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HUMAN P53, MUTATED AT CODON 273, CAUSES DISTINCT EFFECTS ON NUCLEOTIDE BIOSYNTHESIS SALVAGE PATHWAY KEY ENZYMES IN Rat-1 CELLS AND IN THEIR DERIVATIVES EXPRESSING ACTIVATED RAS ONCOGENE

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SUMMARY. The introduction of human p53 with mutation at codon 273 into Rat-1 cells induces changes in the salvage pathway of nucleotide synthesis. In cells expressing the mutant p53 the activities of hypoxanthine phosphoribosyl transferase (HPRT) and thymidide kinase (TK) decrease 3.5- and 2-3-fold, respectively, while the activities of adenosine deaminase and uridine kinase, in contrast, increase correspondingly 2.5- and 1.5-fold. On the other hand, in cells transformed by *ras* oncogene, which causes dramatical reduction in HPRT activity as well as enhancement of TK function, the expression of exogeneous p53 leads to the opposite effects and causes the reversion of activities of both enzymes to the levels found in parental cells.

p53, a nuclear phosphoprotein that possesses properties of transcription *trans*-activator, is involved in negative regulation of the cell cycle progression (for review see [1,2]). Evidently, it functions as a critical part of a DNA damage-induced G_1-phase checkpoint control that maintains genetic stability (3,4). Mutations within the p53 gene are the most common genomic alterations found to date in human cancers (1,5). It is generally accepted that mutant p53 proteins act in a dominant-negative fashion by binding to the wild type protein which results in the formation of oligomeric complexes with inhibited physiological activity (1,2). However, at least in some systems mutant p53 proteins may act in a true dominant mode since the introduction of a mutant p53 gene into cells lacking either wild type, or any p53 expression enhances their neoplastic properties (1,6,7).

Among the possible targets of p53 function are the enzymes involved in nucleotide salvage uptake. Purine nucleotides and nucleosides are considered by many researchers as potential universal regulators of cell growth and differentiation (8-11). The salvage pathway of purine and pyrimidine nucleotides synthesis seems to be especially important for regulation. The activities of its key enzymes show a significant variety in different cell

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types. In many of them, both normal and malignant, the salvage pathway predominates over the *de novo* synthesis (12,13). Recently it was shown (14) that the wild type p53 (unlike the mutant p53 with altered conformation) inhibits activity of one of the enzymes of purine salvage uptake, the inosine-5-monophosphate dehydrogenase (EC 1.2.1.14).

We decided to study the influence of p53 bearing one of the most frequent mutation, substitution of arginine for histidine at codon 273 (p53His273) that does not lead to conformational changes (1), on purine and pyrimidine nucleotides salvage synthesis. Earlier we have found that this mutation modifies, but, in contrast to other mutations tested, does not abrogate the ability of p53 to specific binding to consensus DNA sequences (15). It is well known that mutant p53 cooperates with an activated *ras* in transformation of rat embryo cells (1,16,17). The mechanisms of this cooperation remain unclear. We compared the effects caused by human mutant p53 in Rat-1 cells that express or do not express the *ras* oncogene and have found substantial differences in the activities of salvage pathway key enzymes in these cell systems.

Materials and Methods

Cell lines. RatR4(8)1 and RatR4(8)2, derivatives of Rat-1 cells expressing p53His273 that was introduced by self-inactivating retroviral vector HSG/neo, as well as Rat/neo cells containing the "empty" HSG/neo construction, were described earlier (15). Rat/neo-ras and RatR4(8)2-ras sublines were obtained by infection of Rat/neo and RatR4(8)2 cells, respectively, with amphitropic virions of pPS/hygro construction (18) bearing human mutant *N-ras*^{Asp12} cDNA (19). The sublines containing pPS/hygro construction were obtained as a mixture of several dozens of independent clones grown after selection with hygromycin (200 μg/ml). In the developed cell lines the expression of exogeneous *N-ras* oncogene, (confirmed by RT-PCR analysis; I.A.Grigorian, P.M.Chumakov, and B.P.Kopnin; unpublished data), resulted in a pronounced morphological transformation (Fig. 1). All the cell lines were cultivated in DMEM medium supplemented with 10% of fetal calf serum. For doubling time and saturation density assays 10^4 of cells were seeded onto 35mm dishes. The number of cells were counted daily on duplicated dishes during 10 days.

