Doxorubicin and octreotide induce a 40 kDa breakdown product of p53 in human hepatoma and tumoral colon cell lines

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The chemotherapeutic drug doxorubicin and the anti-proliferative long-acting somatostatin analogue octreotide, both used in cancer treatment, have been shown to increase the expression of the p53 tumour suppressor protein. In the present study, we demonstrate by Western-blot analysis that, in addition to the p53 protein, these molecules were able to induce the expression of a shorter protein with an apparent molecular mass of 40 kDa (p40), recognized by antibodies raised against the N-terminus of p53. This induction was present in tumoral and non-tumoral cells and did not depend on the status of the endogenous p53 protein. The p40 protein was significantly induced after 3 h of cell treatment with doxorubicin or octreotide, remained stable until 24 h and was located in the nuclear extract. Using reverse primers corresponding to each exon of the p53 gene, only one

transcript was amplified by reverse transcriptase-PCR. This suggested that p40 was issued from a post-translational modification and not from an alternative splicing. This protein was not recognized by the PAb421 antibody, suggesting that it was issued from a cleavage of the p53 C-terminal region (p40ΔC). Furthermore, this cleavage was not dependent on caspase activity. In conclusion, these results support the hypothesis that this post-translational modification plays a significant role in the regulation of multiple p53 signalling pathways. These results also suggest that octreotide, a molecule with different signalling pathways, was able as doxorubicin to generate a p53 breakdown product.

Key words: caspase, chemotherapeutic drug, p53 cleavage, somatostatin analogue.

INTRODUCTION

In unstressed cells, the tumour suppressor gene encoding the p53 protein, a tetrameric transcription factor essential for the maintenance of the genomic integrity, is short-lived and expressed at a lower level. A variety of anti-proliferative or pro-apoptotic molecules act as cellular stimuli triggered by DNA-damaging agents and activate p53 through its stabilization and accumulation in the nucleus, and binding to multiple DNA-response elements. Both the chemotherapeutic compound doxorubicin, which induces DNA damage and stimulates apoptosis, and the somatostatin analogue octreotide, which exerts anti-proliferative and pro-apoptotic effects, stimulate the p53 transcriptional activity [1,2]. These two molecules are currently used in the treatment of liver and/or colon cancer, but they are not totally effective, due to a large number of resistance mechanisms being present in the tumoral cells [3–6]. To restore this loss of efficiency, multiple signalling pathways stimulated through the activation of p53 need to be better understood, in particular post-translational modifications that may play a critical role in the regulation of various functions. Phosphorylation–dephosphorylation, acetylation–deacetylation and proteolytic cleavages of p53 appear after cellular stress [7]. The generation of several p53 breakdown products, resulting from the N- or C-terminal truncations, has been characterized according to the type of DNA damage. A previous study [8] suggested that they are involved in the cell-cycle arrest and/or in the induction of apoptosis. The relevance of these signalling pathways remains poorly understood, in particular, in human tumoral hepatoma and colon cell lines, where none of these p53-truncated forms has been examined as yet.

To investigate the presence of such molecular forms, we induced the expression of the p53 protein with a different

endogenous status in human hepatoma and tumoral colon cell lines. We used the chemotherapeutic molecule doxorubicin and the somatostatin analogue octreotide to stimulate expression of the p53 tumour suppressor through different mechanisms.

