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Transcriptional Inhibition of the Human Papilloma Virus Reactivates Tumor Suppressor p53 in Cervical Carcinoma Cells

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Abstract

Inactivation of tumor suppressor p53 accompanies the majority of human malignancies. Restoration of p53 function causes death of tumor cells and is potentially suitable for gene therapy of cancer. In cervical carcinoma, human papilloma virus (HPV) E6 facilitates proteasomal degradation of p53. Hence, a possible approach to p53 reactivation is the use of small molecules suppressing the function of viral proteins. HeLa cervical carcinoma cells (HPV-18) with a reporter construct containing the β -galactosidase gene under the control of a p53-responsive promoter were used as a test system to screen a library of small molecules for restoration of the transcriptional activity of p53. The effect of the two most active compounds was studied with cell lines differing in the state of p53-dependent signaling pathways. The compounds each specifically activated p53 in cells expressing HPV-18 and, to a lesser extent, HPV-16 and exerted no effect on control p53-negative cells or cells with the intact p53-dependent pathways. Activation of p53 in cervical carcinoma cells was accompanied by induction of p53-dependent *CDKN1* (p21), inhibition of cell proliferation, and induction of apoptosis. In addition, the two compounds dramatically decreased transcription of the HPV genome, which was assumed to cause p53 reactivation. The compounds were low-toxic for normal cells and can be considered as prototypes of new anticancer drugs.

Keywords

p53; human papilloma virus; cervical carcinoma; HeLa cells; chemical library; transcriptional reporter construct; β -galactosidase; suppression of cell proliferation; apoptosis

Introduction

Carcinogenesis results from a gradual accumulation of genetic changes mostly affecting the genes whose products are involved in regulating the cell cycle, apoptosis, and morphogenetic

reactions of the cell [1]. When the systems maintaining the genome stability are impaired, mutations accumulate at a higher rate and the disease develops rapidly. Tumor suppressor p53 is responsible for early negative selection of damaged cells, which ensures exceptional stability of the human genome [2]. The p53 function is disturbed in the overwhelming majority of tumors [3]. This is caused by *p53* mutations in half of the tumors [4], while other components of p53-dependent signaling are affected in other cases [5]. Tumors resulting from virus-induced carcinogenesis usually contain structurally intact *p53*, but p53 activity is inhibited to a considerable extent. Such inhibition is due to certain viral proteins whose function is to prevent p53-dependent apoptosis, which can prevent virus replication [6]. When this virus function is preserved and some others are lost, virion replication becomes impossible and some cell properties change, which leads to malignant transformation.

Restoration of p53 function is a promising approach to cancer therapy [7]. A higher sensitivity to p53 is explained by the tumor cell abnormality, permanently activating the proapoptotic function of p53. To restore its function in tumors expressing mutant *p53*, it is necessary to introduce exogenous wild-type *p53* [8,9] or use small molecules partly restoring the conformation of mutant p53 [10,11]. In the case of virus-induced carcinogenesis, the problem is reduced to overcoming the effect of viral proteins, because components of the p53-dependent signaling pathways are intact.

Cervical carcinoma is associated in more than 90% of cases with human papilloma virus (HPV) type 16 and 18 [12]. The product of HPV *E6* binds to p53 and promotes its rapid degradation by the proteasomal system [13,14]. It is clear that *E6* silencing or prevention of the E6-p53 interaction should restore the activity of p53 and enhance its tumor suppressor effect. We searched for small molecules capable of reactivating p53, restoring its transcriptional activity. As a model, we used the HeLa cervical carcinoma cell line, which produces HPV-18 proteins [15].

Experimental

Reporter plasmids

We used p53-responsive reporter plasmids pSIP-ConA-lacZ and pUSTdSB-WafConA-mCMV-lacZ [16,17].

Preparations of vector virions, infection of target cells, and selection of infected cells

To obtain retroviral vector virions, retroviral constructs were transduced into PhoenixAmpho cells [24] with the use of Lipofectamine-Plus (Invitrogen). Infected cells were selected on the standard culture medium supplemented with 1 µg/ml puromycin.

