[CANCER RESEARCH 55, 4540–4543, October 15, 1995]

Advances in Brief

WT1 Induces Expression of Insulin-like Growth Factor 2 in Wilms' Tumor Cells[1]

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Abstract

The Wilms' tumor suppressor gene *WT1* encodes a zinc finger transcription factor, whose expression inhibits the growth of the RM1 Wilms' tumor cell line. Transient transfection of WT1 constructs into 3T3 or 293 cells results in transcriptional repression of a number of cotransfected promoters containing the early growth response gene 1 consensus sequence. We now show that WT1 has properties of a transcriptional activator in RM1 cells, an effect that may be associated with the presence of a mutated *p53* gene in these cells. Stable transfection of wild-type WT1 into RM1 cells results in induction of endogenous insulin-like growth factor 2 (IGF2) but not of other previously postulated WT1-target genes. The induction of IGF2 is dramatically enhanced by WT1 mutants encoding an altered transactivation domain. We conclude that IGF2 is a potentially physiological target gene for WT1 and that its induction may contribute to the growth-stimulating effects of WT1 variants.

Introduction

Wilms' tumor, a pediatric kidney cancer, has been linked to the inactivation of a tumor suppressor gene, *WT1*, at the chromosome 11p13 locus (reviewed in Ref. 1). WT1 encodes a Cys-His zinc finger protein that is normally expressed in cells of the developing genitourinary system (2–4). Mutations inactivating WT1 have been detected in ~10% of sporadic Wilms' tumors (5, 6), and reintroduction of wild-type WT1 into Wilms' tumor cells expressing an aberrant WT1 transcript results in growth suppression (7). WT1 has properties of a transcriptional repressor, based on transient transfection experiments in 3T3 and 293 cells (8). These properties are differentially mediated by alternative splicing variants of wild-type WT1 (9). Whereas the four zinc finger domains of WT1 bind the 5′-CGCCCCCGC-3′ DNA consensus sequence shared by the EGR1[3] gene product, insertion of an alternatively spliced KTS sequence between zinc fingers 3 and 4 abolishes this DNA-binding activity (10).

The EGR1 consensus is found upstream of many transcriptional start sites, leading to the identification of a number of promoters that bind *in vitro*-translated WT1 and are repressed in transient transfection assays. WT1 has, therefore, been identified as a potential transcriptional repressor of genes such as EGR1 (8), IGF2 (11), insulin-like growth factor 1 receptor (12), PDGF-A (13, 14), colony-stimulating factor 1 (15), among others. Although transcriptional suppression of the endogenous genes themselves has not been demonstrated, these experiments have led to the concept that the tumor suppressor properties of WT1 might result from its ability to reduce expression of growth-inducing gene products. This model is supported by the observation that naturally occurring point mutations arising within the transactivation domain of WT1 convert the encoded protein from a transcriptional repressor to a potent activator of the EGR1 promoter in 3T3 cells (7, 16, 17). The nature of the recipient cells themselves is an important factor in these transient transfection studies. We have demonstrated recently that WT1 and p53 proteins are associated *in vivo* and that transfection of WT1 into cells with a deleted *p53* gene results in transcriptional activation, an effect that is suppressed by the reintroduction of wild-type p53 (18). Thus, the transactivational properties of WT1 appear to be complex, involving specific domains within the protein itself, as well as potential interactions with other cellular proteins.

To define further the transactivational properties of WT1 in an appropriate cell type, we analyzed the effect of WT1 expression in the RM1 Wilms' tumor cell line. This cell line, derived from an anaplastic Wilms' tumor, has the unique property of indefinite passage *in vitro*, while retaining the ability to produce tumors with characteristic Wilms' histology following inoculation into nude mice (7, 19). The endogenous WT1 transcript in these cells is expressed at very low levels and is comprised of the WT1/del2 variant, an aberrantly spliced transcript that is found in a subset of Wilms' tumors and encodes a protein with altered transactivational activity (7). We now demonstrate that transient transfection of WT1 into RM1 cells, which harbor a p53 mutation, results in transcriptional activation of a reporter construct containing the EGR1 consensus site, rather than transcriptional repression. Stable transfection of wild-type WT1 in these cells is associated with an increase in the expression of endogenous IGF2 but not of other postulated WT1 target genes with EGR1-containing promoters. Transfection of naturally occurring WT1 variants encoding an altered transactivation domain leads to a dramatic induction of endogenous IGF2 mRNA. These results suggest that only a subset of EGR1-containing, WT1-responsive promoters may be physiologically regulated by WT1. Although we do not detect transcriptional repression of endogenous IGF2 by wild-type WT1, the potent induction of this growth-inducing gene by naturally occurring WT1 mutants suggests that these altered proteins may actively contribute to cell transformation.

