Altered Expression of Wild-Type p53 Tumor Suppressor Gene during Murine Epithelial Cell Transformation

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Abstract

An epidermal cell model in which initiated, benign tumor-producing and carcinoma stages were derived from a cloned parental cell strain was used to examine p53 expression during multistage epithelial carcinogenesis. Increased steady-state levels of p53 RNA were detected in squamous cell carcinomas compared to papilloma and normal epidermal cells. Nontumorigenic initiated cell precursors of the carcinomas exhibited normal p53 expression, localizing altered p53 regulation to the malignant conversion stage. Immunoprecipitation and Western immunoblot analyses demonstrated elevated levels of p53 protein in the moderately differentiated carcinoma compared to normal cells, and negligible levels of p53 in the poorly differentiated carcinoma cells. Sequence analysis of p53 complementary DNA from normal and carcinoma cells revealed no mutations in the coding or 3'- and 5'-untranslated regions, suggesting a novel mechanism of p53 inactivation.

Introduction

The cooperation of two or more activated oncogenes and the inactivation of tumor suppressor genes have been postulated to be required for malignant transformation of keratinizing epithelial cells (1–3). However, it remains uncertain which defects are responsible for carcinoma formation and whether the order of their accumulation is random or specific to transformation stage (initiation, promotion, malignant conversion, phenotypic progression). We sought to identify genes altered at discrete stages of transformation using a mouse keratinocyte model in which independent initiated cell clones (4) were derived from a common normal parental cell strain after 7,12-dimethylbenz[a]anthracene treatment. Three lineages have been characterized by tumorogenicity in athymic nu/nu mice (5, 6) and by differentiation in culture (7). Using this model system, it has been demonstrated previously that loss of regulation of VLS30 (endogenous virus-like 3OS RNA) gene expression by extracellular Ca2+ occurred at the malignant conversion stage and that VLS30 expression was associated with differentiation of normal and papilloma cells (8). In an effort to identify additional genetic changes involved in epidermal carcinogenesis, we examined p53 expression in this transformation model. Frequent allelic deletions, rearrangements and mutations at the p53 locus have been found in many human and animal tumors (9–14), suggesting that wt p53 functions as a "tumor suppressor gene." Transfection studies support a role for p53 as a negative growth regulator (15–19). On the other hand, mutated p53 can function as a dominant transforming factor (15, 20–22). While loss of the normal p53 function occurs by chromosomal deletion or rearrangement at both alleles, it can also occur through complex formation between wt p53 and mutant p53 (23, 24) or, in virus-transformed cells, between wt p53 and viral proteins such as SV40 large T-antigen, adenovirus E1B protein or HPV type 16 and 18 E6 protein (25–27). Furthermore, a mutated p53 can function in a dominant positive manner in the absence of wt p53 (28), suggesting that mutated p53 has additional biological and biochemical activities. Recent studies indicate that wt but not mutated p53 can act as a trans-activator (29) and putative binding sites on DNA have been isolated and characterized (30). To date the functional studies of p53 have been performed primarily in fibroblast cell cultures. However, interacting proteins within the cell and the genes regulated by p53 are likely to vary in different cell types. As a preliminary to functional studies, we sought to determine whether alterations in p53 primary sequence or expression were associated with transformation in the epidermal model. The results show abnormalities in p53 gene expression at the RNA and protein levels which are not due to mutations in the p53 coding sequence or 5'- and 3'-untranslated regions examined, suggesting a novel mechanism of p53 inactivation.

Materials and Methods

Cell Culture. The normal parental cells of strain 291 and the independent normal clone 27le were grown in conditioned "low-calcium" Eagle's medium (i.e., modified to contain 0.02–0.04 mM Ca2+ and 10% fibroblast-conditioned medium) containing 10 ng/ml epidermal growth factor to enrich for basal proliferating epidermal cells, or in "high-calcium" Eagle's minimum essential medium with nonessential amino acids, 5% fetal bovine serum, 1.4 mM Ca2+, and 1% antibiotic-antimycotic for 48 h to enrich for spinous cells committed to terminal differentiation (4, 5, 31). Tumor cell derivatives and their nontumorigenic precursor cells were grown in the above "high-calcium" medium (6).

Northern Blot Analysis. Cells were harvested when approximately 70 or 100% confluent. RNA was isolated by guanidinium/cesium chloride extraction and dissolved in diethylypyrocarbonate-treated water for Northern blot analysis as described previously (8, 32). A 500-base pair ParI fragment of p53–422 was used for p53 detection (33). An 840-base pair EcoRI-SalI fragment of pA6 was used for 7S RNA detection which served as an internal control for RNA loading (34). Probes were labeled with [α-32P]dCTP by the random primer method using a multiprime DNA labeling kit (Amersham, Arlington Heights, IL). 32P-labeled probe was used at the final concentration of 1 to 2 × 106 cpm/ml. Quantitation of relative amounts of RNA was done by densitometry of exposed films using a Quick Scan (Helena Laboratory, Beaumont, TX) and Fastscan computing densitometer (Molecular Dynamics, Sunnyvale, CA). The p53 hybridization signals were compared after adjustment for differences in 7S RNA.

