferation and Tumor Growth of Human Prostate Carcinoma$^1$

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ABSTRACT

**Purpose:** C-Jun NH$_2$-terminal kinase (JNK) has been implicated in numerous functions including stress responses, apoptosis, and transformation. The role in transformation is based largely on studies of isolated cell types with little indication of whether JNK plays a general role in a specific human tumor type or whether this occurs in vivo.

**Experimental Design:** We examined 9 human prostate carcinoma cell lines in vitro and a representative line in vivo.

**Results:** For all of the cell lines proliferation is highly correlated with serum-supported JNK activity ($r_{\text{Pearson}} = 0.91; P = 0.004$), whereas no relationship was observed for 10 human breast cancer cell lines ($r_{\text{Pearson}} = -0.32$). Treatment with characterized antisense oligonucleotides complementary to sequences common to either the JNK1 or JNK2 family of isoforms showed that, whereas antisense JNK1 inhibited growth by a maximum of 57%, antisense JNK2 inhibited proliferation up to 80%. Sense and scrambled control oligonucleotides had little effect (average 3.7 ± 1.5%). Moreover, systemic treatment of mice bearing established xenografts of PC3 prostate carcinoma cells with antisense JNK1 and JNK2 led to inhibition tumor growth by 57% ($P < 0.002$) and 80% ($P < 0.001$), respectively. The difference is significant ($P < 0.012$). Combined antisense treatment led to a significant increase in frequency of tumor regression ($P = 0.022$).

**Conclusion:** These results indicate that JNK is required for growth of prostate carcinoma cells in vitro and in vivo, and additionally indicate that JNK2 plays a dominant role. The JNK pathway is a novel target in the treatment of prostate carcinoma.

INTRODUCTION

The stress-activated protein kinase or JNK$^5$ is one of at least three recognized mitogen-activated protein kinase signal transduction pathways composed of a cascade of protein kinases that ultimately regulate gene expression by phosphorylation and activation of one or more transcription factors (reviewed in Refs. 1, 2). Two genes, JNK1 and JNK2, are commonly expressed in mammalian cells whereas a third gene, JNK3, is principally expressed in brain, testes, and heart tissue. There are at least four isoforms of JNK1 and JNK2, and two isoforms of JNK3 that arise from alternative splicing (3, 4). Numerous stress stimuli including DNA-damaging agents, inflammatory mediators, environmental stresses such as heat shock, osmotic stress, and certain growth factors stimulate JNK activity through multiple pathways leading to activation of one of the upstream kinases such as JNK kinase kinase. Examples at this level include MEKK1 or mixed lineage kinases, and TAK1 (5). MEKK1 in turn phosphorylates the JNK kinases MKK4/SEK1 and/or MKK7, dual specificity kinases that preferentially phosphorylate the threonine and tyrosine residues, respectively, in the T-X-Y motif of the JNK activation loop (6). “Scaffold” proteins bind selected kinase cascade members into modules that may respond to specific stimuli and/or may affect localization (1, 6). JIP-1 binds JNK, mixed lineage kinases, and MKK7, which is preferentially responsive to cytokines (7). Another complex contains JNK, MEKK1, and MKK7, as well as MKK4, which is preferentially responsive to environmental stress and interacts with docking proteins of activated Fas (8–10). Activated JNKs phosphorylate several transcription factors such as c-Jun, ATF2, Elk1, and others (e.g. BCI2, p53, NFATc1, and Smad4; Refs. 11–14) where, at least in the case of c-Jun and ATF2, increased transactivation potential results (15, 16). Thus, JNK potentially regulates a wide variety of genes, many of which have been revealed by expression analyses (17–20).

Numerous studies have shown that JNK is commonly required for the apoptotic response of many cells (12, 21–25). In

$^1$ The abbreviations used are: JNK, c-Jun NH$_2$-terminal kinase; MEKK, mitogen-activated protein kinase kinase kinase; EGF, epidermal growth factor; FBS, fetal bovine serum; GST, glutathione S-transferase.
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contrast, JNK is reported to be required for transformation of a variety of animal cell types. The activation of c-Jun by NH2-terminal phosphorylation is required for the transformation of primary fibroblasts and other model cell systems by activated oncogenes such as Ha-ras (26–28), c-fos (28), v-src, c-raf, and v-sis (27–30). JNK may be an important mediator of transformation by other activated Ras family members (28, 31–37) and the Met oncogene (38, 39).