Materials and Methods

Culture of RM1 Cells. The generation of RM1 cells (also called W4) has been described elsewhere (19). RM1 cells were maintained as tumor explants in nude mice by serial s.c. passage. For growth *in vitro*, tumors were minced and adapted to growth on a collagen matrix and then on standard tissue culture plates and grown in DMEM, 10% FCS, and supplemental glutamine. Sequence analysis of the WT1 transcript has been described previously (7). For p53 mutational analysis, the p53 transcript was reverse transcribed using random oligonucleotide primers and subjected to PCR amplification using primers spanning the coding region. The amplified cDNA was analyzed by automated sequencing (ABI), and the mutation observed was confirmed by sequencing multiple independent clones.

CAT Assays. To test the transactivational properties of WT1 in RM1 cells, cells were transfected by calcium phosphate DNA precipitation with CMV-driven constructs encoding murine WT1 (with or without the KTS alternative splice), along with the p3X-EGR1-CAT reporter, containing three tandem

Received 7/6/95; accepted 8/30/95.

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[1] This grant was supported by NIH Grants CA 37887 (to A. J. G.) and CA 58596 (to D. A. H.).

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[3] The abbreviations used are: EGR1, early growth response gene 1; IGF2, insulin-like growth factor 2; PDGF, platelet-derived growth factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; RT, reverse transcription.

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EGR1 consensus sites upstream of a minimal promoter (10). The total amount of CMV promoter sequence transfected into the cells was equalized with the addition of vector DNA, and transfection efficiency was standardized using a cotransfected human growth hormone reporter construct (Nichols Institute). CAT assays were performed by the method of Gorman *et al.* (20), and CAT activity was quantitated by scintillation counting of appropriate sections of the TLC plate.

Northern Analysis. Total cellular RNA was prepared by extraction with guanidine isocyanate and electrophoresed on a 1% agarose/formaldehyde gel, followed by transfer to a Genescreen membrane (Amersham). Northern hybridizations were performed under standard conditions, using cDNA probes for WT1, IGF2, EGR1, PDGF-A, Pax 2, and phosphoglutaraldehyde dehydrogenase (PGAD).

Results

Transcriptional Activation of a WT1-Target Promoter in RM1 Cells. To examine transcriptional regulation by WT1 in Wilms' tumor cells, we transfected CMV-driven expression constructs into RM1 cells, along with a reporter containing a WT1 target site. WT1 lacking the KTS alternative splice, WT1(−KTS), has been shown to repress transcription from a promoter containing three tandem EGR1 consensus sites (p3X-EGR1-CAT) by 2–3-fold, following transfection into 3T3 cells (10). In contrast, transient transfection of WT1(−KTS) into RM1 cells resulted in 5-fold transcriptional activation of this reporter. Insertion of the KTS splice within the WT1 zinc finger domain, which abrogates binding to the EGR1 consensus, also reduced transactivation of the reporter (Fig. 1).

The transactivational properties of WT1 appear to be modulated by a number of factors, including promoter structure, cellular context, and the status of p53 (18, 21). To determine the sequence of endogenous p53 in RM1 cells, we amplified the endogenous p53 transcript by RT-PCR, followed by direct automated sequencing of the amplified product. A homozygous single base deletion of thymidine at codon 212 was noted, leading to a frame shift and premature termination at codon 246. p53 mutations in Wilms' tumors are rare (estimated at <5%), and an association between these mutations and histological anaplasia has been suggested by recent studies, consistent with a role in tumor progression (22, 23).[4] The absence of wild-type p53 in RM1 cells may affect the transactivational properties of WT1, as demonstrated for fibroblasts expressing a temperature-sensitive *p53* allele (18).

