Up-Regulation of a Mutant Form of p53 by Doxorubicin in Human Squamous Carcinoma Cells

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Abstract
Human squamous carcinoma A431 cells express a high level of epidermal growth factor (EGF) receptor. The cells carry only a mutated form of the p53 gene, the G—A mutation at codon 273 which results in an arginine (CANCER RESEARCH 54, 2834-2836, June 1, 1994)

Introduction
p53 is the most commonly mutated gene in human tumors. There are 5 conserved regions within the p53 molecule; mutation is clustered in all these domains except domain 1. The mutation frequency in certain codons of p53 gene appears to be tumor type specific (1—3). wtP53 is considered to be a transcriptional factor when it binds to specific DNA consensus sequences and a transcriptional repressor when it binds nonspecifically. The biochemical properties of wtp53 in general differ from that of the mutated p53. The half-life of wtp53 is relatively short compared to that of the mutant. Expression of certain genes, such as MDR-1, PCNA, and c-fos, is suppressed by wild-type but is activated by mutant p53 (1–3).

DNA-damaging agents induce wtp53 in cells and cause G1 block (4, 5). The induction is regulated at a posttranscriptional level, requires protein synthesis, and is due to the increased half-life of the protein (6). The expression of wtp53 is upstream of mdm-2 and GADD45 after exposure to radiation (7, 8). Human squamous carcinoma A431 cells overexpressed EGFR (9) and carried a mutated form of the p53 gene (G — A in codon 273). DOX inactivates EGF signal transduction and subsequently induces mp53. The increase in mp53 is coincident with DOX-induced G1-M block in cells.

Materials and Methods
Cell Culture and Drug Exposure Experiment. Human squamous carcinoma A431 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The EGFR density in this cell line is 2 × 10^6/cell (9). In a typical drug exposure experiment, exponential phase cells were exposed to DOX (Sigma Chemical Co., St. Louis, MO) for 1 h, rinsed three times, and then incubated with fresh medium for up to 24 h.

Results
Characteristics of mp53 in A431 Cells. Exons 4—8 of the p53 gene in A431 cells were amplified by PCR and examined by SSCP to detect any mutation. Mutations of the p53 gene detected in the A431 cells were detected only in exon 8 by mobility shift in SSCP analysis (Fig. 1A). Direct sequencing of the mutated exon indicated the G — A mutation at codon 273, which results in an arginine to histidine substitution (Fig. 1B). The mp53 protein was preferably precipitated by antibody specific for wtp53 conformation (PAb 1620) rather than by antibody specific for mutant conformation (PAb 240).
DOX on EGF Signal Transduction and mp53. The results presented in this report are from A431 cells exposed to 5 μg/ml DOX for 1 h; cell survival is about 10% as measured by clonogenic assay. The described changes can be seen but to a lesser extent in cells after treatment with a DOX dose as low as 1 μg/ml; cell survival is about 50%. Furthermore, in addition to DOX, mp53 can also be induced by other DNA-damaging agents, such as cisplatin and ionizing radiation.3

EGFR is the major phosphotyrosine protein in A431 cells. The basal phosphorylation level of EGFR was unchanged immediately following drug exposure, was reduced at 5 min, and became undetectable at 4 h. There are other phosphotyrosine proteins in A431 cells. The tyrosine phosphorylation of some of these proteins is inducible on addition of EGF and appeared not to be altered by DOX (Fig. 2). The EGFR level in A431 cells remained unchanged immediately after exposure to DOX, increased gradually, reached a maximum in 4–8 h, and decayed to the control levels by 24 h (Fig. 3).

Two forms of c-Raf-1 protein are identified on PAGE gel, the slow migrating hyperphosphorylated and the fast migrating underphosphorylated forms. Addition of EGF induced phosphorylation of c-Raf-1; in that case, only the hyperphosphorylated form was seen.3 Immediately after exposure to DOX, there was no change in the levels of both forms of c-Raf-1. After the drug was removed, the level of hyperphosphorylated c-Raf-1 was reduced at 30 min and became undetectable at about 8 h. A slight increase in underphosphorylated c-Raf-1 was seen at the first 30 min after DOX treatment and it may be due to dephosphorylation of the hyperphosphorylated c-Raf-1. Thereafter, the protein decayed and was undetectable at 24 h (Fig. 3).