Although less information is available, Jun kinase may play an essential role in certain human cells (40–50). JNK and c-Jun are required for transformation by the Bcr-abl leukemia oncogene (44, 46). Transformation is accompanied by increased expression of the c-Jun-target genes catalase, glutathione S-transferase π, and multidrug resistance protein 1. The transformed phenotype includes increased drug resistance because of efficient drug efflux and rapid growth (51). All of these changes are reversed on expression of a nonphosphorylatable derivative, c-Jun(S63A,S73A; Ref. 51). In human A549 non-small cell lung cancer cells, JNK is activated by addition of EGF (41, 43) possibly in a phosphatidylinositol 3'-kinase-dependent manner (45, 47). Similarly, expression of the EGF receptor variant type III, a constitutively active naturally occurring mutation that is found in many types of human tumors, leads to transformation of NIH-3T3 cells, activation of JNK, and down-regulation of the mitogen-activated protein kinase/ERK pathway (40). Inhibitors of phosphatidylinositol 3'-kinase reverse these effects (40). In the case of human A549 non-small cell lung cancer cells, EGF causes preferential activation of JNK and a 2-fold enhancement of four characteristics of transformation: proliferation (41, 43), tumor-take rate (41), growth of xenografts (41), and colony formation in soft agar (41). The in vitro effects are completely inhibited by expression of c-Jun(S63A,S73A) or by application of specific antisense JNK2 oligonucleotides but not by antisense JNK1 oligonucleotides indicating that JNK2 may play a role in transformation of these cells (41, 43). Similarly, JNK2 is essential for growth of human T98G glioblastoma cells (52). When treated with antisense JNK2 but not antisense JNK1 or control oligonucleotides, these cells exhibit a marked elevation in the expression of the cyclin-dependent kinase inhibitor p21\(^{CIP1/WAF1}\), which is accompanied by inhibition of the Cdk2/Cdc2 kinase activities suggesting a possible mechanistic basis for the role of JNK2 in these cells (52).

Most studies suggest that JNK1 and JNK2 are functionally redundant (1, 53, 54). However, several studies describe contexts in which JNK1 plays a distinct role (55–61). In several cases, JNK1 preferentially mediates apoptosis (55–58). CD8+ T cells from JNK1-deficient mice challenged in vitro proliferate less than those from wild-type mice (62). Similarly, when challenged in vivo by viral infection, isolated CD8+ cells exhibit decreased mitogenic expansion, which was related to increased apoptosis indicating a role of JNK1 in survival functions in vivo (60). In contrast, CD8+ cells from JNK2-deficient mice exhibited enhanced expansion after a viral challenge taken to indicate a role of JNK2 in the mitotic expansion in vivo (60). Consistent with a role in proliferation, JNK2-deficient mice were observed to be resistant to induction of skin papillomas indicating that JNK2 may play a critical role in papilloma formation (63).

The in vitro studies of isolated cell types suggest a role for JNK, especially JNK2, in cell transformation. Therefore, a critical issue is to know whether JNK functions in this role in vivo. We examined a panel of 9 human prostate cell lines and observed that serum-stimulated growth is highly correlated with JNK activity. By applying antisense oligonucleotides specific for the JNK1 or JNK2 family of isozymes, we observed that antisense JNK2 greatly inhibited in vitro growth indicating that JNK2 is essential for serum stimulation of growth. Similarly, treatment of mice bearing established tumors of PC3 cells with antisense JNK2 and, to a lesser extent, JNK1 inhibited tumor growth, and treatment with combined antisense JNK1 and JNK2 lead to significant tumor regression. This appears to be the first in vivo result indicating that JNK may mediate malignancy and suggests a potential treatment modality.

MATERIALS AND METHODS

Cells and Reagents. Human prostate carcinoma cell lines DU145, LNCaP, and PC3 were obtained from American Type Culture Collection and are described,6 whereas ALVA-31, JCA-1, PPC-1, and TSU-Pr1 were a kind gift of John C. Reed, The Burnham Institute, La Jolla, CA. The 267B1 line is a SV40 immortalized normal neonatal prostate epithelial line, whereas Kiras-267B1 is a Kirsten-Ras transformed derivative, and both were kindly provided by John Rhim (48). JCA-1 (64) and TSU-Pr1 (65) were reported to be derived from T24 bladder carcinoma cells (66). The genetically altered PC3 clonal lines PC3mJun and PC3LHCX were prepared by calcium phosphate-mediated transfection (as described below) with either the “empty vector” retrovirus plasmid pLHCX where L is the retroviral long terminal repeat sequences, H is the hygromycin phosphotransferase encoding sequence, C is an abbreviated human cytomegalovirus promoter/enhancer, and X is a polylinker, and was prepared as described previously (67, 68) or transfected with pLHCmJun where mJun indicates the DNA sequence encoding expression of a mutant-c-Jun, which encodes alanine residues in place of serine residues at amino acid positions 63 and 73 (c-Jun(S63A,S73A); Ref. 49).

Human breast cancer cell line MDA-MB-361 was obtained from American Type Culture Collection and is described,6 Cell lines MDA-MB-231, MDA-MB-435, MDA-MB-453, and MDA-MB-549 were gifts from Joseph Lustgarten (Sloan Kettering Cancer Center, New York, NY), MCF-7, T47D, and ZR-75.1 are long standing achieved lines of this laboratory (49, 68–70). SKBR-3 was a gift of Eileen Adamson, The Burnham Institute, La Jolla, CA.