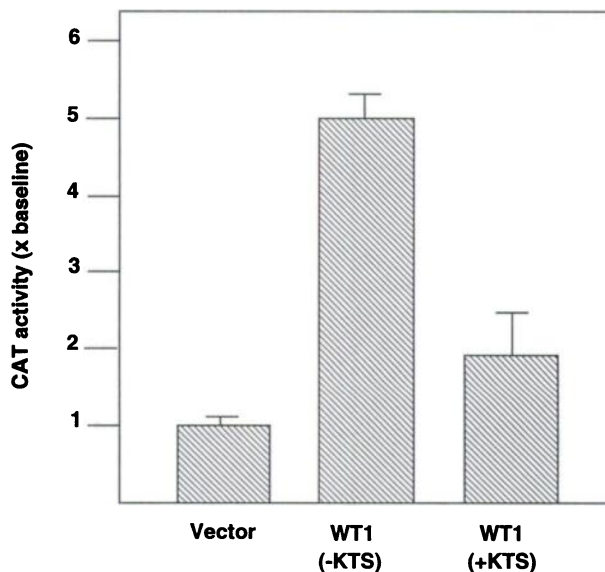

Fig. 1. Transcriptional activation by WT1 in RM1 cells. CAT activity in RM1 cells transiently transfected with the p3X-EGR1-CAT reporter, containing three tandem EGR1 sites upstream of a minimal promoter, along with constructs encoding CMV-driven murine WT1. The WT1 construct lacking the KTS alternative splice, WT1(−KTS), encodes a protein that binds to the EGR1 consensus site, whereas insertion of KTS between zinc fingers 3 and 4, WT1(+KTS), abrogates DNA binding. *Bars,* SDs calculated from multiple independent experiments.

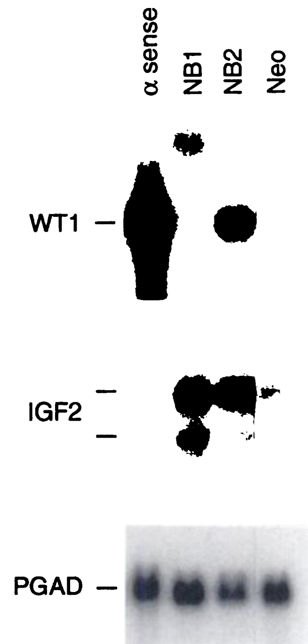

Fig. 2. Induction of endogenous IGF2 mRNA in RM1 cells expressing WT1. Northern blot of total mRNA from four independent RM1-derived clones, stably transfected with antisense WT1, WT1 lacking the KTS splice (*NB1* and *NB2*), and vector alone (*Neo*), and grown as explants in nude mice. Blots were hybridized with probes for WT1, IGF2, and PGAD (loading control).

Induction of IGF2 by Wild-Type WT1 in RM1 Cells. Although WT1 has been reported to repress transcription from a number of promoters containing the EGR1 consensus site, it has not been shown to alter the expression of endogenous genes containing these promoters. Therefore, we examined the expression of putative WT1 target genes in stable RM1 clones, generated by transfection of CMV-driven wild-type WT1, linked to the neomycin resistance (*neo*) gene (7). These cell lines expressed WT1 mRNA that was detectable by Northern blot and did not contain any mutation as determined by RT-PCR and sequencing (7). However, WT1 protein expression was below detection by immunoblot, suggesting that inefficient translation of WT1 mRNA in these cells made it possible for them to remain viable. Stable RM1 transfectants showed a decreased cloning efficiency in soft agar and reduced tumorigenicity in nude mice, consistent with the tumor suppressor properties of WT1 (7).

Two RM1-derived cell lines, NB1 and NB2, expressing WT1(−KTS) were compared with cells transfected with the neomycin resistance vector (Neo) or with an anti-sense WT1 construct. NB1 cells contained a WT1 transcript of increased size, consistent with a read-through transcript. Cell lines were grown both as tumor explants in nude mice (*in vivo*) and in tissue culture (*in vitro*), conditions that alter the levels of endogenous growth factors, and Northern blots were used to examine the expression of postulated WT1-target genes with EGR1-containing promoters. WT1 expression in RM1 cells had no effect on the expression of EGR1, Pax 2, and PDGF-A. However, endogenous IGF2 expression was increased in RM1 cells expressing wild-type WT1 (Fig. 2). The baseline expression of IGF2 in RM1 cells was low compared with that of most Wilms' tumors, and IGF2 mRNA levels were at the limit of detection when cells were grown *in*

[4] R. El Bahtimi and A. J. Garvin, unpublished data.