Enzyme assays. Hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase and purine nucleoside phosphorylase as well as thymidine kinase (TK) and uridine kinase, the key enzymes in salvage synthesis of purines and pyrimidines, respectively (Fig. 2), were studied. Enzyme activities were assayed in lysates of cells obtained after two cycles of freezing and thawing followed by centrifugation at 20000g. The HPRT, TK and uridine kinase activities were determined according to the initial reaction rate by measurement of the convertion of ^{14}C-nucleoside (or base) into ^{14}C-nucleotide (20-22). Samples were incubated for 5, 10, 15, 20 minutes. Aliquotes of 10 μl were placed on DEAE-cellulose (DE-81) paper disks (2x2 cm) and washed twice with 3 mM formiate ammonium pH 4.4, water and alcohol. The filters were dried and their radioactivity was counted. Adenosine deaminase and purine nucleoside phosphorylase activities were assayed according to the initial reaction rate by optical method (21). The enzyme activities were expressed as *nmol* of the substrate converted *for one hour per one milligram* of protein.

Results and Discussion

The introduction into Rat-1 cells of either p53His273, or an activated *N-ras* cDNAs resulted in changes of cell morphology as well as some growth

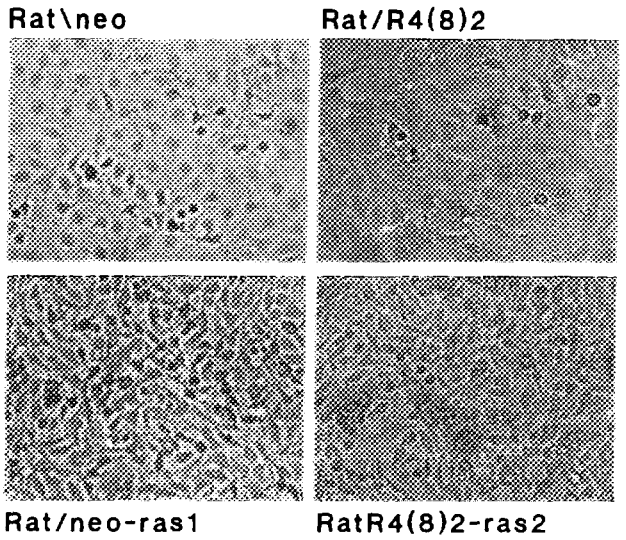

Figure 1. *The morphology of Rat-1 cell sublines expressing the "empty" HSG/neo vector* [Rat/neo], *p53His273* [RatR4(8)2], *activated N-ras* [Rat/neo-ras1] *and both these genes simultaneously* [RatR4(8)2-ras2].

characteristics. Cells that express p53His273 were somewhat more flattened (Fig 1), had increased doubling time and decreased saturation density (Table 1). In addition, they showed an enhanced dependence on serum factors (15). The expression of the *N-ras*^{Asp12} cDNA caused a manifest morphological