All of the cells were maintained in RPMI 1640 (LNCaP) or DMEM (low pyruvate, high glucose) in an atmosphere of 5% or 10% CO2, respectively, both with 5% FCS (Irvine Scientific, Santa Ana, CA). In addition, PC3LHCX and PC3mJun clones were maintained in the presence of 200 μg/ml of Hygromycin-B (Calbiochem, La Jolla, CA). High serum growth curves were determined in similar medium at 20% FCS (Irvine Scientific). Medium was prepared from premixed powder without pyruvate or NaHCO3 obtained from Sigma-Aldrich Co. or Irvine Scientific, Inc., and was used with supplemental NaHCO3 and glu-

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6 Internet address: http://www.atcc.org.
mine according to the manufacturer’s specifications. GST-c-Jun (1–223) was expressed in Escherichia coli kindly provided by Michael Karin, University of California at San Diego, La Jolla, CA. Antisense phosphorothioate compounds were synthesized, purified, and provided by Isis Pharmaceuticals Inc.

**Transfection.** Antisense and scrambled sequence control oligonucleotides were developed and characterized as described (41, 43). The sequence of antisense JNK1 is 5’- CTCTCTGT-TAGGCCCCGCTTGG - 3’ (Isis12539) and the sequence of antisense JNK2 is 5’ - GTCCGGGCCAGCCAAAGTC - 3’ (Isis12560). Both sequences are complementary to sequences that are common to the four family members of JNK1 and JNK2, respectively, and are referred to here as JNK1AS and JNK2AS. For in vivo studies a single-control phosphorothioate oligonucleotide consisting of a scrambled 20 nucleotide sequence used previously as a control in the analysis of antisense protein kinase c was chosen: 5’ - TCGCATCGACCCGCCCA-CTA-3’ (71). Both antisense JNK sequences contain a single CpG sequence, and such sequences are thought to have possible immunoefector cell-activating properties (72). The control oligonucleotide closely approximates the average composition of JNK1AS and JNK2AS but contains three CpG dinucleotides sequences and, therefore, also serves as a control for any antisense and scrambled sequence control oligonucleotide activity in athymic mice. The transfection of cells was performed as described (43, 73). Briefly, oligonucleotide at the concentration indicated in the text was added to DMEM containing 10 μl/ml of Lipofectin (Life Technologies, Inc., Rockville, MD) at 1 mg/ml original concentration. This preparation was added to 50–80% confluent cells seeded the previous day. After 4.5 h, the transfection medium was generally replaced with DMEM with 0.5% or 20% FBS except for TSU-Pr1 and PPC-1 cells, which were exposed to the lipofection mixture for 9 h.

Clonal lines of PC3 were prepared by calcium phosphate-mediated transfection as described previously (74). Briefly, 100-mm tissue culture plates at 75% confluence were prepared 1 day before transfection. For transfection, the medium was removed and replaced by 2 ml of 0.2% serum containing medium with freshly added calcium phosphate precipitated DNA (10 μg), and incubated for 18 h at 3% CO2. The plates were then supplemented with normal medium and, after 48 h, treated with 400 μg/ml Hygromycin-B. Half of the medium was changed to fresh medium every 3 days. Most cells died; however, after 10–20 days visible clones appeared, which were isolated by placing “cloning rings” coated on end with sterile silicon vacuum grease directly through the medium. Medium within the ring was removed, and the clone harvested by addition of 0.05 ml of trypsin (ATV; Irvine Scientific) followed by transfer on an well of a 96-well tissue culture plate with 1 ml of medium supplemented with 200 μg/ml of Hygromycin-B.

**In Vitro Growth Assay.** Growth curves were determined as described (43, 49). Briefly, cells were seeded into 24-well multwell tissue culture plates at 10–30,000 cells/cm2 at 0.5% or 20% FBS, and used for cell counting in quadruplicate. On alternate days, starting 3 days after seeding, four wells were harvested by removal of the medium, washing once with 0.5 ml PBS, and addition of 0.1 ml of trypsin (ATV; Irvine Scientific, Inc.). The resulting suspended cells were transferred to vials containing 9.9 ml of PBS and counted using a Coulter counter. Counting was continued through log-phase growth as defined by the observation of a plateau. The resulting curves were integrated and the integrated value observed in high serum (Gs) is expressed relative to basal growth in low serum (G0) as Gs/G0. Typically cells were counted in quadruplicate on the last 4 days of a 5-day experiment, thereby providing 20 measurements for the determination of integrated area, G, which can therefore determined precisely with SE of G determined from √(Σσ2), where the σ are the SEs estimated for the daily quadruplicate counts.

