Characterization of Sarcoma Cell Lines from v-jun Transgenic Mice

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ABSTRACT

Wounding is a prerequisite for tumor formation in v-jun transgenic mice. The progression from wound to dermal sarcoma is a multistep process which, at some stage, results in an increase in transgene mRNA expression in tumor tissue. However, transgene expression in individual sarcoma cell lines was clonal. We cloned several cell lines from wound-related v-jun transgenic tumors to determine whether a relationship existed between the cellular growth properties and structure, expression, or function of the transgene. Cell lines with very high v-jun expression had a high cloning efficiency in soft agar and tumorigenicity in nude mice. However, for cell lines with an intermediate or low level of transgene expression there was no correlation between transgene expression and the transformed phenotype. There was also no correlation between transgene expression and individual cell line morphologies, growth rates, transgene genomic DNA copy number, or mRNA expression of jun-related genes. The tumor cell subclones (1-20-2, 2-24-3) with very low transgene expression, very poor cloning efficiency, and low tumorigenicity also showed reduced activator protein 1 DNA binding activity and had an increased expression of endogenous c-jun when compared to other tumor cell lines. Transfection of a v-jun expression vector into cell lines with poor cloning efficiency and low tumorigenicity enhanced both in vitro cloning and in vivo tumor formation. However, such overexpressed v-jun had no effect on NIH3T3 cells. Our studies show that expression of the v-jun transgene contributes to the transformed phenotype of tumor cell lines but that there are additional factors that determine growth properties in culture and in the animal.

INTRODUCTION

The v-jun oncogene was originally isolated from avian sarcoma virus 17 (1). This virus induces fibrosarcomas at the site of inoculation in chickens and transforms chicken embryo fibroblasts in vitro (1, 2). The protooncogene c-jun encodes a nuclear protein that is a major component of the AP-1 complex (3, 4). Overexpression of the c-Jun protein is growth stimulatory in avian cell cultures, but it is not tumorigenic (5-7). In primary cultures of mammalian cells overexpressed v-Jun or c-Jun does not induce a detectable change in growth properties, but coexpression with a mutationally activated Ha-ras gene leads to cotransformation (8). Endogenous c-jun expression is enhanced in response to a variety of oncoproteins and growth factors (9, 10), and overexpression of activated Ha-ras enhances c-jun transactivation by serine phosphorylation in the transactivating domain of the protein (11-13). These observations suggest that changes in Jun expression and activity are linked to altered growth properties of the cell.

Wounding is a prerequisite for tumorigenesis in transgenic mice carrying the germline v-jun under the control of the promoter from the murine major histocompatibility complex I gene H2Kk (14). After wounding, 85% of v-jun homozygote mice develop abnormal hyperplastic granulation tissue at the site of the wound, followed 3-4 months later by the formation of a dermal fibrosarcoma in a further 25% of cases. There is a 5-10-fold increase in transgene mRNA expression in sarcoma tissue compared to normal skin in these animals. Tumor cross-sections, however, show a heterogeneous pattern of transgene expression (15).

We have cloned and characterized a series of cell lines from wound-related tumors formed in H2Kk-v-jun transgenic mice. In order to understand the role of v-jun in this model of tumorigenesis we evaluated the relationship between the cellular growth properties, transgene structure, expression, and function.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Clonal cell lines from wound-related dermal sarcomas were cultured in Iscove's modified Dulbecco's medium (Irving Scientific) buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid supplemented with 10% fetal bovine serum, penicillin, streptomycin, nonessential amino acids, and β-mercaptoethanol. The tumor cell lines were all derived by limiting dilution (14). v-jun transgenic embryo fibroblasts were prepared according to the method of Freshney (16) from 17-day-old embryos of the homozygous Tg2 lineage (14). Both the NIH3T3 cells and the v-jun transgenic embryo fibroblasts were cultured in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mM), glucose (4.5 g/liter), 10% fetal bovine serum, and penicillin/streptomycin.

Southern and Northern Analysis. Total genomic DNA was digested, electrophoretically size fractionated, transferred to nylon filters, and hybridized using standard techniques (17). Total cellular RNA was isolated using the method of Chomczynski (18) and then passed several times over an oligo(dT)cellulose column. Two to 5 μg of poly(A)-selected RNA was denatured for 10 min at 65°C in 50% formamide (v/v), 3% formaldehyde, 0.4 μg boaric acid, and 0.02 μl EDTA and separated through a 1.0% agarose/3% formaldehyde gel then transferred in 20× standard saline citrate (NSS), 0.15 μl, Natrium citrate, 0.3 μl) in nylon filters. Hybridization was performed using standard techniques (17). All Southern and Northern hybridizations were washed in stringent conditions (0.2% sodium dodecyl sulfate at 65°C).

