Selective Expression of Mutated p53 in Human cells Immortalized with either 4-nitroquinoline 1-oxide or 60Co Gamma Rays

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ABSTRACT. Many studies on in vitro transformation of human cells indicate that the cells must be immortalized before they can be neoplastically transformed, indicating that immortalization is a critical step in multistep neoplastic transformation of human cells. We immortalized three human cell lines by repeated treatment with either 60Co gamma rays or a chemical carcinogen, 4-nitroquinoline 1-oxide, and found that all three immortalized cell lines have mutations in the tumor suppressor gene, p53. Direct sequencing of the reverse-transcribed mRNA and immunoprecipitation of p53 protein revealed that mutant p53 is selectively expressed in all the immortalized cell lines, whereas the genomic fragments of the immortalized cells contain wild-type and mutated p53 alleles. Although the mutated p53 is selectively expressed in the immortalized cells, expression of the wild-type p53 was induced by treatment of the cells with a hypomethylating reagent, 5-azacytidine, indicating that the wild-type p53 allele might be inactivated by hypermethylation of DNA. Actually, the entire genomic locus including the promoter region of p53 is hypermethylated in all the immortalized cell lines. Expression and phosphorylation of Rb was normal in these three cell lines. Thus, inactivation of both wild type p53 alleles and selective expression of mutated p53 seem to be key factors in the immortalization of human fibroblasts.

Experimental models of in vitro malignant transformation of human cells provide considerable insight into the mechanisms of multistep carcinogenesis of human cells. Normal human cells must be immortalized before they can be neoplastically transformed. However, normal human cells are extremely refractory to immortalization, making in vitro neoplastic transformation of normal human cells very difficult (29). Once these cells have been immortalized, they are transformed into malignant cells fairly easily with a variety of carcinogenic agents, such as oncogenes, chemical carcinogens, and ionizing radiation (4, 27, 28, 31, 32, 35, 37).

More than 50% of the human malignancies of epithelial, mesenchymal, hematopoietic, lymphoid, and neural origin analyzed so far, have been found to contain a mutated p53 gene (14, 21, 30). Therefore, inactivation of the p53 tumor suppressor gene is currently regarded as an almost universal step in the development of human cancers. Although the role of p53 in multistep carcinogenesis of human cells remains unclear, many lines of evidence indicate that the loss of normal p53 function is associated with a disturbance of cell proliferation and/or with instability of the gene (17, 18, 20).

Inactivation of the p53 gene has been observed as a late event in colorectal tumorigenesis (3), but participation of p53 in immortalization of cells has also been reported (25, 36). Fibroblasts from patients with Li-Fraumeni syndrome have a mutation in a single allele of p53 (22, 33), but are phenotypically normal and have a limited life span. Immortalization of Li-Fraumeni fibroblasts has been correlated with loss of the remaining wild-type allele (38). These results suggest that inactivation of both p53 alleles may play an important role in immortalization of human cells.

The other tumor suppressor gene, Rb, has been reported to be involved in cellular aging (34). The addition of Rb antisense oligomers to human diploid fibroblast cultures delayed their senescence, and co-addition of p53 and Rb antisense oligomers showed a greater senescence-delaying effect than the addition of antisense Rb alone (13).

Although human diploid fibroblasts are extremely resistant to immortalization, we previously succeeded in immortalizing three human fibroblast cell lines, KMST-6, SUSM-1 and OUMS-24F, by treatment with either 60Co gamma rays or a chemical carcinogen, 4-nitroquinoline 1-oxide (4NQO) (1, 26, 29). These immortal cell lines continue to grow without cellular aging, whereas their normal counterparts became senescent after 50-80

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population doublings (PDs). The immortal cell lines showed neither tumorigenicity on transplantation into nude mice nor anchorage-independent growth in soft agar, but they became tumorigenic upon the introduction of an activated ras gene (29). These findings indicate that our established immortalized cell lines are valuable for studying the early stage of carcinogenesis of human cells. In the present study, we investigated the roles of p53 and Rb in the immortalization of human cells.