MATERIAL AND METHODS

Cell lines and antibodies

Human hepatoma cell lines HepG2, Hep3B, Chang Liver and simian virus 40 ('SV40')-immortalized human hepatocytes (IHH) were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 1 % penicillin–streptomycin, 1 % gentamycin and 10 % fetal calf serum (FCS; Life Technologies) [9,10]. The human colon adenocarcinoma HCT116 cell line was cultured in McCoy's 5A medium, 1 % penicillin–streptomycin, 1 % gentamycin and 10 % FCS [11]. The human colon cancer LoVo cell line was grown in RPMI medium with 1 % penicillin–streptomycin, 1 % gentamycin and 10 % FCS [12]. Monoclonal anti-p53 antibodies, DO-1 (N-terminus), PAb1801 (N-terminus) and PAb421 (C-terminus) were purchased from Novocastra Laboratories Ltd. (Newcastle upon Tyne, U.K.), Oncogene Sciences (Uniondale, NY, U.S.A.) and Biomol (Plymouth Meeting, PA, U.S.A.) respectively. Polyclonal anti-caspase 3 antibody was obtained from Pharmingen (San Diego, CA, U.S.A.).

Cell treatment with doxorubicin, octreotide and caspase inhibitor

Three days after seeding, cells were incubated for 0–24 h with 0.2–2 µg/ml of doxorubicin (Sigma Aldrich, Basel, Switzerland) or 10 nM octreotide, a gift from Novartis Pharma. To determine the exact role of caspases in the induction of p40, the cells were

Abbreviations used: FCS, fetal calf serum; IHH, immortalized human hepatocytes; RT, reverse transcriptase.

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Table 1 Sequences of forward and reverse primers used for exon by exon RT-PCR analysis of p53 transcript

F, forward exon; R, reverse exon.

Lanes	Position of primers from the translational start codon	Sequence of forward and reverse primers	Size of amplified cDNAs (bp)
1, 2	F 2: 219–237 R 4: 491–512	5'-AGGAGCCGCAGTCAGATC-3' 5'-GGAAGGGACAGATGACAG-3'	293
3, 4	F 2: 219–237 R 5: 602–623	5'-AGGAGCCGCAGTCAGATC-3' 5'-TTGGCAAACATCTTGTGAG-3'	404
5, 6	F 2: 219–237 R 6: 786–806	5'-AGGAGCCGCAGTCAGATC-3' 5'-ACTCGGATAAGATGCTGAG-3'	587
7, 8	F 2: 219–237 R 7: 972–993	5'-AGGAGCCGCAGTCAGATC-3' 5'-AGTCTTCCAGTGTGATGG-3'	775
9, 10	F 2: 219–237 R 8: 1031–1140	5'-AGGAGCCGCAGTCAGATC-3' 5'-CAGGACAGGCACAAACATG-3'	833
11, 12	F 2: 219–237 R 9: 1141–1161	5'-AGGAGCCGCAGTCAGATC-3' 5'-GAGAGGGCTGGTGTGTTG-3'	943
13, 14	F 2: 219–237 R 10: 1228–1247	5'-AGGAGCCGCAGTCAGATC-3' 5'-CAGCTCTCGGAACATCTCG-3'	1029
15, 16	F 2: 219–237 R 11: 1340–1359	5'-AGGAGCCGCAGTCAGATC-3' 5'-TTTTATGGCGGGAGGTAGA-3'	1136

incubated in the presence or absence of 10 μ M of Z-VAD(OMe)-fluoromethylketone caspase inhibitor (Alexis Corporation, Coger, France).

Isolation of total and nuclear extracts

Cells were incubated with doxorubicin or octreotide for 0–24 h, washed with PBS, scraped and finally centrifuged for 5 min at 2000 rev./min. Cell pellets were then resuspended in 200 μ l of lysis buffer containing 50 mM Hepes, 0.1 % Nonidet P40, supplemented with a protease inhibitor cocktail (Sigma Aldrich), and 1 mM PMSF. Lysates were incubated for 20 min on ice, centrifuged for 15 min at 12000 rev./min and the supernatants were maintained at -80°C . Nuclear and cytosolic proteins were extracted from cell lysates using 0.2 % Nonidet P40 as described by Sadowski and Gilman [13]. Protein assay was performed using the Bradford method (Bio-Rad protein assay kit).