Polymerase Chain Reaction. Cell protein extract preparation, Western blotting analysis, and gel shift mobility assays were performed as described in the accompanying paper (19).

Anchorage-independent Growth. The efficiency of colony formation in soft agar was measured by the method of McPherson and Montagnier (20). 10 medium supplemented with 10% fetal bovine serum was mixed with agarose at a final concentration of 0.3% and 0.02 μl EDTA and separated through a 1.0% agarose/formaldehyde gel then transferred in 20× standard saline citrate (NSS), 0.15 μl, Natrium citrate, 0.3 μl) in nylon filters. Hybridization was performed using standard techniques (17). All Southern and Northern hybridizations were washed in stringent conditions (0.2% sodium dodecyl sulfate at 65°C).

Plasmids and Probes. The v-jun probe used on Northern and Southern analyses was a 0.9-kilobase BamHI-EcoRI fragment from pgS-5-1 containing the v-jun coding sequence from avian sarcoma virus 17 (1). The following probes were used in Northern analysis: a 1.0-kilobase Psrl/HindIII fragment from the human c-fos cDNA and a 1.4-kilobase Styl fragment from the murine c-jun cDNA, both kindly provided by Dr. I. Verma; a 1.3-kilobase Psrl fragment from the murine c-ets-2 cDNA, kindly provided by Dr. T. Papas; a 1.5-kilobase EcoRI fragment from the murine JunB cDNA and a 1.7-kilobase EcoRI murine JunD cDNA, both kindly provided by Drs. D. Nith and K. V. B. S.

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RESULTS

Wound-related Tumors of v-jun Transgenic Mice Give Rise to Various Continuous Cell Lines. Clonal cell lines were derived from wound-related dermal sarcomas forming in the tail of v-jun transgenic mice of two different parent transgenic lineages (14) known as Tg2 and Tg4 (Table 1). The F3-1-1 line was obtained from a mouse of the Tg2 lineage. The L2 and L1-1 lines were derived from 2 different tail fibrosarcomas which formed at different times in the same mouse of the Tg4 lineage. The L1-1 line was injected into athymic nu/nu mice, and the tumor that formed was used to generate the N2a line. The clonal L1-1 cell line was further subcloned by limiting dilution to generate different lines. Therefore, differences between L1-1 subclones reflect mutations occurring in cell culture. All these cell lines represented both the population of cells heterogeneous for v-jun expression during in vivo tumorigenesis and cells with acquired mutations conferring a proliferative advantage in vitro. We went on to characterize the tumor cell lines and cells derived from Tg2 mouse embryos for the relationship between the v-jun transgene and the transformed phenotype.

Growth Properties of v-jun Transgenic Tumor Lines Are Heterogeneous. Cloned and subcloned cell lines exhibited diverse morphologies, from flat cells with minimal cytoplasmic processes (L2) to more compact cells with longer cytoplasmic extensions (3-24.3). Some cell lines also demonstrated myogenic abilities and were able to fuse into myotubes. This diversity of morphological appearance was also present among subclones of L1-1.

Growth Properties of v-jun Transgenic Cells Are Imperfectly Correlated to Transgene Copy Number or Expression. Because of the wide heterogeneity in biological properties we investigated transgene structure and expression in the tumor-derived cell lines. Southern blot analyses of genomic transgene fragments are presented in Fig. 1. They show that the transgene copy number varies between 35 and 130 across all of the cell lines. There was no evidence of deletion or rearrangement of transgene DNA. The tumor cell line F3-1-1 from the Tg4 transgenic lineage had a 3- to 4-fold higher transgene copy number than the other cell lines. It may be significant that this line also showed the highest efficiency of agar colony formation, was very tumorigenic, and showed relatively high AP-1 DNA binding activity (vide infra).

Growth Properties of v-jun Transgenic Cell Lines Are Revealed in Northern Blot Analyses. Northern blot analysis of the transgenic cell lines revealed wide heterogeneity in the level of transgenic messenger RNA expression (Fig. 2). The amount of poly(A)-selected RNA hybridizing with the v-jun probe varied by a factor of 100 among the cell lines. There was a 1.8-kilobase EcoRI fragment from the murine stromelysin cDNA, kindly provided by Dr. L. Matrisian. The v-jun expression vector pRc/RSV v-jun was constructed by cloning the 1.8-kilobase XbaI fragment from the previously described vector RCAS-VJ-0 (21) into the XbaI site in the expression vector pRc/RSV (Invitrogen), which also contains the selectable marker gene for Neomycin (G418) resistance. Expression of the gag-jun coding sequence vector pRc/RSV into the XbaI site in the expression vector pRc/RSV (Invitrogen), which also contains the selectable marker gene for Neomycin (G418) resistance. Expression of the gag-jun coding sequence was then under the control of the promoter from the Rous sarcoma virus long terminal repeat. All double-stranded DNA probes were labeled with [α-32P]dCTP by the random primer labeling method.