**In Vivo Growth and Kinase Assays.** Athymic Harlan Sprague mice were inoculated s.c. with 2 × 106 freshly harvested PC3 cells. On the development of visible palpable tumors, treatment was started (Fig. 4A, arrow). Two groups of 10–11 animals each were treated 6 days/week with 25 mg/kg i.p. or 0.2 ml of PBS of the control oligonucleotide or vehicle alone, and three groups of 15 animals each were treated with equal amounts of JNK1AS, JNK2AS, or half-concentrations of both antisense oligonucleotides. Tumor volumes (Fig. 4) were estimated by measurement (calipers) of the greatest dimension, l, and the perpendicular dimension, w, and evaluated using the formula V = (π/6)(lw2; Ref. 75). The volumes of all tumors of a group were averaged for construction of a growth curve. Growth curves are intended to estimate solid tumor volume, and any tumor that became discolored, pitted, or ulcerated was excluded from the group (see text). All of the growth curves were statistically compared with each other using all of the tumor volume estimates measured during the treatment period (Fig. 3, arrow) but excluding measurements of any tumors with visible defects by ANOVA as implemented using Systat (Evanston, IL) software and including corrections for multiple comparisons (Bonferroni correction). Jun kinase activity of the tumors was determined exactly as described previously (41, 73).

**RESULTS**

**Serum-supported Growth of Human Prostate Cancer Cell Lines Correlates with Jun Kinase Activity.** A major goal of this study was to test the role of JNK in human prostate cells in vivo. Thus, the most appropriate medium for examination of the in vitro counterpart was restricted to serum-containing medium without other growth-promoting supplements. Nine human prostate cell lines were examined in basal (low serum, 0.5%) and stimulating (high serum, 20%) conditions, and the resulting JNK activity and growth were compared. When compared with their growth in low serum, all of the lines exhibited readily detectable JNK activity even in low, 0.5% serum, suggestive of constitutive activity (Fig. 2A, top). As for growth, when the cell lines were treated with medium containing 20% serum, the cell lines exhibited a range of serum-stimulated JNK activities from 1.2 to >9-fold (Fig. 2A, bottom). To compare serum-supported growth (Gs) and JNK activity, both the growth (integrated growth curve) and JNK activity were expressed as a multiples of the parallel determinations.
made in the basal medium with 0.5% serum ($G_B$; Fig. 1). Serum-stimulated growth of all of the carcinoma cell lines appeared to be directly proportional to the serum-inducible JNK activity (Fig. 1A). In contrast, one normal cell line of embryonic prostate epithelium, 267B1, exhibited the maximum observed serum-inducible JNK activity, which was not linked with proportional growth, whereas the Ki-ras-transformed 267B1 cells behaved similar to the human prostate carcinoma-derived cell lines (Fig. 1A). Thus, a consistent relationship was observed indicating that proliferation of prostate carcinoma cells in serum is proportional to JNK activity. Indeed, the relationship is precise with a Pearson correlation coefficient of 0.91 ($P = 0.004$, Bartlett $\chi^2$; Fig. 1A, legend).

Recently, it has been argued that two lines, JCA-1 and TSU-Pr1, are derived from bladder carcinoma T24 cells (66). The results for these lines are closely clustered consistent with a high degree of relatedness (Fig. 1A). When excluded from the linear analysis, the $r_{\text{Pearson}}$ is not greatly altered ($r_{\text{Pearson}} = 0.86$), and the probability remains significant although higher ($P = 0.027$). Thus, similar conclusions follow when the results for JCA-1 and TSU-Pr1 are excluded. Nevertheless, to examine the specificity of the relationship to prostate cells, similar studies were carried out with a panel of 9 human breast cancer cell lines (Fig. 3). The breast cancer cell lines also exhibited a range of serum-stimulated JNK values similar to the prostate carcinoma cells. These cell lines also exhibited a range of serum-stimulated growth values. However, with one exception, the serum-stimulated growth values were considerably less than those observed for the prostate carcinoma lines. Moreover, even within this range, growth appeared to be largely independent of JNK activity. Indeed the Pearson correlation coefficient is negative ($r_{\text{Pearson}} = -0.32$). Thus, the linear relationship between serum stimulation of JNK activity and growth demonstrated by the prostate cell lines does not appear to be an inevitable or common association to transformed cells of differing origins.