Western-blot analysis

Solubilized proteins were loaded on to a 10–15% SDS-polyacrylamide slab gel in the presence of 2-mercaptoethanol. Proteins were electroblotted on to nitrocellulose paper using a semi-dry apparatus (Bio-Rad) and stained with Ponceau Red for protein normalization. Western-blot analyses were performed using anti-p53 monoclonal antibodies and horseradish peroxidase-labelled sheep anti-mouse secondary antibody revealed by Signal Chemiluminescence West Pico Substrate according to Pierce procedure (Interchim, Montluçon, France).

Reverse transcriptase (RT)-PCR analysis of p53 transcript

First-strand cDNA synthesis was performed using the SuperScript™ Pre-amplification kit (Invitrogen) and 4 μ g of total RNA was extracted from untreated HepG2 cells and also from HepG2 cells treated with doxorubicin. PCR was performed in the presence of 0.2 mM dNTP, 2.5 units of platinum *Taq* polymerase (Invitrogen) and 25 pmol of forward primer corresponding to exon 2 and 25 pmol of reverse primers corresponding to exons 4–11 of the p53 gene (see Table 1). After incubation of

the sample for 3 min at 94°C , PCR was run for 35 cycles, 30 s at 94°C , 30 s at 60°C and 90 s at 72°C . This was followed by a final extension step for 10 min at 72°C .

RESULTS AND DISCUSSION

Induction of a p53 shorter molecular form in several cell lines expressing a different endogenous p53 protein status

Western-blot analysis was performed using the DO-1 antibody (epitopes 21–25) on human hepatoma HepG2 and Chang Liver

Figure 1 Induction of a 40 kDa protein by doxorubicin in liver and colon cell lines expressing different endogenous p53 protein status

Human hepatoma cell lines HepG2, Chang Liver, human tumoral colon cell line HCT116 and IHH expressing a wild-type p53 protein or human tumoral colon cancer LoVo cell line displaying a mutant p53 or the p53-negative human hepatoma Hep3B cell line were treated or not treated with 0.6 μ g/ml doxorubicin (Dox) for 24 h. Lysates were prepared from these cells as described in the Material and methods section, and Western-blot analysis was performed using the anti-p53 DO-1 monoclonal antibody.

Figure 2 Dose-response effect of doxorubicin on stimulation of p40 induction

HepG2, Chang Liver and HCT116 cell lines were treated for 24 h with increasing concentrations of doxorubicin from 0.1–3 $\mu\text{g/ml}$ and were added to the culture medium. Lysates were prepared from these cells as described in the Material and methods section, and Western-blot analysis was performed using the DO-1 anti-p53 monoclonal antibody.

cell lines expressing a wild-type p53 protein [14–18]. We observed by SDS/PAGE the presence of a p53 shorter molecular form of 40 kDa (p40) being induced, in equal proportion to p53, when 0.6 $\mu\text{g/ml}$ of doxorubicin was added to the culture medium (Figure 1). The induction of p40 was also observed in the human colon cancer cell line HCT116 known to possess a functional wild-type p53 protein [19]. The expression of the p40 protein was also detected in IHH, a non-tumoral hepatocyte cell line. In addition, the induction of p40 seems to be independent of the p53 status, since the 40 kDa protein was detected in a p53-mutant human tumoral colon cell line LoVo (Figure 1). Consistently, no p40 expression was observed in the p53-negative Hep3B cell line supporting the hypothesis that this protein is probably released from p53 (Figure 1) [18]. The following two conclusions can be drawn from these findings. First, the p40 induction was observed in non-tumoral cells and secondly, it was not dependent on the endogenous p53 status. The latter is in agreement with the observation that p40 was detected in the human gastric carcinoma cell line HGT-1 [20], displaying a p53 mutant (codon 266; results not shown). The presence of p40 in p53-mutant cell lines can be explained by the fact that, if the conformational structure remains intact, p53 mutants retain their ability to undergo proteolytic cleavage [21].