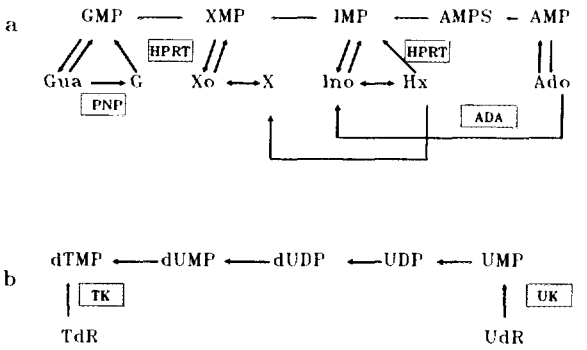

Figure 2. *Salvage pathways for purines (a) and pyrimidines (b).*
a: ADA - adenosine deaminase (EC 3.5.4.4), HPRT - hypoxanthine phosphoribosyl transferase (EC 2.4.2.1), PNP - purine nucleoside phosphorylase (EC 2.4.2.1), TK - thymidine kinase (EC 2.7.1.21), UK - uridine kinase (EC 2.4.2.3), IMP - inosinic acid, GMP - guanylic acid, XMP - xanthanylic acid, AMPS - adenylosuccinate, AMP - adenylic acid, Gua - guanosine; G - guanine, Xo - xanthosine, X - xanthine, Ino - inosine, Hx - hypoxanthine, Ado - adenosine.
b: UMP - uridine 5'-monophosphate, UDP - uridine diphosphate, dUDP - deoxyuridine diphosphate, dUMP - deoxyuridine monophosphate, UdR - uridine, dTMP - deoxythymidine monophosphate, TdR - thymidine.

Table 1. Influence of Exogeneous p53His273 and Activated N-ras on Growth Characteristics, Thymidine Kinase and Hypoxanthine Phosphoribosyl Transferase Activities

Cell Subline	Introduced p53His273	Introduced N-ras^{Asp12}	Doubling Time (h)	Saturation Cell Density (10^4/cm^2)	TK[a] (nmol/h×mg)	HPRT[b] (nmol/h×mg)	Km Hx[c] (μM)
Rat-1	-	-	18.5	11.9	5.8±1.0 (8)	9.7±0.5 (8)	ND[d]
Rat-HSG/neo	-	-	19.2	11.1	3.8±0.5 (12)	9.7±0.5 (12)	70
RatR4(8)2	+	-	24.9±0.7 (2)	8.8	2.5±1.1 (12)	2.5±0.2 (12)	100
RatR4(8)1	+	-	24.6	7.9	1.5±0.5 (8)	2.9±0.1 (8)	ND
Rat/neo-ras1	-	+	14.5±0.1 (2)	21.5	9.1±0.7 (8)	0.5±0 (8)	ND
RatR4(8)2-ras2	+	+	17.2±0.2 (2)	14.2	3.3±0.3 (4)	11.0±0.9 (4)	83

[a] *Incubation mixture for thymidine kinase (TK) assay contained (in mM):* [2-^{14}C]thymidine (specific radioactivity 370 MBq/mmol) - 0.4; $MgCl_2$- 3; ATP - 10; NaF - 30; tris HCl, pH 8.0 - 100.

[b] Incubation mixture for hypoxanthine phosphoribosyl transferase (HPRT) assay contained (in mM): [8-^{14}C]hypoxanthine - 0.1; (specific radioactivity 1.7 TBq/mol), 5-phosphoribosyl-1-pyrophosphate (PRPP) - 1; $MgCl_2$ - 5; tris HCl, pH 7.4 - 50.

[c] Measurements of apparent Michaelis constant for hypoxanthine (Km Hx) were performed by varying hypoxanthine concentrations (300-3 μM) in the presence of 5 mM $MgCl_2$ and 1 mM PRPP.

[d] ND - not determined.

In parentheses ( ), the numbers of experiments performed are indicated.

transformation and an increase in both proliferation rate and saturation density (Fig. 1, Table 1). In comparison with Rat/neo-ras1 the cells expressing both constructions simultaneously [RatR4(8)2-ras2] demonstrated less pronounced features of transformation *in vitro* (Table 1).

We observed that the thymidine kinase (TK) activity directly correlated with the proliferation rate of the cells studied: the introduction of the *ras* oncogene resulted in its increase while the expression of p53His273, in contrast, led to the decrease in the enzyme activity. These findings are consistent with the data showing that TK activity increases in the S-phase (23,24). It is noteworthy that simultaneous function of the both introduced human genes was accompanied by the reversion of the TK activity to the level that is characteristic of control Rat-1 and Rat/neo cells (Table 1). Another enzyme of pyrimidine salvage synthesis, the uridine kinase, showed equal activity in Rat/neo and Rat/neo-ras1 cells (about 400 nmol/h x mg), however its function was augmented 1.5-1.7-fold in cells expressing p53His273 and was independent on additional expression of the *ras* oncogene.

