Increased Oncogenicity of Subclones of SV40 Large T-induced Neuroectodermal Tumor Cell Lines after Loss of Large T Expression and Concomitant Mutation in p53

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ABSTRACT

A model for medulloblastoma-like primitive neuroectodermal tumors was established in rat using retrovirally transduced SV40 large T antigen (LT) as an inducing agent (O. D. Wiestler et al., Brain Pathol., 2: 47–59, 1992). A cell line isolated from such a tumor and clonal derivatives thereof were biologically and molecularly characterized. In the parental tumor cell line, TZ870, which had been selected for G418 resistance, virtually all cells expressed LT and wild-type p53, which were complexed to each other. When plated in soft agar, these cells grew relatively slowly and formed disperse colonies. However, when grown without selection pressure, these cells reproducibly gave rise to LT-negative and G418-sensitive derivatives, LT-0 cells. Surprisingly, these latter cells exhibited a higher degree of malignancy both in vitro, growing readily to large colonies in soft agar, and in vivo, where they gave rise to a rapidly growing malignant tumor. Clonal selection from TZ870 cells revealed two types of clones; in one type, LT expression was stably maintained, even without selection pressure, whereas the other type lost the LT coding sequences. All LT-negative clones exhibited the same phenotype as the LT-0 cells. Reexpression of LT had no effect. However, LT no longer formed complexes with p53, and p53 was metabolically stable, suggesting that it had been mutated. Sequence analyses and diagnostic restriction digestions of the p53 gene revealed that (a) both the parental LT-transformed cells and their derivatives contained only one complete p53 allele and (b) all LT-positive clones expressed wild-type p53, whereas all LT-negative clones expressed a mutant allele with a common mutation at Cys-174→Tyr, indicating their clonal origin. We assume that the loss of LT coding sequences is the reason why the other type lost the LT coding sequences. All LT-negative clones exhibited the same phenotype as the LT-0 cells. Reexpression of LT had no effect. However, LT no longer formed complexes with p53, and p53 was metabolically stable, suggesting that it had been mutated. Sequence analyses and diagnostic restriction digestions of the p53 gene revealed that (a) both the parental LT-transformed cells and their derivatives contained only one complete p53 allele and (b) all LT-positive clones expressed wild-type p53, whereas all LT-negative clones expressed a mutant allele with a common mutation at Cys-174→Tyr, indicating their clonal origin. We assume that the loss of LT coding sequences is the consequence of the p53 mutation, perhaps by inducing genomic instability, and that both the p53 mutation and additional genetic alterations that accompany the loss of LT coding sequences might contribute to enhanced malignancy.

INTRODUCTION

Medulloblastomas, which are a subclass of so-called PNETs, are among the most frequent and aggressive brain tumors of early childhood. The genetic basis of this tumor is as yet poorly defined. Two familial cancer syndromes show an increased incidence of medulloblastomas, relative to the normal population: Gorlin’s syndrome, associated with basal cell carcinoma (1), and Turcot’s syndrome, associated with colorectal polyposis, basal cell carcinoma, and tumors of the central nervous system (2). The genetic lesions that seem to predispose to these syndromes have recently been identified as mutations in the homologue of the Drosophila patched gene (3) and in genes involved in β-catenin/APC signaling (4, 5), respectively. Mutations in these genes have, indeed, been found in familial as well as sporadic medulloblastomas (4, 6, 7). Other alterations found in various medulloblastomas are amplifications in chromosomal regions 5p15.3 or 11q22.3; amplifications of c-myc; isochromosome 17q; and losses in chromosomes 8p, 9q22, 10q, 11, 16q, and 17p13, the latter being rather frequent (Ref. 8; for review see Ref. 9). The 17p13 locus contains a number of candidate genes, Lix-1 (10), Rox (11), HIC-1 (12), and p53 (13), the latter two of which have been found to be down-regulated and mutated, respectively; however, these alterations occurred in only 5–10% of the cases (14–16). Thus, it appears that the genetic lesion that is common among or even responsible for most medulloblastomas has yet to be identified.