MATERIALS AND METHODS

Cells. Three normal human embryo fibroblast cell strains, HSF-412, KMS-6 and OUMS-24 and their immortalized counterparts (SUSM-1, KMST-6 and OUMS-24F) were used in the present experiments. SUSM-1 and OUMS-24F cells were immortalized with 4NQO, and KMST-6 was immortalized with 60Co gamma rays. The details of the immortalization process are described elsewhere (1, 26, 29).

5-Azacytidine treatment. 5-Azacytidine (Sigma, St. Louis, MO) was added to cultures at 4 \( \mu \)M for OUMS-24F and 8 \( \mu \)M for SUSM-1 and KMST-6. The cells were harvested after 9 days of treatment which included three changes of medium containing 5-azacytidine.

PCR and sequencing. Cellular RNA was extracted by the acidic guanidine isothiocyanate method. PCR fragments from the reverse transcribed and polymerase chain reaction (RT-PCR) and the genomic DNA were purified by gel filtration (Chroma Spin 30, Clontech Laboratories, Inc., Palo Alto, CA). The purified PCR fragments were directly sequenced using an AmpliTaq cycle sequencing kit (Perkin-Elmer Cetus, Branchburg, NJ). To obtain specific PCR amplification from genomic DNA or mRNA, intron sequences for genomic DNA or exon-intron junction sequences for RT-PCR were used to produce PCR primer sets.

Immunoprecipitation. Randomly proliferating cells or 5-azacytidine-treated cells were harvested, and proteins were extracted with 400 \( \mu l \) of lysis buffer [0.2% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM 2-mercaptoethanol, 2 \( \mu \)g/ml aprotinin, 2 \( \mu \)g/ml pepstatin, 2 \( \mu \)g/ml leupeptin and 100 \( \mu \)g/ml phenylmethylsulfonyl fluoride]. Cell lysates were clarified by centrifugation at 12,000 \( \times \) g for 10 min at 4°C. Mouse monoclonal IgG against both the wild-type p53 (clone 1620, Oncogene Science, Inc., Manhasset, NY) (7) and the mutated p53 (clone 240, Oncogene Science) (11), was immobilized on Affi-Gel Hz hydrazide gel (Bio-Rad, Hercules, CA) which covalently couples the Fc region of IgG. Cell lysates were gently mixed with the gel beads. Proteins were denatured and fractionated using SDS polyacrylamide (10%) gel electrophoresis, and transferred to a nitrocellulose membrane filter (Amersham). The filter was first blocked for 1 hour with TBST (20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 0.1% Tween-20) containing 5% dry milk, incubated for 1.5 hr with a 1:100 dilution of p53 antibody (clone 1801, Oncogene Science, Inc.), which recognizes both mutant and wild-type p53, and finally washed four times. The filter was then incubated for 1 hr with a secondary antibody (1:1,000 dilution of horse-radish peroxidase-conjugated sheep anti-mouse IgG, MBL, Nagoya, Japan), and p53 was detected using an enhanced chemiluminescence system (ECL, Amersham).

Rb protein was detected in the same way using rabbit IgG (against synthetic peptide P5) for immunoprecipitation, 6% polyacrylamide gel for electrophoresis, and rabbit IgG (against synthetic peptide P3) to detect blotted Rb protein (23).

RESULTS

Detection of p53 mutations in immortal cells. Direct sequencing of RT-PCR-amplified p53 mRNA revealed that mutations had occurred in the p53 gene in all three immortalized cell lines (Fig. 1, A and B). SUSM-1 cells, immortalized with 4NQO, had a mutation in exon 5 at codon 179, where CAT (histidine) was changed to AAT (asparagine). KMST-6 cells immortalized with 60Co gamma rays had a mutation in the same amino acid codon, resulting in the replacement of CAT (histidine) by CCT (proline). The other cell line, OUMS-24F, which was immortalized with 4NQO, had a mutation in exon 7 at codon 248, with a change from CGG (arginine) to CAG (glutamine). There were no other mutations within exons 4 to 9 in these three cell lines.