JNK2 Is Preferentially Required for Serum-stimulated Growth. To determine whether a causal link exists between JNK activity and the growth of prostate cancer cells, we used characterized antisense oligonucleotides (41, 52, 73, 76). These oligonucleotides are complementary to sequences common to all four of the alternate splice variants that make up both the JNK1 or JNK2 isoform families, and are termed JNK1AS and JNK2AS, respectively. They have been shown to function at low concentrations (0.2–0.4 $\mu$M) to preferentially reduce JNK mRNA and protein. Protein suppression occurred for 48–72 h after a single treatment or approximately two cell-doubling periods leading to a decrease in cumulative growth that, in the case of A549 cells, was detectable up to 14 days after treatment (73). Thus, these reagents would be expected to substantially reduce growth of cells that use either JNK1 and/or JNK2. Cells plated 24 h previously were treated with JNK1AS, JNK2AS, control oligonucleotides composed of a scrambled sequence of each antisense oligonucleotide, the lipofection agent alone (mock transfection), or were not treated. For proliferation studies the cells were treated 1 day after plating to generate a nadir of JNK target protein levels through log-phase of growth, days 2–4 (“Materials and Methods”). Growth was monitored for an additional 4 days after treatment. Net growth for the >60 observed curves was characterized by calculation of a single number, the integrated value of the growth curves termed $G_n \pm SE$ for basal growth and $G_s \pm SE$ for serum-supported growth as described (“Materials and Methods”).

Treatment with either scrambled sequence control oligonucleotide had little effect. When expressed relative to mock-transfected cells, the average inhibition of proliferation for treatment of all of the prostate carcinoma cell lines with the scrambled control oligonucleotides was low (3.7 ± 1.5%). Thus, for Fig. 1B, inhibition by the antisense oligonucleotides is expressed relative to their respective scrambled control oligonucleotide. As for previous studies (41, 43, 52), preferential...
suppression of protein expression by JNK1AS and JNK2AS was examined (Fig. 2B). For example treatment of cells with JNK1AS preferentially reduced steady-state protein by >80%, whereas JNK1 protein was decreased by 25%. Conversely, treatment of cells with JNK2AS also preferentially reduced steady-state JNK2 protein by >80% (Fig. 2B). Although JNK1 protein was also reduced, there was a preferential reduction of JNK2 protein. These results indicate that for each antisense compound a relative multiple of 3–4-fold less steady state protein may be achieved by a single treatment.

Treatment with JNK1AS (Fig. 1B) produced little additional inhibitory effect on the growth of any cell line relative to scrambled control, and the average inhibition is 7.0 ± 4.1% (Fig. 1B, blue). In contrast, transfection with the JNK2AS oligonucleotide led to a marked inhibition of growth (Fig. 1B, red). First, a clear trend is apparent. The sensitivities of the various cell types to inhibition by JNK2AS increased in proportion to the ability of serum-containing medium to activate JNK (compare Fig. 1, A and B). Therefore, a very similar rank-order distribution of cell types along either the growth inhibition or the JNK activity axis (Fig. 1B) is observed as for the order along the respective axes in Fig. 1A. Thus, LNCaP cells are the most growth responsive cells and the most sensitive to inhibition by JNK2AS leading to >75% growth inhibition of a 4-day growth curve after a single treatment on day 1 (Fig. 1B). Conversely, KiRas-267B1 and DU145 were among the least responsive to serum (Fig. 1A), and least sensitive to inhibition of JNK2 expression by JNK2AS (Fig. 1B). Second, each cell line, including those of low responsiveness to serum, exhibits a statistically significant (P < 0.007, Student’s t test) increase in inhibition on treatment with JNK2AS compared with treatment with JNK1AS (Fig. 1B). Again cell lines TSU-Pr1 and JCA-1 exhibit similar results to each other (Fig. 1B).

Treatment of cells with JNK2AS leads to markedly decreased JNK2 protein level and to a much lesser extent, decreased JNK1 protein. Because treatment with JNK1AS had little effect on growth of any cell line, whereas treatment with JNK2AS is effective at promoting a significant reduction in growth of all of the cell lines examined, these results indicate that one or more JNK2 isoforms and likely none of the JNK1 isoforms are commonly required for the proliferation of prostate carcinoma cells in vitro.

Jun Kinase Is Required for Prostate Tumor Growth as Xenografts. To determine whether the results are indicative of the roles of JNK1 and JNK2 in tumor growth, in vivo tumor studies were undertaken (“Materials and Methods”). The biodistribution of phosphorothioate oligonucleotides in the mouse have been studied extensively and include the recovery of intact oligonucleotides from s.c. tumors (77) indicating that the use of antisense oligonucleotides in vivo after an established regimen may be useful for determining the role of JNK in tumor growth. For this test, human prostate carcinoma PC3 cells were chosen as representative, because they are known to be tumorigenic and because PC3 cells exhibit near average serum-inducible JNK activity and proliferation values (compare Fig. 1, A and B). After inoculation of athymic male mice, tumors were allowed to establish and grow to readily palpable and visible xenografts before beginning systemic treatment (Fig. 4A, down arrow). Systemic treatment was achieved by daily i.p. injections of JNK1AS, JNK2AS, JNK1AS + JNK2AS, a single scrambled sequence oligonucleotide (“Materials and Methods”), or vehicle (PBS) alone for 27 days (Fig. 4A).