Transfections. Transfections were performed as described in the accompanying paper (19).
a rough correlation between mRNA transgene expression and agar colony formation. Very low (1× basal level on Fig. 2) mRNA expressers were all very poor agar colony formers. There was less correlation between cell morphology and transgene mRNA expression. Some cell lines with flat morphology (transgenic embryo fibroblasts, F3-1-1, L2) showed a high level of jun mRNA, while the transgene mRNA was undetectable in other cell lines (e.g., 3.24.2) with a more rounded, transformed morphology.

A semiquantitative determination of Jun protein expression in Western blotting assays (Fig. 3) showed a correlation between mRNA levels and Jun protein quantities in individual cell lines. Surprisingly, cell lines with low transgene expression (1× basal level in Fig. 2), very poor efficiency of anchorage-independent growth, and low tumor formation (i.e., 1-20.2 and 3-24.3) had an increased expression of the endogenous cellular Jun protein. This inverse relationship between transformation and expression of cellular Jun has also been noted in other cell systems (22).

AP-1 Activity in Transgenic Tumor Cell Lines Is Partially Correlated with Growth. The v-Jun protein would be expected to form homodimers or heterodimers with endogenous Jun-related proteins, bind DNA at the AP-1 consensus sequence, and influence the transcriptional regulation of target genes. We tested the possibility that loss of Jun expression might correlate with loss of DNA binding or altered protein DNA complex mobility. We performed gel mobility shift assays using equal amounts of nuclear protein extracts of transgenic cells grown in log phase bound to the AP-1 consensus sequence (Fig. 4). Cell lines 1-20.2 and 3-24.3 with low transgene expression (1× basal level) and very poor cloning efficiency had a low AP-1 DNA binding activity. There was otherwise no correlation between transgene expression or cloning efficiency and AP-1 DNA binding activity.

Expression Levels of jun-related and jun Target Genes Do Not Reflect Growth Properties of jun Transgenic Tumor Cell Lines. Differential expression of genes known to influence the oncogenicity of jun in other systems might be expected to affect the relationship between v-jun expression and the transformed phenotype in our cell lines. AP-1 activity might be influenced by the altered expression of endogenous genes such as junB, junD, c-jun, c-fos, and c-ets, the protein products of which are capable of interacting in a positive or negative manner with v-jun at the level of gene regulation (23-26). Alternatively, oncogenicity in this system may relate to the altered expression of endogenous AP-1 target gene such as stromelysin. We measured the mRNA expression of these genes in the tumor cell lines by hybridizing the same two Northern blots from Fig. 2 sequentially with probes for each of the genes (in each case following removal of the previous probe) (Fig. 5). As controls, these blots also carried

Fig. 2. v-jun transgene expression in sarcoma cell lines. Northern blot analysis of poly(A)-selected RNA from transgenic sarcoma cell lines, transgenic mouse embryo fibroblasts (transMEF), NIH3T3 mouse fibroblasts as negative control, and chicken embryo fibroblasts infected with avian sarcoma virus 17 (CEFv-jun) as the positive control. A v-jun-specific probe was used. Two bands were observed at 1.9 and 2.1 kilobases. The blots were scanned with an LKB ultroscan XL laser densitometer; numbers below each lane, fold expression of v-jun mRNA as compared to the level of the lower expressor (3-24.3) set at 1.0.

Fig. 3. Jun protein expression in sarcoma cell lines. Western blot analysis of proteins extracted from sarcoma cell lines, transgenic mouse embryo fibroblasts (transMEF), murine embryo carcinoma P9 cells (known to have a low endogenous jn expression), murine NIH3T3 fibroblasts (used as a negative control for v-jun expression), and chicken embryo fibroblasts infected with viruses expressing viral jun (CEFv-jun) or mouse c-jun (CEFmc-jun). Proteins were separated by electrophoresis, transferred, and probed with a polyclonal antiserum made against the carboxy terminus of Jun.
Fig. 4. AP-1 DNA binding activity in the sarcoma cell lines. Band-shift assay using a labeled synthetic AP-1 oligonucleotide and cell extracts from the sarcoma cell lines, transgenic mouse embryonic fibroblasts (transMEF), murine embryonal carcinoma F9 cells, murine NIH3T3 fibroblasts, and chicken embryo fibroblasts infected with viruses expressing viral jun (CEFv-jun), or mouse c-jun (CEFmc-jun). Gels were run for 6 to 7 h to allow separation between the v-jun- and c-jun-containing complexes. Parallel shorter runs were conducted to ensure that the probe was present in large excess (results not shown). The specificity of the complex was verified by showing that the addition of an excess of nonlabeled AP-1 oligonucleotide or polyclonal anti-Jun anti-serum in the reaction disrupted its formation.