Although we treated the normal OUMS-24 cells 59 times with \( 10^{-4} \) M 4NQO to immortalize them, p53 remained normal at the 49th PD level in the cells that were treated 49 times with 4NQO (data not shown). However, just after these cells were immortalized by 4NQO (at the 69th PD level), only the mutated p53 was detected by RT-PCR sequence analysis. This result indicates that the mutated p53 was expressed immediately after immortalization of the cells and that the mutation of the p53 was not due to extensive passage of the immortalized cells.

Selective expression of the mutant p53. Only the mutant p53 was expressed in all three immortalized cell lines. This was shown by sequencing the expressed p53 mRNA (Fig. 1, A and C), and by the fact that transcription of the normal alleles of p53 was completely inhibited. However, the sequencing analyses showed that the genomic fragments of the immortalized cells contained both wild-type and mutated p53 alleles (Fig. 1, B and D).

To eliminate the possibility of low-level expression of the wild-type p53 in the immortalized cell lines, we used the wild-type-specific anti-p53 monoclonal antibody PAb 1620 and the mutant-specific anti-p53 monoclonal antibody PAb 240. Immunoprecipitation demonstrated that normal fibroblasts expressed only the wild-type p53 (Fig. 2, lane 1) and that immortalized cell lines ex-
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Fig. 1. RT-PCR and genomic sequencing analysis of cells containing mutations of p53. Only the mutated p53 was detected in reverse-transcribed mRNA (panels A and C). The sequence from genomic DNA (panels B and D) contains both normal and mutated p53 sequences (two sequences shown in the parentheses). The outlined letters indicate mutated p53 sequences and the bold letters indicate the corresponding wild-type sequences. Reverse and complementary sequences of the p53 coding frame are shown in panels A and B.

pressed only the mutant p53 (Fig. 2, lanes 3, 5 and 7). The amount of cell lysates used in the wild-type p53 lanes was 5 times more than that in the mutated p53 lanes, but the expression of the wild-type p53 in the immortalization cell lines was under detectable level under these conditions.

**Induction of wild-type p53 by 5-azacytidine treatment.** Genetic alterations in one of the wild-type p53

![Graph](image-url)

Fig. 2. Immunoprecipitation (IP) of p53 using wild-type (W)- and mutant (M)-specific monoclonal antibodies. Cell extracts used for detecting the wild-type p53 were 5-fold more concentrated than for the mutant p53. Immunoprecipitated proteins were eluted with SDS sample buffer, fractionated on SDS-polyacrylamide gel (10%), and transferred to a nitrocellulose membrane. A pantropic antibody, clone PAb 1801, was used as the primary antibody for Western blotting. p53 proteins were precipitated with the monoclonal antibodies immobilized on hydrazide beads, but trace amounts of IgG were seen by chemiluminescent autoradiography.
alleles caused selective expression of the mutated p53, but no genetic changes in the other wild-type allele were found in the three immortalized cell lines using Southern blotting and RT-PCR sequencing (data not shown). We considered the possibility that hypermethylation might be involved in down-regulation of the wild-type p53. To test this hypothesis, we treated the immortalized cells with a deoxycytidine analog, 5-azacytidine, which inhibits methylation at cytosine sites in DNA. The RT-PCR products from 5-azacytidine-treated SUSM-1 and KMST-6 cells contained both wild-type and mutated p53 sequences (Fig. 3), but the RT-PCR products from OUMS-24F treated with this same reagent, contained only mutant p53.