Characterization of the p40 protein induction

As shown in Figure 2, the induction of the protein p40 in HepG2, Chang Liver and HCT116 cell lines was observed when the concentration of doxorubicin was increased from 0.1 to 3 $\mu\text{g/ml}$ added for 24 h in to the culture medium. In HCT116 and Chang Liver cells, a decrease in the p40 protein content was observed when high concentrations of doxorubicin were used (1–3 $\mu\text{g/ml}$ respectively).

In HepG2 as well as in Chang Liver or HCT116 cell lines, the p40 protein became strongly induced after 3 h of treatment with doxorubicin. It reached a maximum and remained stable around 18–24 h (Figure 3). The facts that p40 was recognized by an anti-p53 antibody, was induced with the same doxorubicin concentrations as p53 and was not detected in the p53-negative cell

Figure 3 Time course of p40 protein induction stimulated by doxorubicin

HepG2, Chang Liver and HCT116 cell lines were treated from 0 to 18–24 h with 0.6 $\mu\text{g/ml}$ doxorubicin. Lysates were prepared as described in the Material and methods section and immunoblotted with the anti-p53 DO-1 monoclonal antibody.

line Hep3B support the hypothesis that this form could be either a breakdown product generated by proteolytic cleavage or an alternative splicing of the p53 mRNA.

To investigate the presence of an alternative splicing of the p53 mRNA, we performed RT-PCR using a forward primer corresponding to exon 2 and reverse primers corresponding to exons 4–11 of the p53 gene (Table 1) on template cDNAs generated from total RNA prepared from doxorubicin-treated (even lanes) and untreated (uneven lanes) HepG2 cells. As shown in Figure 4, with each reverse primer used, a unique transcript was amplified in control and doxorubicin-treated cells corresponding to p53, with expected sizes of 293 bp (lanes 1 and 2), 404 bp (lanes 3 and 4), 587 bp (lanes 5 and 6), 775 bp (lanes 7 and 8), 833 bp (lanes 9 and 10), 943 bp (lanes 11 and 12), 1029 bp (lanes 13 and 14) and 1136 bp (lanes 15 and 16). The non-existence of a second transcript to encode p40 suggests that this protein was not released from an alternative splicing, but probably generated from a p53 proteolytic cleavage. This is in agreement with the previous reports [8,22,23], where p40 was generated by an autoproteolytic cleavage of p53, appearing after its translocation to the nucleus and its binding to damaged DNA. It is further supported by our data showing that the doxorubicin-stimulated induction of p40 was located in the nuclear extract and not in the cytosol (Figure 5).

Figure 4 Amplification of the p53 transcript, exon by exon, by RT-PCR

RT-PCR was performed on HepG2 cells treated with 0.2 $\mu\text{g/ml}$ doxorubicin (lanes 2, 4, 6, 8, 10, 12, 14 and 16) and untreated cells (lanes 1, 3, 5, 7, 9, 11, 13 and 15) for 24 h. PCR DNA markers (Sigma) were used (M).

Figure 5 Localization of p40 in the nuclear extracts

HepG2 and HCT116 cells were treated from 0 to 18–24 h with 0.6 or 0.1 µg/ml doxorubicin respectively. Cytosolic (c) and nuclear (n) extracts were isolated according to the procedure given by Sadowski and Gilman [13]. Western-blot analysis was performed using DO-1 antibody as described previously.

Figure 6 Western-blot analysis of p40 using anti-p53 antibodies with different epitopes

Total lysates prepared from untreated (—) HepG2 cells and cells treated (dox) with 0.6 µg/ml doxorubicin were immunoblotted with anti-p53 antibodies DO-1, PAb1801 or PAb421.

Several shorter forms generated according to the site of the p53 truncation (N- or C-terminus) have been reported previously [8,22,23]. To determine the region of the p53 cleavage that could generate p40 in our cells, we performed the Western-blot analyses using anti-p53 antibodies recognizing various epitopes.