Hypoxanthine phosphoribosyl transferase (HPRT) activity did not correlate with growth characteristics of the cell cultures (Table 1). It was reduced in cells bearing the exogeneous p53 gene (about 3.5-fold) that possess the longer

doubling time as well as in cells transformed by *ras* (about 20-fold) showing the increase in proliferation rate. Surprisingly, simultaneous expression of p53 and *ras* results in enhancement of HPRT activity, restoring it to the level found in the control sublines (Table 1). It should be emphasized that cells containing p53His273 showed a decreased sensitivity to the cytotoxic action of 8-azaguanine (Fig. 3), an antimetabolite converted by HPRT. Evidently the suppression of this enzyme in cells bearing p53His273 or *ras* resulted in a reduced synthesis of both, guanylic and inosinic acids from guanine and hypoxanthine, respectively. This might explain slightly increased sensitivity of cells containing one of the introduced constructions, to methotrexate which blocks *de novo* pathway of nucleotide synthesis. However, the cells with simultaneous expression of both human genes, characterized by restoration of normal HPRT activity as compared with their parental RatR4(8)2 cells, retained the enhanced sensitivity to methotrexate (Fig. 4) that indicates more complex regulation of methotrexate sensitivity.

HPRT activity was further studied in respect to its kinetic properties. KmHx values in all the cell lines tested were similar (Table 1). This indicates that the p53His273 (and possibly ras) expression lead to quantitative changes in HPRT protein, rather than alterations of the enzyme affinity to the substrate.

It should be noted that in all the cases the reduced HPRT function was accompanied by an increase in adenosine deaminase activity (1.5- and 2.5-fold in cells expressing *N-ras* and p53His273, respectively) while the activity of purine nucleoside phosphorylase did not show any changes in all the sublines tested (Fig. 5).

So after introduction of p53His273 into Rat-1 cells we have observed a set of changes in nucleotide synthesis salvage pathway. The most prominent of

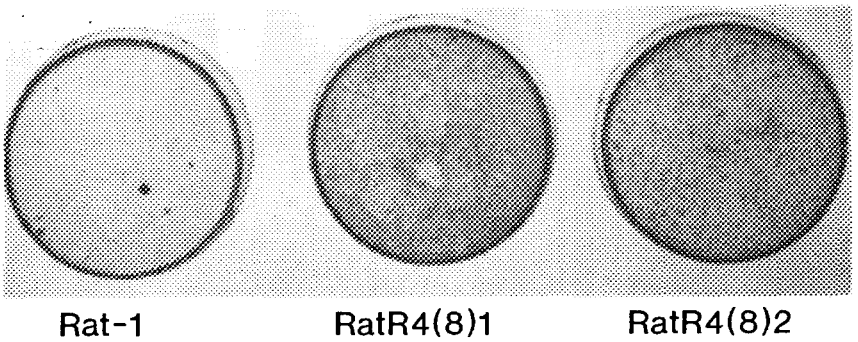

Figure 3. *Decreased sensitivity of cells containing p53His273 to 8-azaguanine.* 10^5 cells were seeded on 60 mm dishes in the medium containing 10 μg/ml of 8-azaguanine. After cultivation for 8 days the dishes were stained by Giemsa. In contrast to control Rat-1 cells which formed single resistant colonies, the sublines bearing p53His273 showed thousands of dividing cells.

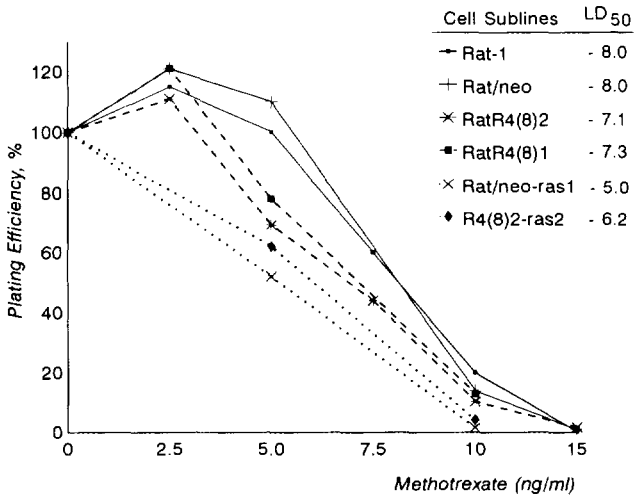

Figure 4. *The sensitivity of different sublines to methotrexate.*
$2x10^2$ cells were seeded on 60 mm dishes in media with different methotrexate concentrations. After cultivation for 9 days the dishes were stained by Giemsa and the numbers of colonies grown were scored. In the right upper corner along with the designations of the cell lines tested their LD50 (the dosages of methotrexate in ng/ml that diminished on a half the number of colonies as compared with control) are given.