The SV40 system provides an excellent model system that revealed detailed insights at the molecular level into the mechanisms of growth control and malignant transformation. The transforming activity resides in the viral coded LT (17). According to the current view, transformation by LT is mainly achieved by interaction with cellular tumor suppressor proteins of the Rb family (pRb, p107, and p130) and p53, resulting in their inactivation (18, 19). In accordance with this, Rb and p53 are found mutated in a large proportion of various spontaneous tumors. However, a number of studies indicate that the interaction of LT with Rb and p53 is required but not sufficient for transformation and that additional activities are involved such as modulation of p300 (20), protein kinases (21, 22), insulin-like growth factor-I receptor (23), and perhaps other factors that have yet to be identified (24, 25). Although this system had long been regarded solely as a model, it has acquired new attention by recent reports showing that a considerable portion of human mesotheliomas and other tumors exhibit expression of LT in a functional form, as deduced from its interaction with Rb and p53 (for review see Ref. 26).

To gain insight into the mechanisms that lead to induction and progression of medulloblastomas, we established an animal model system using LT as inducing agent. PNETs were induced in rat brain by infecting fetal brain cells with recombinant retroviruses encoding LT and Neo, as a selectable marker, and subsequently inoculating the infected cells into the brain (left caudoputamen) of adult rats. After 8–10 months, ~80% of the animals developed brain tumors with characteristics of PNETs, the equivalent of medulloblastomas (27, 28). The specificity with which LT induces PNETs is striking, particularly because LT is capable of inducing a large variety of tumors, depending on the route of delivery and the promoter governing its expression (29–31). It is assumed that LT-induced PNETs have arisen from a small subpopulation of precursor cells, which were susceptible to transformation by LT during a certain time period (32). It is conceivable that, through inactivation of Rb, these cells retain their proliferative and migratory potential (32) and that inactivation of p53 might allow for the establishment and accumulation of genetic alterations and at the same time prevent apoptosis of cells with altered DNA (33, 34). Additional genetic alterations occurring during the latency period are presumably required to achieve the fully trans-
formed and, finally, malignant state. Such alterations are as yet poorly characterized.

Here, we studied the properties of a LT-induced PNET-derived cell line TZ870. A characteristic feature of these cells was their tendency to cease LT expression and, unexpectedly, to convert to a more malignant state. This property can, therefore, be used as a model for tumor progression. We found that cessation of LT expression was due to disintegration of retroviral sequences and that this disintegration event was accompanied by loss of adjacent cellular sequences. Additionally, all LT-negative cells carried a mutation in p53. Both of these alterations might contribute to increased malignancy.

MATERIALS AND METHODS

Retroviral Vector. The retroviral vector pZIPNeoSV, used for induction of PNETs, was derived from the Moloney murine leukemia virus, in which the SV40 LT is expressed under the control of a strong viral long terminal repeat promoter (35) that also encodes the neomycin phosphotransferase gene (Neo), which confers resistance to geneticin (G418; Life Technologies, Inc., Egggenstein, Germany).

Establishment of Cell Lines. The tumor cells were dissected from the host brain, mechanically dissociated, and then plated in tissue culture flasks with DMEM containing 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). Permanent tumor cell lines were selected in the presence of G418 (0.8 µg/ml). Both LT expression and morphology were found to be stable under these conditions. Cells were grown as monolayer cultures in DMEM supplemented with 10% FCS.

Cloning in Soft Agar. Cells were plated in duplicates at 1 × 10³ cells per 60-mm-diameter culture dish in DMEM containing 10% FCS and 0.3% (w/v) agar (Seaplaque agar; FLM Biozyme) onto a bottom layer of 0.6% (w/v) agar in DMEM. Colonies were scored and photographed 14 days after plating.

Actin Filament Staining. Cells were seeded on coverslips and grown for 2 days. Coverslips were rinsed in PBS and fixed for 15 min at room temperature in 3% formaldehyde in PBS. Actin filaments were stained with FITC-phallodin (0.05 mg/ml; Sigma Chemical Co., St. Louis, MO) according to the method of Wulf et al. (36). The cells were examined with an Axioshot fluorescence microscope (Zeiss) equipped with a ×63 immersion objective.