Immunoprecipitation Analysis. Rapidly growing cells (5 to 10 × 106 cells) were labeled with 200 μCi of [35S]methionine (Amersham) for 3 h in 3 ml of methionine-free RPMI 1620 containing 2% dialyzed calf serum before lysis in 1% Nonidet P-40-150 mM NaCl-50 mM Tris, pH 7.4.

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3 The abbreviations used are: wt, wild type; HPV, human papillomavirus; PCR, polymerase chain reaction; nt, nucleotide; cDNA, complementary DNA.
were obtained as the supernatant of hybridoma tissue culture from Dr. Amersham) in Tris-saline buffer containing 5% bovine serum albumin. The dried blot was exposed to film at -80°C with intensifying screens.

Immunoprecipitations were carried out by using nonimmune serum as a control or murine p53-specific monoclonal antibody PAb421 (35) or PAb246 (36) as described (9). Immunoprecipitated proteins were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described (37) and fixed in 7.5% acetic acid/25% methanol. The gel was soaked in enhancer solution (NEN, Boston, MA) for 30 min and dried before exposure to XAR film (Kodak, Rochester, NY), digested with EcoRI and BamHI, isolated from 3% low melting temperature agarose (FMC, Cetus, Norwalk, CT). Amplified fragments were desalted by Centricon 100 ultrafiltration (Amicon, Beverly, MA), digested with EcoRI and BamHI, isolated from 3% low melting temperature agarose (FMC, Rockland, ME) using GeneClean II (BIO 101, La Jolla, CA), cloned into pGEM3zf(+) (Promega) for sequencing of the sense strand of p53 cDNA isolated from 291 normal, 05RAT or 03RAT tumor cells and transfected into Escherichia coli strain JM109 (41). Single-stranded DNA isolated after transfection with helper phage R408 (Promega) was used as a template for dideoxynucleotide sequencing with M13(-40) forward or T7 primers and [α-32P]dATP (NEN), using Sequenase Version 2.0 (United States Biochemical Co., Cleveland, OH) according to manufacturer’s instructions. Internal primers were also used for better resolution of RS23: CCGTGCCTCCATGGTAGT for the sense strand sequencing and TCGGGTGGCTCATAAGGT for the antisense strand sequencing. Three or more clones derived from each segment of 5p3 cDNA isolated from 291 normal, 05RAT or 03RAT tumor cells were sequenced. The reaction products were loaded on 6% polyacrylamide/8 M urea gels and electrophoresis was carried out at 70 watts. Gels were soaked in 15% methanol/5% acetic acid for 45 min, dried at 80°C and exposed to XAR film.

Results

Since it has been reported previously that the level of p53 transcript is elevated in certain tumors and transformed cells lines (42, 43), the steady-state level of p53 RNA was examined first by Northern blot analysis. In an attempt to distinguish altered gene expression due to normal functional states from altered gene expression due to transformation stage, proliferating or differentiating normal parental cells (291L, 291H) along with an independent normal clone (271Lc, 271Hc) were compared to each of three tumor cell derivatives. As shown in Fig. 1, there was no difference at the level of p53 in proliferating and differentiating normal cells. In contrast, the steady-state level of p53 RNA was increased 3- to 5-fold in moderately (05RAT) and poorly differentiated squamous cell carcinoma (03RAT) but not in well differentiated papilloma (09RAT) compared to normal cells (Fig. 1A). To determine at which stage of tumorigenesis the altered p53 expression was first detectable, nontumorigenic initiated cell precursors of each carcinoma line were examined (Fig. 1B). The p53 transcript levels in initiated cell lines (03C, 05C, 09C) were the same as in normal cells, suggesting that overexpression of p53 RNA is associated with malignant conversion. In a previous study we found that the expression of differentiation-associated gene VSL0 is dependent on cell culture density (8). As shown in Fig.
1B, the levels of p53 RNA were not different in preconfluent (70%) and confluent (100%) cultures. To determine whether overexpression of p53 might be the result of gene amplification or rearrangement in carcinoma cells, Southern blot analysis was performed. Genomic DNA digested with EcoRI, BamHI, HindIII, or XbaI from normal and carcinoma cells displayed no rearrangement or amplification of the p53 gene (data not shown).