them were the alterations in HPRT activity. Our results along with the data showing the suppression of 5'-inosine monophosphate dehydrogenase by wild type but not by mutant p53 (14) indicate a strong influence of p53 on nucleotide

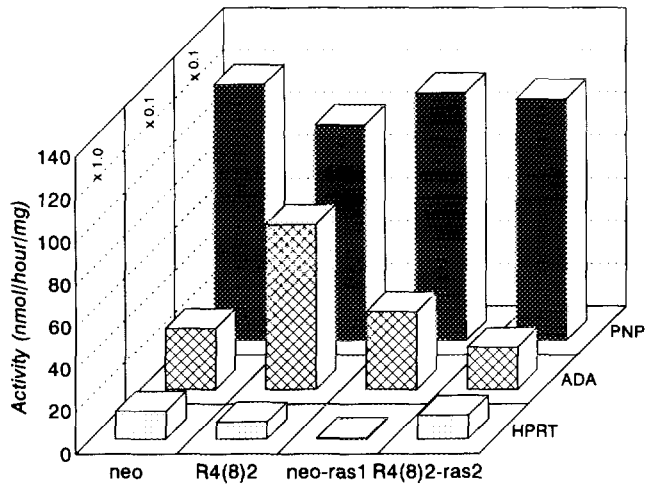

Figure 5. *Effect of p53His273 and N-ras on hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP) activities.*
The reaction mixture for ADA and PNP assays contained $8x10^{-5}$ M adenosine and $8x10^{-5}$ M guanosine, respectively, in 0.1 M potassium phosphate buffer, pH 7.0. The initial velocity of decrease in the absorbance at 265 nm of adenosine and at 252 nm of guanosine was followed at 37° C.

metabolism. However the data obtained do not allow to distinguish whether the altered genes are the primary targets of p53 action, or some of the alterations could be induced to compensate primary changes. In fact, the inherited deficiency in HPRT (Lesch-Nyhan syndrome) is accompanied by activation of *de novo* pathway as well as by stimulation of inosine 5'-monophosphate dehydrogenase activity (25). It can not be excluded that p53 influences on either salvage or *de novo* pathway enzymes, or on some targets from both pathways. It is also unclear whether the observed changes are due to the inactivation of endogeneous wild type p53 by exogeneous mutant protein or they represent the direct effects of p53His273 on transcription of some genes. The wild type p53 can activate gene transcription by binding to consensus DNA sequences in regulatory regions (1,2) and inhibit it by interaction with TATA-binding protein (26). Thus, the inactivation of wild type p53 can lead to both, an enhancement and a reduction of gene expression. p53His273, unlike the most of p53 mutants, retains some ability to specific DNA-binding (15) and therefore, may also directly affect p53 responcive elements.

At present it is difficult to explain the surprising finding of opposite effects of p53His273 on salvage enzymes activity in cells that express or do not express the activated *ras* oncogene. p53 and *ras* may act on salvage enzymes via different pathways. According to recent data (27) both, the wild type and His273 p53, but not the conformationally altered p53His175, can reppress a transcription from the human *hsp70* promoter by direct protein-protein interaction with other transcription factor, CBF, that itself causes the *hsp70* gene activation. Similar mechanism might function in cells transformed by *ras*, which evidently induces the production of a set of transcription factors. In rat REF52 cells mutant p53 releases the cell cycle growth arrest in G_1/S or G_2/M boundaries induced by overexpression of activated *ras* (28). It seems important to determine whether this ability to overcome the cell cycle restriction might be connected with the normalization of the activities of the enzymes involved in nucleotide synthesis.

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