The effects of antisense treatment on tumor JNK activity were examined in comparison to cultures of pure PC3 cells. As indicated in Fig. 1A, the JNK activity of these cells varies with the growth condition leading to a >6-fold decrease in activity for cells maintained in 0.5% serum when compared with serum-supported cells (Fig. 2C). In the case of tumors, extracts of tumors from control animals treated with the scrambled sequence oligonucleotide exhibited half the maximum value observed for a culture of PC3 cells maintained in high serum in vitro (e.g., Fig. 2C, “scrambled”). Treatment of tumor-bearing animals with JNK1AS or JNK2AS leads to additional reductions of 2.5- and 5-fold, respectively, compared with extracts of
scrambled sequence-treated animals (Fig. 2C). Treatment with an equimolar mixture of JNK1AS and JNK2AS at the same total dose as for the separate treatments led to a >5-fold decrease compared with an expected 6.25-fold decrease. These observations are consistent with previous studies of the biodistribution and efficacy of phosphorothioate oligonucleotides in mouse models (77), and provide supporting evidence that systemic treatment with antisense JNK oligonucleotides affectively reduced target protein activity in vivo.

On the basis of the integrated values of the tumor growth curves (Fig. 4A), treatment with both JNK1AS and JNK2AS lead to substantial inhibition of tumor growth of 57% and 80%, respectively. To examine the significance of the differences between growth curves in this study, all of the measurements of tumor volume for each growth curve from the start of treatment to the end of treatment, typically ~60 observations, were analyzed by ANOVA analysis (“Materials and Methods”). Thus, the inhibitions of 57% and 80% observed for treatment with JNK1AS and JNK2AS, respectively, are significant when compared with either control curve with probabilities of <0.0002. Moreover, the increased inhibition of JNK2AS compared with JNK1AS is also significant (P = 0.012). Combined antisense treatment lead to an inhibition of 78% compared with the average of JNK1AS alone and JNK2AS alone of 70%. Thus, these results support a role for both JNK1 and JNK2 in tumor growth with JNK2 playing a dominant role.

During the course of treatment, from approximately day 20, it was observed that an increasing number of tumors developed small darkened spots or blemishes invariably at the most distal site of the tumor. These sites became small breeches in the epithelium (ulcers) followed by progressive and massive caviation of the tumors. This process is illustrated in Fig. 4C. These defective tumors, whether discolored or ulcerated, were not used for additional volume averaging for tumor growth plots of Fig. 4, A and B. The occurrence of frank ulcers was readily recognized and, for quantification, the cumulative occurrence is summarized in Fig. 4D. By day 38 only three groups remained with tumors that were free of either ulceration or the preulceration discoloration (Fig. 4B). Thus, after the end of treatment, two populations, intact tumors and ulcerated tumors, were followed.

For the intact tumors, after a lag phase of approximately 1 week after the end of treatment, the tumors of the antisense JNK1-treated animals exhibited a burst in tumor growth leading to a final growth rate (slope; Fig. 4B) considerably greater that of either control group. These observations suggest that viable but arrested tumors of the JNK1AS-treated group retained the ability to grow on cessation of treatment.

Treatment with Combined Antisense JNK1 and JNK2 Is Associated with Regression in High Frequency. Of the discolored tumors, all progressed to develop ulcers that expanded leading to regression of the tumor masses. The regression process can be followed by measuring the diameter of the ulcer cavities and by measuring the frequency of the development of ulceration for each experimental group (Fig. 4D). As the average ulcer sizes approached the maximum observed values, the visible tumor mass retreated to become a low circumferential rim, and typically completely vanished leading to flat dry lesions (Fig. 4D, inset). In some cases tumor regression was preceded by undermining the skin around a small ulcer mouth leaving opposite edges of the circumference in proximity to each other (Fig. 4C), and in one case the ulcer edges reunited to form a healed site and apparent tumor-free animal (Fig. 4C, circle).

Histology sections of tumors at the various stages of regression show that distal ulceration is preceded by subepithelial microabscess formation (Fig. 5) together with an infiltrate of acute inflammatory polymorphonuclear leukocytes (neutrophils) in the surrounding dermis suggesting that devitalization stimulates an acute inflammatory response.

Focal infiltrates of these cells are evident around the base of the expanding ulcer (data not shown) indicating that these cells are responsible for the progressive ulceration. The time course of regression (Fig. 4D) also indicates that the process, once started, typically extended well beyond the end of the antisense treatment period (“end” arrow, Fig. 4, A, B, and D) suggesting an inflammatory mechanism of continued erosion. Therefore, it is the frequency of initiation of ulceration, i.e., Fig. 4D, rather than the actual erosion that is taken as the more relevant variable in relating regression to the treatment regimen. Remarkably, all 14 of the extant animals of the combined JNK1AS + JNK2AS-treated group developed distal ulcers,
which rapidly lead to complete regression of tumors characterized by flat, encrusted sores with an average diameter of 1.4 cm (e.g., Fig. 4D, inset). \( \chi^2 \) analysis of the increased frequency of regression in the JNK1AS + JNK2AS-treated group compared with the next most commonly regressing group indicates that the increase is significant \( (P = 0.022) \).