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vitro. However, growth of these cells *in vivo* led to an increase in baseline expression, and WT1-transfected cells had a 5-fold increase in IGF2 mRNA, compared with vector-transfected cells. RM1 cells expressing high levels of an antisense WT1 transcript had no detectable expression of IGF2 mRNA. Of the two major IGF2 mRNA species, the increased expression following WT1 transfection was observed with the 6-kb IGF2 transcript originating primarily from the P3 promoter, which has been shown to have WT1-responsive sites (11).

Enhanced Induction of IGF2 by WT1 Transactivation Mutants. We have described previously naturally occurring WT1 proteins with an altered transactivation domain that display potent transcriptional activation of reporter constructs in transient transfection assays. The aberrantly spliced WT1/del2, lacking WT1 exon 2, is observed in a subset of Wilms' tumor specimens (7), and mutant WT1/201, encoding a gly to asp substitution in WT1 exon 3, was found in a tumor specimen from a patient with WAGR, a Wilms' tumor predisposition syndrome (17). Stable RM1 transfectants were generated with CMV-driven WT1/del2 and WT1/201. Expression of the transfected gene in these cells was only detectable by RT-PCR, suggesting selection against high levels of expression of these transactivating WT1 variants. WT1/del2 and WT1/201 transfectants were grown *in vitro* and compared with RM1 cells transfected with the different wild-type isoforms of WT1: WTA (lacking both the KTS splice and the alternatively spliced exon 5); WTB (containing exon 5 and lacking KTS); and WTD (containing both exon 5 and KTS). IGF2 mRNA expression in RM1 cells grown *in vitro* was low, and no significant change was detected in cells transfected with wild-type WT1 isoforms. However, WT1/del2 and WT1/201-transfected cells expressed dramatically increased levels of IGF2 mRNA (Fig. 3). The 6-kb IGF2 transcript induced was that derived primarily from the P3, WT1-responsive promoter. No other previously postulated WT1-target genes with EGR1-containing promoters were found to be induced in RM1 cells transfected with the WT1/del2 and WT1/201 variants.

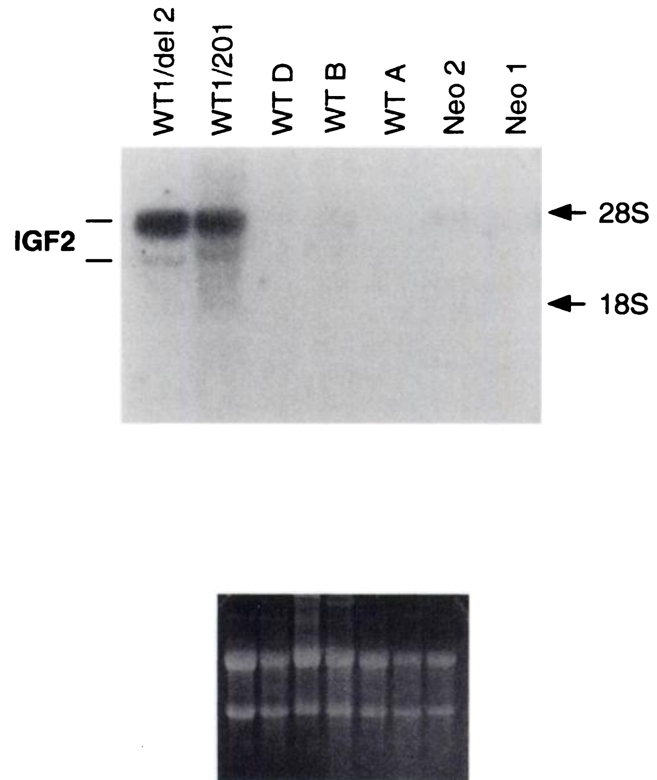

Fig. 3 Induction of endogenous IGF2 transcript by WT1 variants. Northern blot of total mRNA from RM1 cells grown *in vitro* and stably transfected with vector (*Neo 1* and *Neo 2*), wild-type WT1 lacking both alternative splices (*WTA*), WT1 containing exon 5 but lacking KTS (*WTB*), WT1 containing both exon 5 and KTS (*WTD*), or two WT1 variants: a gly to asp mutation at codon 201 within exon 3 (*WT1/201*; Ref. 17) and an aberrantly spliced product with an in-frame deletion of exon 2 (*WT1/del2*; Ref. 7). Blots were hybridized with a probe for IGF2. An ethidium bromide-stained gel is shown to demonstrate equal loading and RNA integrity.