β-Galactosidase activity

In the case of X-Gal staining, cells were fixed with cold 0.5% glutaraldehyde, 1 mM MgCl₂ in PBS and stained with 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.02% NP-40, 0.2% X-Gal (Sigma) in PBS at 37°C for several hours. In the case of ONPG staining, we used 2 mg/ml ONPG, 250 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 0.02% NP-40 at 37°C. Staining intensity was measured as A₄₀₅ on a Victor 1420 Multilabel counter (Perkin Elmer).

Western blotting

Cells were lysed in buffer RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS) supplemented with Complete Mini protease inhibitors (Roche). Protein extracts (20 µg of total protein) were examined by PAGE in 4-20% gradient gel (Invitrogen). Proteins were transferred onto nylon membranes and stained with the use of specific antibodies against p53 (DO-1, Santa Cruz) or p21 (F-5, Santa Cruz).

Northern blotting

Total RNA was isolated using Trizol as recommended by Invitrogen. Electrophoresis was carried out in 1% agarose gel. As probes, we used the *CDNK1*, *GAPDH*, and HPV *E6* cDNAs, which were obtained by RT-PCR.

Cell counting

Cells treated with chemicals were counted every 24 h for 6 days, using a Goryaev chamber. Statistical analysis of the results was performed using Microsoft Excel spreadsheets.

Portion of apoptotic cells

was estimated by flow cytometry after cells were stained with Annexin V-FLUOS and a Roche kit, as recommended by the manufacturer.

Results And Discussion

Construction of a Reporter System to Assay the Transcriptional Activity of p53 in Cervical Carcinoma Cells

The main tumor suppressor effects of p53 are due to p53-dependent transcriptional activation of several genes triggering apoptosis or arresting the cell cycle [19]. The transcriptional activity of p53 is favorable to assay with reporter constructs containing a reporter gene under the control of a p53-responsive promoter [20]. Since transcriptional activity of p53 in HeLa cells is dramatically inhibited [21], the reporter gene should be expressed at a low level to allow detection of p53 activation in response to various factors. With this approach, we used HeLa cells to obtain a sensitive test system suitable for searching for small molecules that activate p53. As reporters, we used retroviral vectors pSIP-ConA-lacZ [17] and pUSTdSB-WafConA-mCMV-lacZ [16], which expressed *Escherichia coli lacZ* under the control of a minimal promoter (*Hsp70* or cytomegalovirus), which was combined with three p53-binding elements: one from a ribosome gene cluster [22,23], an artificial 20-bp high-affinity p53-binding segment [24], and the *CDKN1A* element [25]. Introduction of a reporter cassette with a retroviral vector is advantageous over plasmid transfection, as single copies are usually integrated and expression only slightly varies among individual clones [16,17,26]. With *lacZ* used as a reporter, expression can be assayed both qualitatively with X-Gal and quantitatively by β -galactosidase activity in the reaction with ONPG [17].

When HCT116 colorectal carcinoma cells were infected with the constructs and then treated with increasing doses of 5-fluorouracil (5-FU), the background level of the reaction with ONPG in the case of pSIP-ConA-lacZ was half as high as in the case of pUSTdSB-WafConA-mCMV-lacZ and increased by a factor of 3-3.5 upon 5-FU treatment with the latter construct (Figure 1a), suggesting its higher sensitivity. Hence, further experiments were performed with reporter cell lines obtained with pUSTdSB-Waf-ConA-mCMV-lacZ.

Suppression of stress-induced p53-dependent transcription was observed when 5-FU was used at various doses to treat HeLa and SiHa cells, expressing HPV. In parallel, similar treatment was performed with control reporter cell lines, which possessed (HEF and HCT116) or lacked (H1299 and A431) p53 activity. Induction of β -galactosidase activity was completely suppressed in HeLa and SiHa cells, as was the case with H1299 cells, carrying a *p53* deletion, and A431 cells, expressing *p53* with an inactivating mutation in codon 273 (Figure 1b).