Spontaneous ulceration in tumors of control animals also occurred occasionally (Fig. 4D, controls); however, this process may be distinct. Spontaneous ulceration occurred late, day \( \geq 26 \) (Fig. 4D), at time when these control tumors were very large, \( >1000 \text{ mm}^3 \) (Fig. 4A), whereas treatment-induced ulceration occurred in tumors commonly \( \sim 300 \text{ mm}^3 \) (compare Figs. 4, A and D). The spontaneous ulceration of large tumors involved fewer animals with a maximum incidence of \( <30\% \) (scrambled sequence control, Fig. 4D). These results indicate that, unlike cytostasis (Fig. 4A), both antisense JNK1 and JNK2 treatment are required for efficient tumor devitalization and regression of small tumors. Conversely, no tumors of the control groups that were of comparable small size, \( \sim 300 \text{ mm}^3 \), ulcerated or regressed. Thus, the sum of observations indicates that systemic treatment of tumor-bearing mice with of combined antisense JNK1 and JNK2 is specifically and significantly associated with induction of a process that culminated in tumor regression for all of the extant animals (14 of 15) of the treatment group. These results provide evidence for a role of JNK in growth and survival of the human PC3 prostate cell as xenografts in vivo.
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DISCUSSION

JNK2 Mediates Growth of Prostate Carcinoma Cells in Vitro. Previous in vitro studies implicate JNK2 as mediating transformation of certain human tumor cell lines suggesting that JNK may be of importance in the development of cancer. The principal goal of this study is to test whether JNK plays this role in vivo. A second goal is to determine whether there is a distinction between the JNK1 and JNK2 families. Finally, the generality of the growth-promoting role was examined in prostate carcinoma cells. For the in vitro studies, a simple serum-based regimen was examined, because the use of defined system with defined growth factors would complicate comparison to the in vivo setting where the tumor cell environment is an extravascular ultrafiltrate of blood closely approximating serum. The in vitro studies revealed that the serum-supported proliferation rate of the human prostate carcinoma cell lines studied here is highly correlated with JNK activity ($r_{\text{Pearson}} = 0.91; P < 0.004$), whereas a panel of 10 human breast carcinoma lines show no such pattern ($r_{\text{Pearson}} = -0.32$) suggesting a specific mechanism. The comparison of multiple cell lines even when related to a single origin such as malignant prostate epithelium is difficult as basal growth rates in serum vary. Therefore, we examined their long-term response to the addition of serum to a basal condition, 20% serum versus 0.5% serum. To determine whether JNK is causally linked to the proliferation response, the expression of JNK1 and JNK2 were preferentially inhibited by the use of characterized antisense oligonucleotides that have been used in previous studies (41, 43, 76). It was observed that serum-supported growth is specifically inhibited by JNK2AS but is nearly unaffected by two different scrambled sequence control oligonucleotides and only minimally affected by JNK1AS. Maximum inhibition of proliferation was $\sim 75\%$. This limit may reflect the fact that for a single treatment, the decrease in target mRNA and protein levels is limited to about 80–90% completion (e.g., Fig. 2). In addition, proliferation is measured over the course of 5 days, whereas the effective “window” of the antisense treatment was days 1 through 4 (73). Thus, some recovery of growth after day 4 is possible; however, this was not reflected in the shape of the growth curves, which typically approach the plateau phase of growth. Furthermore, it cannot be excluded that JNK-independent pathways exist, which mediate growth even in the most JNK-dependent cell line observed here.

All of the cell lines exhibited a significantly increased inhibition of growth when treated with JNK2AS compared with JNK1AS. Furthermore, when considered collectively the rank order of cell types for growth inhibition after treatment with JNK2AS (i.e., Fig. 1B) is similar to that for serum stimulation of growth before treatment (i.e., Fig. 1A). These results are expected if the activation JNK2 is causally related to growth. The results are supported by observations of the control cell line. Although only a single nontransformed prostate epithelial cell line was available for comparison (267B), the 9 unrelated breast cancer cell lines are informative. Two lines, MDA-MB-231 and MCF-7, exhibited the highest serum-induced growth consistent with previous studies indicating that c-Jun functions in transformation (18, 50). However, no trend was apparent, and indeed the overall correlation is negative ($r_{\text{Pearson}} = -0.32$). Preliminary studies indicate that treatment in antisense JNK had no significant effect (data not shown). The sum of results indicates that JNK-dependent cell and tumor growth is associated particularly with prostate carcinoma and that these carcinoma cells exhibit a common but individual level of sensitivity to JNK2-mediated growth.