Discussion

We have used a Wilms' tumor-derived cell line to study the transactivational properties of WT1 and to confirm the identity of IGF2 as a potentially physiological target gene for WT1. The presence of the EGR1 consensus sequence in a promoter, together with transcriptional suppression of that promoter in transient transfection assays, has been taken as evidence that a gene is a potential target for WT1. However, the relative loss of binding specificity associated with overexpression of WT1 in transient transfection assays may lead to the identification of putative target genes that are not physiologically regulated by WT1. Furthermore, the cell type-specific expression of WT1 may affect the identity of putative target genes. Thus, the observation that endogenous IGF2 expression is altered in Wilms' tumor cells stably expressing WT1 confirms that IGF2 is a genuine target gene for WT1. Our results, however, do not exclude the possibility that other genes with WT1-responsive promoters may be affected by WT1 expression in other appropriate cell types.

The induction of endogenous IGF2 by WT1 was dramatic in cells transfected with transactivating variants, WT1/del2 and WT1/201. These mutant proteins may prove particularly useful in identifying bona fide WT1 targets, since their effect does not appear to be dependent on the baseline expression level of the target gene. In contrast, the induction of IGF2 by wild-type WT1 was more modest and only evident when the baseline IGF2 expression was enhanced by growth *in vivo*. Our observations also suggest a potential physiological consequence of the potent IGF2 induction by naturally occurring WT1 mutants. A substantial fraction of WT1 mutations observed in Wilms' tumors are heterozygous, and WT1/del2 is an aberrant splicing variant that is often coexpressed with the wild-type WT1 transcript in these tumors (5–7). Thus, the altered transactivation domain of these mutant WT1 proteins may have dysfunctional, potentially dominant properties. The induction of growth-inducing genes, such as IGF2, provides an attractive mechanism for their function in tumorigenesis. An analogous mechanism may be inferred from the fusion of the Ewing sarcoma gene *EWS* to zinc fingers 2–4 of WT1 that defines the mesothelial cancer desmoplastic small round cell tumor (24). However, the altered WT1 DNA binding domain in the EWS-WT1 fusion appears to result in distinct DNA target specificity.[5]

The characterization of WT1 as a repressor of transcription is supported by transient transfection experiments using a number of EGR1-containing promoters, recipient cell lines, and chimeric Gal4 constructs (8, 10–15). However, wild-type WT1 has also been shown to act as a potent transcriptional activator, depending upon promoter and cellular contexts (18, 21). It is possible that IGF2 induction by WT1 in RM1 cells results from the absence of wild-type p53 in these cells. We have shown previously that WT1 activates an EGR1-containing reporter when transfected into Saos-2 cells lacking p53 or into rat embryo fibroblasts expressing a temperature-sensitive p53 in the mutant conformation (18). Alternatively, WT1-mediated transcriptional activation could be affected by the low baseline expression level of IGF2 in RM1 cells, with transcriptional repression by WT1 dependent upon competition with other transcription factors that normally regulate IGF2

[5] K. E. Nichols and D. A. Haber, unpublished data.

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expression. Nonetheless, our observations suggest that wild-type WT1 induces expression of IGF2 in RM1 Wilms' tumor cells in which it exerts a tumor-suppressor effect (7). Thus, it is unlikely that WT1 suppresses the growth of RM1 cells by inhibiting an autocrine growth factor-signaling pathway involving IGF2. However, in addition to IGF2, WT1 may either activate or repress transcription of additional target genes. Identifying these genuine target genes of WT1 and demonstrating whether their expression is induced or repressed in appropriate cell types will be required to understand the tumor suppressor properties of WT1.

Acknowledgments

We thank Dr. V. Sukhatme for providing the p3X-EGR1-CAT reporter construct, Drs. S. Friend and P. Romero for their assistance in p53 sequencing, S. Park for expert technical assistance, and Dr. J. Settleman for helpful criticism.

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