Study of the cytotoxic effect of a peptidic inhibitor of the p53–hdm2 interaction in tumor cells

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Abstract Inhibitors of the p53–hdm2 interaction are attractive molecules for stimulating the p53 pathway in tumors. In this report, an inhibitor of the p53–hdm2 interaction, the AP peptide, is used to activate p53 in tumor cells expressing various levels of hdm2 protein. It induces apoptosis only in cells expressing high endogenous levels of hdm2 protein. The absence of apoptosis in tumor cells with low hdm2 levels is due not to alterations in the p53-dependent apoptotic pathway but to a different regulation of this pathway. The peptide is also less toxic for non-tumor cells than for tumor cells overexpressing the hdm2 protein.

Key words: p53; mdm2; Protein–protein interaction; Apoptosis

1. Introduction

The hdm2 protein regulates the activity of the p53 protein in at least three different ways [1]. First, it binds to the p53 transactivation domain, inhibiting its transcriptional activity [2]. Second, upon binding to p53, it induces p53 nuclear export [3]. Third, it stimulates p53 degradation [4,5]. Several cellular pathways target the p53–hdm2 interaction. For example, oncogenic proteins such as ras or myc induce the expression of the $p14^{ARF}$ protein, which upon binding to hdm2 prevents p53 degradation [6,7]. Similarly, genotoxic stresses induce the phosphorylation of p53 within the hdm2-binding site, preventing an interaction between the two proteins [8]. The disruption of the p53–hdm2 interaction is therefore a mechanism used by the cell to modulate the p53 pathway. Since hdm2 is amplified in various tumors [9], inhibitors of the p53–hdm2 interaction could be used as anticancer agents to treat these cancers, therefore having potential applications in medicine. Recently, we showed that a small synthetic peptide that inhibits the p53–hdm2 interaction in vitro stimulates the p53 pathway in tumor cells [10]. When applied directly to the cell culture medium, this 8-mer untagged peptide [11], the AP peptide, activates the p53 pathway only in the presence of wild-type p53 protein.

In this report, we analyze the biological consequences of AP peptide treatments in cells that express different levels of hdm2 protein.

2. Materials and methods

2.1. Cell lines

The HCT 116, JAR and SJSA-1 cells express wild-type p53. The H1299 cells express the $p14^{ARF}$ protein and lack the p53 protein [12], the SK-BR-3 cells express a mutated p53 protein [13] and the Saos-2 cells express a truncated pRb protein [14]. The NHDF 710 (normal human dermal fibroblasts) and the HMEC 2595 (human mammary epithelial cells) are non-tumor cells.

2.2. Preparation of the peptides

The sequences of the peptides are:

AP peptide: Ac-Phe-Met-Aib-Pmp-*6-Cl*-Trp-Glu-Ac_3c-Leu-NH_2

IP peptide: Ac-Phe-Met-Aib-Pmp-Ala-Glu-Ac_3c-Leu-NH_2,

where Aib is α-aminoisobutyric acid, Pmp is phosphonomethylphenylalanine, *6-Cl*-Trp is 6-chloro-tryptophan and Ac_3c is 1-aminocyclopropanecarboxylic acid. The synthesis, the analytical and biological characterization of the AP and IP peptides have been previously reported [10,11]. Unless otherwise indicated, the AP and IP peptides were used at the concentration of 500 μM in the cellular assays.

2.3. Western blot and ELISA

The hdm2, pRb, $p14^{ARF}$, $p21^{Waf1/Cip1}$, procaspase-9 and β-tubulin proteins were detected by Western blot using the IF2, clone LM95.1, p14ARF Ab-1, EA10, polyclonal rabbit anti-caspase-9 and TUB 2.1 antibodies, respectively. The cellular amounts of the p53 protein were determined by ELISA as previously described [10].

2.4. Cell proliferation assay, measurement of caspase-3 activity and FACS analysis

The cell proliferation assay and the measure of caspase-3 activity have been conducted as previously described [10]. The FACS analyses were performed as follows. The cells, treated during 72 h with 500 μM AP peptide, were harvested and stained with propidium iodide. The FACS analysis was realized with an Excalibur flow cytometer (Becton Dickinson) equipped with CellQuest software.

2.5. Measurement of cytochrome-c release

After 24 h seeding the cells were incubated in the presence of AP or IP peptide for 48 h. The release of cytochrome-*c* from the mitochondria was measured using an ApopAlert Cell Fractionation kit (Clonetech) according to the instructions provided by the manufacturers.