mRNA from NIH3T3 cells transformed by activated Ha-ras. Allowing for differences in mRNA loading, there was an increase (3-5X) in c-jun mRNA expression in 1-20.2 and 3-24.3 cell lines with low transgene expression (1X) and very poor cloning efficiency. Endogenous c-fos was undetectable in all analyzed cell lines. Endogenous junB and junD were expressed at similar levels across all cell lines, while stromelysin and ets-2 mRNA expression did not correlate with either the transformed phenotype or v-jun expression levels.

Overexpressed v-jun Transforms Transgenic Cell Lines. Our analysis of the relationship between transgene expression and cloning efficiency suggested that v-jun expression might enhance the transformed phenotype in these tumor cell lines. To confirm this impression, we overexpressed v-jun in transgenic cell lines with low levels of transgene expression (3-24.3 and 1-20.2). We transfected each cell line with a v-jun expression vector, pRc/RSV v-jun, which contained a neomycin (G418) resistance marker gene. Following transfection, 300 to 500 G418 resistant clones were pooled, and transcription from the expression vector was confirmed by Northern analysis. Agar colony formation was enhanced by a factor of 100 to 1000 in all of the transgenic tumor cell lines transfected with v-jun (also see accompanying paper) (19). NIH3T3 cells transfected with the same construct had no increase in cloning efficiency, despite similar transcriptional levels. This result suggests that the presence of the transgene is required for the stimulation of growth by transfected v-jun.

DISCUSSION

Tumorigenesis in v-jun transgenic mice is initiated by wounding. Transgene expression increases in wound tissue at some stage during the progression from benign to malignant tumor histology (14). However, tumor sections show a highly heterogeneous expression of the v-jun transgene (15). We show that there is also a wide variability in v-jun expression and growth properties among different transgenic sarcoma cell lines in culture. Our cell lines, derived from different

Fig. 5. Expression of jun-related, Fos, ets-2, and stromelysin genes in transgenic sarcoma cell lines. Northern blot analysis of poly(A)-selected RNA extracted from transgenic sarcoma cell lines, transgenic mouse embryonic fibroblasts (transMEF), and murine NIH3T3 fibroblasts transformed by an activated human Ha-ras oncogene (NIH3T3). Probes specific for the indicated genes were used. Bands were observed at the expected indicated size.
tumors as well as subclones from one sarcoma cell line, represent both selected components of the wound-related tumor and biological characteristics acquired in culture. They show widely differing morphologies, transgene expression levels, growth characteristics, and transformation level. This heterogeneity allowed an investigation of the correlation between the level of v-jun transgene expression and growth characteristics. We found a correlation between v-jun mRNA expression and anchorage independence and tumorigenesis at one extreme of each characteristic; sarcoma-derived cell lines expressing high levels of transgene (F3-1-1) are anchorage independent and tumorigenic in nude mice. However, cells expressing low or moderate level of v-jun show a wide range of transformed properties; cells expressing moderate levels of v-jun may clone in agar and be tumorigenic or they may fail to do so. Low expressors (1X) are very poor cloners as a rule; we found only one clone (results not shown) that has an undetectable level of v-jun but is very efficient in cloning in soft agar and tumor formation.

Cloning efficiency and tumorigenicity of v-jun transgenic tumor cell lines were enhanced by the overexpression of v-jun from a transfected expression vector. High expression remains in some cells as an imprint of the tumorigenic phenotype, but some other cells lose all or part of transgene expression with or without losing the transformed phenotype. Our observations confirm the previous observation that v-jun is unable to confer a fully transformed phenotype on mammalian cells that have not been subjected to wound-related tumor progression. v-jun transgenic embryo fibroblasts were nontumorigenic. Immortalized mouse NIH3T3 fibroblasts transfected with a v-jun expression vector remained anchorage dependent and nontumorigenic. This is further supported by in vivo observations. Although the transgene was overexpressed in adult thymus and testes, tumors did not arise in these tissues (14).

Taken together our observations suggest that wounding provides a unique context in which transgenic v-jun becomes an element in the process of tumorigenesis; increased transgene expression is one of several factors in malignant progression in vivo. Additional events, probably of a genetic nature, are needed for full oncogenicity. Subsequent genetic changes may eventually make the transformed phenotype of these cells Jun independent.

We also report an enhanced expression of endogenous c-jun in transgenic cells with low v-jun expression and very poor transformed properties. Such an inverse correlation between transformed phenotype and expression of endogenous c-jun has been observed in several other systems (6, 22).^5^  

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