To check whether the reporter lines derived from HeLa cells were suitable for searching for small molecules restoring the transcriptional activity of p53, it was necessary to select a treatment protocol that would allow detection of p53 activation. In other words, a search for activating molecules needs a positive control to serve as an activation reference. Actinomycin

D has been reported to activate (stabilize) p53 in HeLa cells [21]. However, we did not detect an increase in p53-dependent *lacZ* expression when the reporter HeLa cell line was treated with various doses of actinomycin D (data not shown). To select an activation control, we tested several compounds known to activate p53 in cells with intact p53-dependent pathways (Figure 2). Unexpectedly, the DNA-modifying agent methyl methanesulfonate (MMS) strongly activated β -galactosidase (Figure 2a). MMS lacked such an effect in p53-negative H1299 cells; in HeLa cells, MMS stabilized p53 and increased transcription of *CDKN1*, a p53 target coding for the cyclin-dependent kinase inhibitor p21 (Figure 2b). These findings indicate that the MMS effect is associated with p53 activation. Although the mechanism of action is unknown for MMS, we used cell treatment with 30 μ g/ml MMS as a positive control in screening chemical compounds.

Search for Chemical Compounds Capable of Reactivating p53-Dependent Transcription in HeLa Cells

A screening of 46,000 compounds from a chemical library (ChemBridge) was carried out with our reporter HeLa cell line by Quark Biotech (Israel); its results will be detailed elsewhere. A compound was considered active if β -galactosidase activity increased more than twice after cells were exposed for 24 h to its 5 μ g/ml concentration. About 60 active compounds were further tested with the HCT116 and H1299 control cell lines carrying p53-responsive pUSTdSB-WafConA-mCMV-lacZ; the objective was to reject the compounds that either activated the reporter non-specifically or induced p53 via activation of stress-related signaling pathways. Many of the compounds identified as active were similar in chemical structure and, in total, belonged to more than ten chemical classes. This suggests a multiplicity of targets that can be affected to activate p53-dependent mechanisms in HPV-expressing cells.

We focused on the effects of two active compounds from different chemical classes (Figure 3). Added to reporter HeLa cells, the compounds each induced β -galactosidase activity in a dose-dependent manner (Figure 4a), but compound I was more effective ($IC_{50} = 2 \mu$ g/ml vs. 7.5 μ g/ml of compound II). Tested with the control reporter cell lines (p53-positive HCT116 and p53-negative H1299), the compounds did not appreciably activate β -galactosidase (Figure 4b). At the same time, the compounds activated the reporter in SiHa cells, carrying HPV-16, although the activation was lower than in HeLa cells, carrying HPV-18 (data not shown). This result suggests that both of the compounds specifically activate p53 inhibited by the products of HPV, especially HPV-18.

Reactivation of p53-Dependent Genes in HeLa Cells Treated with the Selected Compounds

We compared the levels of p53 and its target p21 in HeLa cells examined before and after treatment with compounds I and II at concentrations corresponding to IC_{50} . The p53 level increased after 24-h exposure, especially with compound I. Even a greater increase was observed for p21, suggesting substantial activation of p53 (Figure 5a). A similar treatment did not increase the p53 and p21 levels in HCT116 cells compared to the control. Likewise, changes in p53 and p21 production were not observed in A431 cells, expressing mutant p53 (Figure 5a). This result testifies again that the compounds specifically activated the p53-dependent pathways, which were suppressed by HPV. An increase in the expression of p21-coding *CDKN1* was also observed at the transcriptional level by Northern blotting (Figure 5b).

One of the mechanisms activating p53 in HPV-expressing cells is inhibition of viral products by chemical compounds. To check this possibility, the level of the specific HPV-18 *E6* transcript was studied before and after cell treatment with the two compounds. As Northern blotting showed, the compounds used at IC_{50} dramatically decreased the level of *E6* transcripts (Figure 5b). It is clear that inhibition of *E6* expression releases p53 from its interactions with *E6*, which facilitates proteasomal degradation of p53.