The mp53 level did not change for up to 8 h after drug treatment and increased significantly (20-fold) at about 24 h (Fig. 3).

Cell Cycle Distribution. There was no significant change in cell cycle distribution in A431 cells at 8 h after DOX treatment, whereas at 24 h, most of the cells were in S and G2-M phases (Fig. 4). The cell cycle distribution in control culture was 68% G1, 16% S, and 16% G2-M phases, and it is 3% G1, 43% S, and 54% G2-M phases in culture at 24 h after exposure to DOX. DOX treatment induces G2-M block in A431 cells and the effect is reversible.3

Discussion

DOX up-regulates mp53 by inactivating EGF signal transduction. Inactivation of EGFR, dephosphorylation of c-Raf-1, and induction of mp53 in A431 cells occur temporally after exposure to DOX (Figs. 2 and 3). c-Raf-1 is downstream to EGFR in EGF signal transduction. EGFR activates EGFR, increases c-Raf-1 phosphorylation, and suppresses mp53 expression in A431 cells with or without DOX pretreatment.4 The growth factor also suppressed the expression of mp53 in human breast carcinoma MDA-468 cells (11). Although it is not clear how EGF signal transduction regulates mp53 expression, the likely candidates should be those shared by both pathways and are downstream to c-Raf-1 in EGF signal transduction, such as c-Myc (12, 13). The DNA-binding motif for helix-loop-helix transcriptional factor, such as c-Myc, is located on the promoter region of mp53 gene. c-Myc transactivates the mp53 gene while the expression of c-myc itself is suppressed by p53 (1, 3, 14).

4 T. T. Kwok, manuscript in preparation.
Induction of mp53 by DOX correlates with drug-induced G2-M block in A431 cells, whereas wtp53 is thought to cause G1 block in other cell lines. DNA-damaging agents induce both G1 and G2-M block in cell lines carrying wtp53 protein and induce only G2-M block if the protein is mutated or inactivated (4, 5). The results from A431 cells are therefore compatible with those reported. Blocking cell cycle progression by p13, at least for wtp53, may allow cells after exposure to DNA-damaging agents to repair damage (4, 5). Both wtp53 and mp53 bind RPA and inhibit RPA binding to single-stranded DNA. RPA is involved in the elongation and initiation step for DNA synthesis and is thought to be essential in DNA excision repair (15, 16). Induction of wtp53 suppresses the enzyme activity of IMP dehydrogenase (17). The enzyme is a key enzyme in purine metabolism and regulates the level of GTP, which is an essential element in signal transduction. Altered activity of several major enzymes in the nucleotide metabolism pathway has been reported in Rat-1 cells transfected with the mp53 gene (18). Nucleotide metabolism pathway is known to be important in cell growth and cell damage repair. Although the mechanisms for p53-related damage repair thus far are not clear, the importance of RPA and nucleotide metabolism should not be overlooked.

A key molecule shared by both EGF- and DOX-induced signal transduction is the serine/threonine kinase, PKC. An increase in the level but no change in the affinity of EGFR by chronic exposure to DOX has been reported in HeLa and 3T3 cells. However, the mechanism for induction are not clear and the level of receptor tyrosine phosphorylation was not described (19). Activation of PKC by DOX has been demonstrated in various cell systems (20). The enzyme can be activated in EGFR signal transduction through activation of phospholipase C-γ 1. PKC phosphorylates serine and threonine residues in EGFR and one of those is Thr-654. Phosphorylation of Thr-654 from EGFR by PKC desensitizes ligand-stimulated EGFR tyrosine kinase activity, retards ligand-induced receptor internalization, and induces internalization and recycling of unoccupied EGFR (21). Activation of PKC by DOX will likely suppress the basal phosphorylation and also increase the level of EGFR.

Results from the present study support the notion that EGF signal transduction regulates the expression of mp53. It is tempting to speculate that mp53, and possibly other forms of p53, may be one of the major downstream elements in growth factor signal transduction. Further studies to explore the possibility are currently under way.

Acknowledgments

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