Radiolabeling, Preparation of Cell Extracts, and Immunoprecipitation. Cells were labeled with [35 S]methionine (250 µCi per 9-cm plate) in methionine-deficient medium for the times indicated. After labeling, cells were washed with PBS and lysed with isotonic lysis buffer (pH 8) containing 0.5% NP40; immunoprecipitation was performed with monoclonal antibodies specific for LT (PAb419; Ref. 37) or p53 (PAb122; Ref. 38), as described previously (39, 40).

Primers for PCR and Sequencing. PCR was used to confirm the presence or absence of LT in TZ870 and LT-0 cells or to amplify LT or p53-specific segments encompassing their interaction domains. LT sequences were amplified with the upstream primer 5'-CAAGAGCTTCTCCAGAATTG-3' and the downstream primer 5'-CAATTTTGTCCTTAAAAACGCCAG-3'. These primers amplify a 881-bp segment of LT (bp 4414–3533). p53 sequences were amplified with the upstream primer 5'-AACGATGTCCAGAATTG-3' and the downstream primer 5'-CAATTTTGTCCTTAAAAACGCCAG-3'. These primers amplify a 881-bp segment of LT (bp 4414–3533). p53 sequences were amplified with an Axiophot fluorescence microscope (Zeiss) equipped with a ×63 immersion objective.

Southern Analysis. Genomic DNA was prepared according to standard procedures (43). The DNA was digested with restriction endonucleases as indicated in the text, separated on 0.6% agarose gels, and blotted onto Hybond-N filters. The filters were probed with DIG-labeled or 32P-labeled DNA.
due to epigenetic changes. Inspection of actin filaments by staining with FITC-labeled phalloidin revealed no significant alterations in this respect (Fig. 2, bottom), although the staining pattern of LT-0 appeared slightly more diffuse. Together, these unexpected results suggested that LT-0 cells had suffered genetic alterations, which not only helped to maintain their transformed state in a LT-independent way but rendered them even more malignant. Precedents showing that LT-transformed cells or even LT-induced tumors can become LT independent have been described (46, 47). But the finding that loss of LT expression may lead to increased malignancy appears to be paradoxical and, thus far, unique. Assuming that this was an exceptional case, we investigated whether this phenomenon was reproducible.

**Loss of LT Expression Is Reproducible.** To see whether the tendency to lose LT sequences was an inherent property of these cells, perhaps due to integration at an unstable chromosomal locus or a general genetic instability, and whether the consequence (increased malignancy) was the same, we kept G418-selected and 100% LT-positive cells in the absence of G418 for some passages. Expression of LT was monitored by immunofluorescence. After ~10 passages, 20–30% of the cells were again LT negative. At this stage, the cells were subcloned by serial dilution in 96-well plates. Thirty-five single-cell clones were isolated and established in the absence of G418, and some of them were characterized for LT expression and cloning efficiency in soft agar.

Of 16 individual clones, 7 were LT positive and 9 were LT negative. These clones exhibited growth properties very similar to those found for TZ870 and LT-0 cells, respectively. Eight of the nine LT-negative cell clones grew faster in monolayer cultures, compared to their LT-positive counterparts, and seven of nine formed large compact colonies in soft agar (data are summarized in Table 1). In all cases, loss of LT expression was due to loss of the coding sequences, as confirmed by PCR (data are shown for clones 5, 10, 22, 30, 31, and 33 in Fig. 1, Lanes 3–7 and 9, respectively). Surprisingly, the LT-positive clones exhibited stable LT-expression for >30 passages, even in the absence of G418, without any detectable loss of LT expression. All LT-positive clones exhibited a slower growth rate and formed disperse colonies in soft agar like TZ870 cells. Thus, both the loss of the retroviral insert and the phenotypic consequence, the acquisition of a more malignant state, were reproducible.

These findings suggested that the parental TZ870 cell line contained at least two subpopulations of cells: in the vast majority, the LT coding sequences appeared to be stably integrated and expressed, and these LT-positive cells exhibited a relatively low degree of transformation in vitro as well as in vivo. A very minor fraction of cells, however, appeared to have a high probability of losing the retroviral insert with the phenotypic consequences described above. Under selective growth conditions, we could eliminate these latter cells, but in the absence of the selection agent, they would expand due to their faster growth and finally overgrow the LT-positive cells.