Reactivation of p53 in HeLa Cells Is Accompanied by Inhibition of Cell Proliferation and Induction of Apoptosis

Possible consequences of p53 activation are inhibition of cell proliferation or induction of apoptosis. To study the potential specific cytostatic effect of the two compounds, we estimated the growth rate of cells cultured in their presence (Figure 6). Compound I exerted a greater cytostatic effect on HeLa cells, which correlated with its higher activity in the reporter system. Used at 2 µg/ml (IC₅₀ estimated in the reporter system), compound I significantly inhibited proliferation of HeLa cells (Figure 6a) and had no effect on proliferation of HCT116 cells, expressing wild-type *p53* (Figure 6b). Inhibition of cell growth was accompanied by a considerable increase in the portion of Annexin V-positive apoptotic cells after 48-h exposure to compound I (Figure 7). The portion of apoptotic cells did not increase in HCT116, H1299, and A431 control cell cultures, supporting the specific cytostatic effect of compound I on cells expressing HPV. Thus, the two compounds representing two out of ten chemical classes activate the transcriptional activity of p53 by inhibiting the expression of virus-specific transcripts in HeLa cells. The structural difference between the compounds examined suggests several mechanisms of their action. The p53 function in HPV-positive cells can be restored via suppression of the viral functions at the protein level, i.e., by inhibition of p53-E6 interactions or E6AP ubiquitin ligase. It cannot be excluded that the compounds affect other viral or cell components involved in suppressing the p53 function in cervical carcinoma cells. Further studies may identify such compounds as potential prototypes of new anticancer drugs.

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Figure 1.

Tests for expression of the lacZ reporter gene from different vectors in different cell lines. (a) Induction of β -galactosidase activity in HCT116 cells after the introduction of p53-responsive pSIP-ConA-lacZ and pUSTdSB-WafConA-mCMV-lacZ and cell treatment with increasing concentrations of 5-FU for 16 h. (b) Production of β -galactosidase in several human cell lines carrying p53-dependent pUSTdSB-WafConA-mCMV-lacZ after treatment with 5-FU for 16 h. Cell lines: HEF, HCT116 (wild-type p53), H1299 (deletion of p53), A431 (inactive mutant p53), HeLa, and SiHa (*p53* expression is inhibited by HPV E6). The β -galactosidase activity was inferred from A_{405} , reflecting the yield of the colored ONPG derivative produced by β -galactosidase.

Figure 2.

Tests for lacZ activation by various DNA-damaging agents. (a) Detection of β -galactosidase activity with X-Gal in HeLa, HCT116, and H1299 cells exposed to DNA-damaging agents (indicated at the top). EMS, ethyl methanesulfonate; DOX, doxorubicin. (b) Induction of *p53* and its target, *CDKN1*, in response to cell treatment with 50 μ g/ml 5-FU or 30 μ g/ml MMS for 24 h. Left panel, Western blotting of p53 with monoclonal antibody DO-1; right panel, Northern analysis of *CDKN1* expression.

#5254239

2-Amino-4-(1,3-benzodioxol-5-yl)-5-hydroxy-10-methyl-8-oxo-3,4-dehydro-2H,8H-pyrano[2,3-f]chromen-3-carbonitrile

Compound I

#5228473

1-(2-methyl-4-aminobenzene-3,4-dehydro-2H-quinolone)-ethanone

Compound II

Figure 3.

Structural formulae of compounds I and II (ChemBridge cat. nos. 5254239 and 5228473), which represented two chemical classes and were selected from a chemical library by the capability of restoring the transcriptional activity of p53 in HeLa cells.

Figure 4. Dose-dependent induction of reporter P-galactosidase activity in (a) HeLa, (b) HCT116, and H1299 cells treated with compounds I and II. Ordinate, A_{405} , reflecting the production of the color OPNG derivative by β -galactosidase; abscissa, concentration of compounds I and II.

Figure 5.

Compounds I and II increase the level and transcriptional activity of p53 and decrease the level of virus-specific *E6* transcripts. (a) Western blotting of p53 and its target p21 in HeLa and two control (HCT116 and A431) control cell cultures exposed to compounds I (2 µg/ml) and II (7.5 µg/ml) for 24 h. Monoclonal antibody DO-1 was used for detection. (b) Northern analysis of the *CDKN1* (p21), HPV-18 *E6*, and *GAPDH* transcripts in HeLa cells treated as above. Control cells were not exposed to compounds I and II

Figure 6. Growth of (a) HeLa and (b) HCT116 cells in the presence of compounds I (2 $\mu\text{g/ml}$) and II (7.5 $\mu\text{g/ml}$) and in their absence (control).

Figure 7. Quantitation of apoptotic cells in HeLa, HCT116, H1299, and A431 cultures 48 h after treatment with 2 µg/ml compound I. Ordinate, percent cells stained with Annexin V.