JNK1 Is Required from Growth in Vivo. In contrast to expectations based on the present (Fig. 1) and previous in vitro results (43, 52, 73), treatment of tumor-bearing animals with JNK1 alone revealed a significant decrease in tumor growth compared with treatment with vehicle alone or with the scrambled sequence control oligonucleotide. Moreover, this is likely to be an underestimate of the role of JNK1 in vivo as the elimination of JNK1 expression by antisense is likely to be incomplete. The basis of the difference of the role of JNK1 in...
vitro and in vivo is not known but may reflect the fundamentally different nature of growth of cells on a treated plastic substratum in vitro and growth in vivo. Autonomous growth in vivo requires the development of a stroma composed of connective tissue-based support network, a vascular network, and, variably, a lymphatic network. Furthermore, the environmental factors and signals present in vivo may not be provided by the use of serum-supplemented medium in vitro (78). These distinctions may lead to differences in genes expression by PC3 cells in vitro and in vivo. We have used Affymetrix-based expression analysis to compare the expression of PC3 cells in vitro to those in vivo as s.c. tumors, orthotopic implants, or as regional lymph node metastases (79). For example, 214 genes were found to be significantly differentially expressed by PC3 cells when compared with normal prostate epithelial cells both grown in vitro. However, when orthotopic tumors of PC3 cells were analyzed, >40 of the genes exhibit expression in the opposite direction (79). These observations provide strong evidence that the growth requirements of PC3 cells in vitro and growth in vivo are distinct from those in vivo. Some of these differences may be attributable in part to transcription factors activated by JNK1. Other differences may lead to protein expression, which, directly or indirectly, alters the activity of JNK1 and its role in vivo. Thus, there are a number of potential distinctions between the roles of JNK1 in vivo and in vitro. Additional mechanistic studies will be required to distinguish these possibilities.

Both JNK1 and JNK2 Have Distinct Roles during in Vivo Growth of PC3 Prostate Cells. A role for JNK1 in vivo is additionally illustrated by the observation that induction of the regression of the growth-arrested tumors with high frequency (14 of 15 animals) required both antisense JNK1 and antisense JNK2, whereas the frequency of regression for either treatment alone was not significantly greater than that associated with spontaneous regression of large tumors. These results also indicate that the induction of regression of established tumors is likely distinct from the mechanism of tumor cytostasis, which was promoted efficiently by JNK1AS alone. Because inflammatory cells are present in the affected tumors even before the first visible signs of tumor breakdown, we speculate that combined antisense JNK1 and antisense JNK2 treatment promotes a cytoidal event, and that the resulting dead cells stimulate a cellular inflammatory response leading to a regression process. Consistent with this, we have observed that JNK2 but not JNK1 plays a survival role in human glioblastoma T98G cells (52). Inhibition of JNK2 by JNK2AS leads to permanent growth arrest with features of a senescence-like state, whereas treatment with JNK1AS leads to decreased JNK1, which is entirely reversible. These results indicate that JNK2 is required for survival of T98G cells (52). Furthermore, recent studies indicate that JNK1 isoforms also plays a survival role by inhibiting apoptosis, and that elimination of JNK1 leads to an increase in apoptosis (55, 56). Thus, it appears possible that the combined elimination of JNK1 and JNK2 may promote cell death in greater proportion than observed by either treatment alone. In vivo, apoptotic and dead cells liberate potent chemo-attractants of acute inflammatory cells leading to the infiltration of tissues bearing dead cells by neutrophils (80). Accordingly, the neutrophil-mediated regression process itself may not be specific, whereas the high frequency with which the process is initiated appears to be specific to the combined use of JNK1AS and JNK2AS. The sum of results indicates that JNK1 and JNK2 play distinct but essential roles in the growth of PC3 cells as xenografts. It will be of interest to learn whether specific mechanisms such as apoptosis are involved in determining the frequency of regression.

We conclude that JNK and, in particular, one or more isoforms of JNK2 is commonly required for growth of prostate carcinoma cells in vitro and as xenografts in vivo. Moreover, in vivo JNK1 is also required. Furthermore, NH2-terminal phosphorylation of the JNK substrate c-Jun appears to be required for the initiation of tumor growth. The results indicate a novel role for JNK as a mediator of tumorigenesis and growth in prostate carcinoma, and suggest that antisense strategies merit additional examination as a novel treatment modality.

ACKNOWLEDGMENTS

We thank John Reed for providing ALVA-31, JCA-1, and TSU-Pr1 cells; Eileen Adamson, for commenting on preliminary manuscripts and Connie White for clerical assistance.

REFERENCES


45. Reddy, P., Brown, P. H., and Birrer, M. J. cJun overexpression in...