**Analysis of the Integration Locus.** On the basis of the existence of stable and unstable LT-positive cells, one would expect at least two integration sites for the retroviral sequences, one representing the stable site and one representing the unstable locus. Therefore, we wanted to determine the number of integration sites of the retroviral insert in chromosomal DNA of TZ870 and different cell clones by Southern analysis. Genomic DNA was isolated and cleaved with BamHI, BclI, or BgII, which cleave once in the retroviral insert between the LT and Neo coding region and in the adjacent cellular DNA in an unknown distance. The blot was probed with a DIG-labeled DNA fragment from the LT or Neo genes, respectively. In all LT-positive cases (TZ870 and clones 17, 23, 28, and 32), only one single band hybridized with the probes, as shown in Fig. 3a. The LT probe revealed signals for PstI-, BamHI-, BclI-, and BgII-digested samples of 4.2, 2.23, 8.5, and 4.5 kb, respectively (Fig. 3a, Lanes 1–20; the BgII samples are not shown). The Neo probe hybridized with 5.2- and 4.7-kb BamHI- and BgIII-digested fragments (Fig. 3b). The BcII fragments are not visible because this digest generated a large fragment of >13 kb, which was poorly transferred to the membrane. No signal was detected with DNA from LT-0 cell clones 4, 5, 7, and 10. The sensitivity was about 1 copy per 20 cells. Thus, within the limits of detection, only one integration site was found. However, the existence of a second locus in a very small subpopulation of cells cannot be excluded.

**Reintroduction of LT Uncovers a p53 Mutation.** Because the behavior of LT-0 cells and clones was not typical of LT-transformed cells, we asked whether it was a peculiarity of this type of brain tumor that loss of LT might, paradoxically, result in a higher degree of malignancy, perhaps due to a particular genetic context. In this case, reintroduction of LT should revert the phenotype of the LT-negative cells to that of the parental TZ870 cells. To investigate this possibility, we reexpressed LT from a cytomega-

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<tr>
<th>Cell clone</th>
<th>LT expression</th>
<th>Growth rate (doubling time, in h)</th>
<th>Cloning efficiency in soft agar (%)</th>
<th>p53 status</th>
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<tr>
<td>TZ870</td>
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<td>LT-0</td>
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*IF, immunofluorescence; ND, not determined.*
loivirus promoter-driven expression vector in a tetracycline-dependent manner (Tet-off system; Ref. 41). Cells were pretransformed with the expression vector coding for the tetracycline-repression VP16 transactivator and puromycin resistance. Several clones were selected on the basis of high induction of transiently expressed luciferase reporter gene. The degree of induction was 170-fold for cotransfected LT-0 cells and 78-, 51-, and 7-fold for the pretransformed clones ID4, IIC3, and IB3, respectively (Fig. 4a). Clones ID4 and IIC3 were used for reintroduction of the LT gene. These clones exhibited 10- and 5-fold differences in LT expression after induction (withdrawal of tetracycline; Fig. 4b). These experiments revealed that (a) reexpression of LT did not change the phenotypic properties of the cells, as monitored by growth in soft agar, and (b) the reexpressed LT did not complex with p53, as revealed by immunoprecipitation (Fig. 4c). This latter result suggested that either LT (through the cloning procedure) or p53 (during establishment of the LT negative cell clones) had undergone mutations. Sequence analyses of both LT and p53 coding sequences spanning the interaction domains, indeed, revealed a mutation in p53, with a G→A transition at nucleotide 544 in codon 174, resulting in a Cys→Tyr exchange. No mutation was found in LT. The fact that the p53 mutation was located within the domains that interact with LT (48) explains the failure of complex formation between LT and p53. Pulse chase experiments confirmed that the mutant p53 was metabolically stable, with a half-life of ~3 h (see Fig. 4c).