To further demonstrate induction of the wild-type p53 in the immortalized cell lines, we used the wild-type-specific anti-p53 monoclonal antibody. In this case, all three immortalized cell lines expressed the wild-type p53 following treatment with 5-azacytidine (Fig. 2, lanes 9–11). Thus, although the wild-type p53 was undetectable in OUMS-24F cells by RT-PCR sequence analysis, it became detectable by use of immunoprecipitation.

Rb protein in three immortalized cell lines. We analyzed the expression of Rb protein in the three immortalized cell lines by immunoprecipitation and subsequent Western blotting. Since the HSF-412 cells were near cellular aging, phosphorylated Rb protein was hardly observed. However, expression and the phosphorylated state of Rb protein were normal in all three immortalized cell lines (Fig. 4). However, we could not exclude the possibility of small deletion(s) and/or mutation(s) in the Rb protein in these cell lines.

DISCUSSION

Our sequencing analyses demonstrated that all three human cell lines immortalized with either 4NQO or 60Co gamma rays had mutations of the p53 gene. These mutated points are associated with the T-antigen-binding domain of p53 as well as the hot spots for p53 mutation (16). The same mutation points have also been observed in many other human cancers (14, 30).

Other researchers have postulated that the mutant p53 inhibits the antiproliferative activity of the wild-type p53 via oligomerization (9, 19, 24). However, the universality of the dominant negative phenotype for all p53 mutations is questionable owing to the high frequency of loss of heterozygosity at the p53 locus in many human tumors, resulting in the retention of only the mutant p53 allele (2, 3, 30). A study on transcription and cell growth regulation using bicistronic vectors, which equally transcribed the wild-type and the mutant p53, indicated that the mutant protein could not completely suppress wild-type functions (10).
Direct sequencing of the genomic fragments showed that all three immortalized cell lines contained both wild-type and mutated p53 alleles. Although we did not detect any genetic alterations in the wild-type allele by Southern blotting and RT-PCR sequencing, these three immortalized cell lines expressed only the mutated p53. Our results indicate that inactivation of both p53 alleles may play an important role in immortalization of human cells.

Interestingly, expression of the wild-type p53 was induced in all the immortalized cell lines by treatment with 5-azacytidine, which causes hypomethylation of DNA in many mammalian systems (12). Hypomethylation is one of the known mechanisms of allele-specific gene expression. Actually, Southern blotting using a methylation-sensitive restriction enzyme Hpa II indicates that the entire locus of p53 including the promoter region was hypermethylated in all the immortalized cell lines (data not shown). To demonstrate that down-regulation of the wild-type p53 is due to hypermethylation, experiments showing that the wild-type p53 allele was selectively hypermethylated must be done. However, we were unable to determine the precise methylation state in the wild-type p53 allele using Hpa II, because there are no appropriate restriction enzyme polymorphism markers to identify the wild-type p53 allele by Southern blotting.

Although the precise mechanism of selective expression of the mutated p53 in the three immortalized cell lines remains unknown, selective expression seems to be essential for the immortalization of human fibroblasts. If hypermethylation is one of the mechanisms for inactivation of the p53 gene, then only one mutation or deletion appears to be sufficient for inactivation of p53 function when the other p53 allele has been inactivated by hypermethylation. If the wild-type p53 can be inactivated by hypermethylation in tumors, then, perhaps, treatment with chemotherapeutic agents that inhibit DNA methylation will be effective for suppression of tumor growth.

Rb, which functions in the cell cycle, is regulated by phosphorylation (5, 6, 8, 23). Using the immunoprecipitation method we detected no abnormalities in Rb in the three immortalized cell lines. Furthermore, among cell cycle regulatory genes, oncogenes and tumor suppressor genes, the only common genetic change that we have observed in the three immortalized cell lines is inactivation of p53 (15), although our three immortalized cell lines are obtained from the genetically independent individuals. Thus, inactivation of both wild type alleles of p53 and selective expression of mutated p53 seem to be one of the key factors in the immortalization of human fibroblasts, though in addition to inactivation of p53, some other genetic changes may be necessary for the immortalization of human cells.

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