To examine whether an elevated level of p53 protein is also found in carcinoma cells, Western immunoblotting and immunoprecipitation analyses were performed using two mouse monoclonal antibodies (PAB421 and PAB246) which recognize different epitopes of the murine p53 protein. Normal p53 can bind to both PAB421 and PAB246 while protein encoded by p53 mutated at various codons binds to PAB421 but not to conformation-sensitive antibody PAB246 (44). First, we tested p53 protein expression in carcinoma cells by Western immunoblotting using PAB421 (Fig. 2A). As expected, the p53 protein levels were elevated approximately 2- to 3-fold in 05RAT compared to normal parental cells. However, the level of p53 protein in 03RAT carcinoma cells was extremely low, approximately one-tenth of that found in normal parental cells. Further analysis of labeled cell lysates by immunoprecipitation with PAB421 or PAB246 confirmed that the p53 protein level was increased in 05RAT carcinoma cells and decreased in 03RAT cells (Fig. 2B). Reactivity with PAB246 suggests that the p53 protein overexpressed in 05RAT carcinoma cells has a wild-type configuration. However, the presence of mutations cannot be completely excluded since the same mutated p53 has been reported to produce proteins in a conformation recognized by PAB246 depending upon the transfected cell line (45).

Since an altered steady-state level of p53 in carcinoma cells could be due to mutations in coding sequences or in regions of mRNA regulating translation, we sequenced p53 cDNA from 05RAT and 03RAT carcinoma and 291 normal cells. Three overlapping segments including the entire coding sequences (1170 base pairs), 111 of the 5'-untranslated (105 base pairs downstream of the putative major cap site) and 369 base pairs of the 3' untranslated (75 base pairs upstream of the polyadenylate tail) regions were amplified by PCR and both strands were sequenced. No mutations were detected in any of the sequenced regions (data not shown). Attempts to further sequence the 5'-untranslated region were prevented by the presence of a stem and loop structure (46) which interfered with PCR amplification.

Discussion

Evidence to date indicates that wt p53 has a negative regulatory function for cell growth while mutant p53 promotes cell transformation in vivo and in vitro (47). The p53 found to be elevated in many tumors and transformed cells has been reported to be associated with either missense mutations at the coding sequences or complex formation with viral proteins such as SV40 large T-antigen and adenovirus type 5 E1B proteins (25, 26). The results presented in this report indicate that p53 expression is altered in chemically induced squamous cell carcinomas in the absence of mutations at the coding sequences or 5' and 3'-untranslated regions examined. In addition, the altered regulation is localized to the malignant conversion stage of epidermal cell carcinogenesis. Abnormal p53 gene structure or expression tends to be a late event in human tumors (48). Taken together these findings suggest that abnormal expression of wt p53 is an early event in malignancy, perhaps significant in local invasion or primary carcinoma growth, but that mutations at the coding region or gross chromosomal defects are later events associated with phenotypic variation or metastasis.
p53 REGULATION AND FUNCTIONAL STUDIES OF WT AND MUTANT p53 IN p53 can show different expression levels in different tumors. p53 expression differs in various cell types and the same mutant cells. In cervical carcinomas infected with HPV types 16 and 18 as well as in HPV 16- and 18-transformed cells, the levels of p53 protein degradation might be disturbed in carcinoma cells. Indeed, it has been demonstrated that the wt p53 has a different quaternary structure and stability in transformed cells compared to nontransformed normal cells (53, 54); (c) regulation at the level of p53 protein degradation might be disturbed in carcinoma cells. In cervical carcinomas infected with HPV types 16 and 18 as well as in HPV 16- and 18-transformed cells, the levels of p53 are undetectable even in the presence of translatable mRNA (27). In vitro assays indicate that the binding of E6 viral oncoprotein facilitates degradation of wt p53 through a ubiquitin-mediated protease system (55). Altered posttranslational modification of p53 could change its ability to form complexes with itself or other cellular proteins, giving rise to low levels of p53 protein. Conversely, if the cellular components important for p53 degradation or stabilization were altered or deregulated, it could lead to abnormally low levels of wt p53. The 03RAT cells may provide a means to identify novel mechanisms of p53 inactivation in neoplasia. Although many human tumors originate from epithelial cells, most studies on p53 have been performed using fibroblast cell types. However, each cell type has different responses to growth and differentiation factors. While there is evidence for tissue-specific p53 mutations in tumors, it is not clear whether these are mutagen specific or cell type specific. Indeed, the level of p53 expression differs in various cell types and the same mutant p53 can show different expression levels in different tumors (48), suggesting that p53 is regulated in a cell type-specific manner. Thus studies of the mechanisms responsible for altered p53 regulation and functional studies of wt and mutant p53 in epithelial cells should be facilitated by this epidermal cell model.

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