As shown in Figure 6, the presence of a p40 protein in HepG2 cells was detected only with antibodies DO-1 (epitopes 21–25) and PAb1801 (epitopes 46–55), but not with PAb421 (epitopes 371–380). This suggests the presence of a cleavage in the C-terminal domain of the p53 protein. The results are in agreement with other studies describing the presence of shorter p53 forms deleted from their C-terminal region [8,22,23].

Stimulation of the p40 induction by octreotide

The long-acting somatostatin analogue octreotide is used in the treatment of hepatocarcinoma and liver metastasis [5,6]. Octreotide exerts anti-proliferative effects and stimulates apoptosis by activating the p53 tumour suppressor and pro-apoptotic protein bax [2,24]. As shown in Figure 7(A), a significant stimulation of the p53 protein expression was observed with 10 nM octreotide (no effect was obtained with 1 nM; results not shown) in the three cell lines HepG2, LoVo and HCT116. In addition, the p40 induction was observed in the same proportion and with a time course (after 3 h of treatment) as that observed in the presence of doxorubicin (Figure 7B). It has been suggested that the presence of DNA breaks was necessary to obtain proteolysis of p53 [23].

Figure 7 Dose–response effect (A) and time course (B) of p40 induction stimulated by octreotide

(A) Three days after seeding, untreated (—) HepG2, LoVo or HCT116 cells and cells treated (oct) with 10 nM octreotide. (B) For time-course study, HepG2 cells were treated for 0–18 h with 10 nM octreotide. Western-blot analysis was performed using DO-1 monoclonal antibody.

However, octreotide has never been known to exert DNA-damaging effects. Therefore our observations suggest the hypothesis that p40 induction is independent of the presence of DNA breaks. Probably, p40 is involved in p53 signalling pathways that are not linked directly to DNA repair.

Lack of caspase involvement in the p40 induction

The presence of proteolytic cleavages, generating fragments with biological activities, has been described for proteins other than p53. For instance, the auto-antigen Ku86 belonging to the DNA-repair machinery complex was converted into 18 and 69 kDa forms [25]. Recently [26], the poly(ADP-ribose) polymerase-1, involved in the DNA-damage repair, was shown to be cleaved by caspase 3 and subsequently generate fragments that induce apoptosis. Mechanisms involved in the proteolytic cleavage need to be extensively investigated: autoproteolysis of p53 has been shown, but an effect of caspase 3, a cysteine protease, has also been suggested [27]. However, we showed that the Z-VAD(OMe)-fluoromethylketone caspase inhibitor had no effect on the p40

Figure 8 Effect of Z-VAD(OMe)-fluoromethylketone (FMK) caspase inhibitor on p40 induction (A) and of doxorubicin treatment on caspase 3 expression (B)

(A) Three days after seeding, doxorubicin (0.6 µg/ml) was incubated or not with HepG2 cells for 24 h in the absence (—) or in the presence of 10 µM Z-VAD(OMe)-FMK caspase inhibitor (+) and Western-blot analysis was performed using DO-1 antibody. (B) HepG2 cells were treated for 24 h with 0–1 µg/ml doxorubicin and Western-blot analysis was performed using an anti-caspase 3 antibody.

induction (Figure 8A) and that caspase 3 expression was not modified by the doxorubicin treatment (Figure 8B). A cleavage of p53 by calpains has been described previously, but it is distinct from the proteolytic cleavage that generates p40 after the interaction of p53 with DNA [28].

In conclusion, we suggest that p40 is a novel transactivation factor capable of forming a heteromer with native p53 and modulating its effects in a cell-specific manner. Further insights into p40 involvement in apoptosis induction will help to characterize the biological activity of this protein, in particular using transfected cells expressing exogenous p40. It offers a model for novel therapeutic approaches using combinations of anti-cancer molecules and gene therapy.

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