To determine whether this mutation was common to all LT-negative cells, the genomic sequences from exons 5 to 7, encompassing coding sequences from codons 127 to 297, were amplified by PCR and sequenced. Indeed, all other LT-negative clones (clones 4, 5, 7, 10, and LT-0 cells are shown as examples) carried the same G→A transition, whereas the LT-positive clones (clones 13, 17, 23, 25, 28, and 32) contained wt p53, as shown in Fig. 5 (the only exception was the LT-negative clone 10, which revealed wt p53 in this particular experiment but proved to have mutant p53 in all other experiments; see also data in Fig. 6). Interestingly, no wt allele was detected in the LT-negative cells. These results suggest that all LT-negative cells arose from a common ancestor, that the mutation was already present in a small fraction of the original TZ870 line, and, finally, that the parental cells contained only one p53 allele.

To further investigate this latter conclusion, we performed the following Southern analysis. Genomic DNA from the different clones was completely digested with PstI, BamHI, or BglII, separated on a 0.7% agarose gel, blotted, and probed with a DIG-labeled probe comprising part of the LT coding sequence. The samples used in a were subjected to hybridization with a DIG-labeled Neo probe. DNA digested with BamHI, BclI, or BglII was used in b, and the samples used in a were subjected to hybridization with a DIG-labeled Neo probe. DNA digested with BamHI and BclI was used in c.

Fig. 3. Southern blot analysis detects only one integration site. a, genomic DNA from LT-positive and -negative cell clones was digested with PstI (Lanes 1–5), BamHI (Lanes 6–11), BclI (Lanes 13–19), or BglII (Lanes 21–26), separated on a 0.7% agarose gel, blotted, and probed with a DIG-labeled probe comprising part of the LT coding sequence. Lanes 12, 20, and 27, size markers. b, the samples used in a were subjected to hybridization with a DIG-labeled Neo probe. DNA digested with BamHI (Lanes 1–6) and BclI (Lanes 9–14, and BglII (Lanes 16–20). Lanes 7 and 15, size markers.
showed in Fig. 6, LT-positive cells (clones 13, 17, 23, 25, 28, and 32) revealed a single cleavage product of 761 bp, representing the wt allele (Fig. 6, Lanes 1, 3–7, and 9–11), whereas LT-negative cells (clones 4, 5, 7, and 10) revealed only an Ital-resistant fragment of 950 bp, representing the mutant allele (Fig. 6, Lanes 2 and 13–15). Additional bands seen in all samples represent pseudo-genes that can be used as reference. Thus, both LT-positive and LT-negative cells contained only one complete p53 allele. Moreover, if the mutation was present already in the TZ870 cell line, then it was present in only a very small subfraction of the cells, the limit of detection being ~1%. 

Disintegration of the Retroviral Insert Is Accompanied by Deletion of Adjacent Sequences. Because the p53 mutation alone could not be responsible for the phenotypic alterations seen in LT-0 (see “Discussion”), additional genetic changes must be claimed. One likely possibility was that disintegration of the retroviral insert included deletions of flanking chromosomal sequences. To investigate this possibility, we isolated and sequenced ~1600 and 1200 bp of cellular sequences flanking both sides of the integration site. These fragments were used as probes for Southern or PCR analyses. Comparative Southern analysis of BamHI-digested genomic DNA from TZ870 or LT-0 clone 4 cells revealed that disintegration of the retroviral sequences was, indeed, accompanied by a deletion that exceeded the region covered by the available probes. Work is in progress to determine the extent of this deletion and to identify the gene(s) affected.

DISCUSSION

This study revealed a reproducible conversion of a SV40 LT-induced medulloblastoma-like tumor cell line from a state of low to one of high malignancy. This conversion must be due to genetic alterations, two of which were discovered in this study: a mutation in p53 and deletion of the retroviral insert including LT/Neo coding sequences and adjacent cellular sequences.

Significance of Loss of LT Expression. Transformation by LT is mainly achieved by interaction with and inactivation of Rb (and related proteins) and p53, thereby bypassing restriction point control and preventing apoptosis. Other events related to transformation are deregulation of protein kinases, transcription factors, and other regulatory factors (18–25). However, stable and malignant transformation is a relatively rare event and requires additional genetic alterations that accumulate during the latency period. These alterations may result, in part, from inactivation of p53. Additionally, LT can induce tetraploidy and a variety of other chromosomal aberrations (49–51). Despite these alterations, transformed cells generally remain LT dependent, as shown in vitro with tsA mutants (44) or in animal models with inducible LT expression (47). However, variants may emerge that become LT independent in vitro as well as in vivo (46, 47), probably due to mutations in cellular genes, that are targeted by LT. Again, this is a rare event and may become apparent only if cells gain a growth advantage over their parental cells. LT-independent tumor cells might actually develop more easily in vivo, where LT-expressing cells are targeted by the immune system. In our in vitro model, however, selection was for and not against the presence of LT.

Significance of the p53 Mutation. Loss of LT/Neo sequences occurred spontaneously and reproducibly after release from selection pressure. This event seems to be causally related to mutant p53 because cells expressing wt p53 retained LT, even without selection pressure. The p53 mutation must have occurred before deletion of the retroviral insert; otherwise, cells carrying this mutation would have been eliminated by G418 selection. On the other hand, it is unlikely that the p53 mutation occurred prior to transformation by LT. In this case, all tumor cells should carry the mutation. Clearly, p53 mutations are not a prerequisite for and are only rarely found upon LT-induced transformation (52, 53). However, it is interesting that all tumor cells, LT-positive TZ870 cells as well as LT-negative derivatives, contained only one p53 allele. Loss of the other allele may have occurred before or during the process of transformation and may have facilitated both transformation and evolution of LT independence through inactivation of the remaining allele.
Is the p53 mutation responsible for the malignant conversion? The respective mutation in human p53 (Cys-176) is only rarely found in human tumors (~0.3%; Ref. 54) and is, thus far, poorly characterized. A homologous mutant in mouse p53 with a Cys-173→Ser exchange has, indeed, been shown to enhance transformation by Ras (55). However, in our case, the p53 mutation did not provide a growth advantage per se, otherwise LT+/p53mut cells would have outgrown LT+/p53wt cells even under selective conditions. It is more likely that the mutant conferred genetic instability (56, 57), as indicated by the reproducible rearrangement at the retroviral integration site, and, as a consequence, LT independence and enhanced malignancy.

Is the Rearrangement at the Integration Site Related to Malignant Progression? If mutant p53 induced a general genetic instability, this could lead to numerous aberrations even under selection pressure, where LT/Neo expression is maintained. Thus one might expect malignant variants to evolve that are still LT/Neo positive. Because this was not observed, we assume that the rearrangement at the retroviral integration site is directly related to malignant progression, but additional alterations may also contribute. The extent of the deletion has not yet been determined. It appears to affect the expression or function of an unknown gene. Preliminary Northern analyses revealed that the locus is expressed and the transcripts from TZ870 and LT-0 cells differ in size.5 Isolation and characterization of the respective gene(s) encoded in the vicinity of the integration locus will perhaps allow new insights into tumor progression.

What Is the Clinical Significance of this Model System? Until recently, SV40 has not been implicated in human carcinogenesis. Between 1955 and 1962, millions of people were inadvertently vaccinated with SV40-contaminated poliovirus vaccine; they showed no significant increase in the general tumor incidence, compared to people that received uncontaminated vaccine later on (see Ref. 26 for review and references therein), and this was taken as proof for the harmlessness of SV40. However, in a long-term follow-up study, slight but significant differences were seen for some rare cancers, including medulloblastomas (79 versus 61 cases in ~800,000 people; Ref. 58). More recently, SV40 sequences and, more specifically, LT sequences have been implicated in human carcinogenesis, in Burkitt’s lymphoma and nasopharynx carcinoma, hepatitis B virus in hepatocarcinoma, and human papilloma viruses in cervix carcinoma. Thus, the possibility of SV40 being a potential risk factor in specific genetic or environmental contexts should be reevaluated. On the other hand, the presence of LT in some tumors offers the possibility of immunotherapy. However, the development of LT-independent variants with even higher malignancy must be considered.

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