3. Results

3.1. Determination of p53, hdm2, $p14^{ARF}$ and pRB endogenous levels

Low levels of p53 protein are found in the HCT 116 and JAR cells (Fig. 1A). These levels are higher than the background values obtained in the p53-negative cells, H1299, but lower than in the p53-mutant expressing cell line SK-BR-3. In SJSA-1 cells, the p53 levels are low and comparable to the

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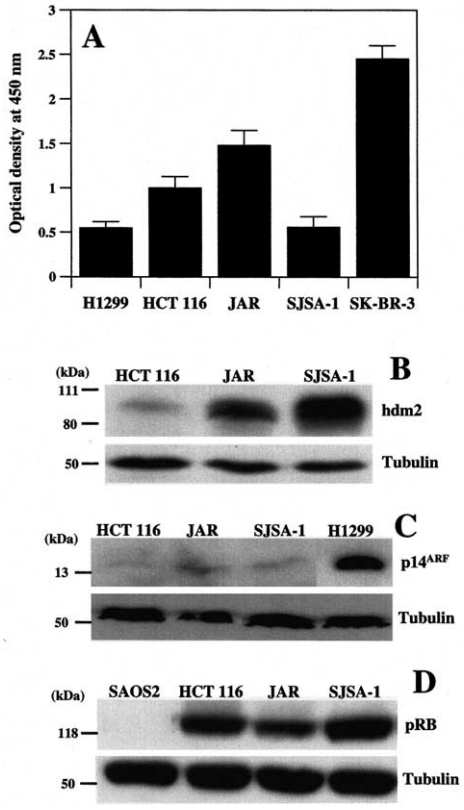

Fig. 1. Endogenous levels of the p53, hdm2 $p14^{ARF}$ and pRb proteins. The endogenous levels of the p53 (A), hdm2 (B), $p14^{ARF}$ (C) and pRB (D) were determined by ELISA (A) or Western blot. The H1299 cells were used as p53-negative and $p14^{ARF}$-positive control cells, the Saos-2 cells as pRb-negative cells and the SK-BR-3 cells as p53 overexpressing cells. The tubulin content of the different extracts was also detected by Western blot. The molecular weights are indicated.

background value measured in the H1299 cells. The hdm2 protein is detected in the three cell lines but is overexpressed in the JAR and SJSA-1 cells, as previously described [15] (Fig. 1B). In agreement with a previous report [12], the three wild-type p53 expressing cell lines contain lower amounts of $p14^{ARF}$ protein than the p53-negative cells, H1299 (Fig. 1C). The pRb protein is detected in the three cell lines but not in the pRb-negative cell line Saos-2 (Fig. 1D).

3.2. Induction of p53 and $p21^{Waf1/Cip1}$ proteins by AP peptide

The AP peptide, but not its inactive analog the IP peptide, induces a weak but reproducible accumulation of p53 in the three cell lines (Fig. 2A). Since the AP peptide induces p53 accumulation, the question was raised as to whether it also stimulates its transcriptional activity. Induction of the $p21^{Waf1/Cip1}$ protein, which is transcriptionally regulated by p53 [16], was measured (Fig. 2B). The AP peptide, but not the IP peptide, induces the accumulation of the $p21^{Waf1/Cip1}$ protein in all the cell lines. The AP peptide did not induce the accumulation of the $p21^{Waf1/Cip1}$ protein in the p53-negative cell line Saos-2. Similar results were obtained with the hdm2 protein (data not shown), another gene transcriptionally regulated by p53 [17], in agreement with previous data [10].

3.3. Effect of AP peptide on cell proliferation and viability

The AP peptide, but not the IP peptide, inhibits the proliferation of the HCT 116, JAR and SJSA-1 cells (Fig. 3A). The AP peptide-mediated inhibition of tumor cell proliferation can be the consequence of an induction either of cell cycle arrest or of apoptosis. The activation of caspase-3 during p53-mediated apoptosis [18] offers the possibility of determining whether the AP peptide induces p53-mediated apoptosis. The experimental results show that caspase-3 is activated in JAR and SJSA-1 cells but not in HCT 116 cells (Fig. 3B). This, together with the proliferation experiments (Fig. 3A), suggests that the AP peptide induces a cell cycle arrest in the HCT 116 cells and apoptosis in the JAR and SJSA-1 cells. To confirm these data, the effect of the AP peptide on the cell cycle of the three cell lines was analyzed by FACS (Fig. 3C). The results show that the AP peptide induces an increase in the G0/G1 and G2/M populations in HCT 116 cells, suggesting a cell cycle arrest. In contrast, there is an increase in the sub-G1 population in both the JAR and SJSA-1 cells revealing the induction of apoptosis.

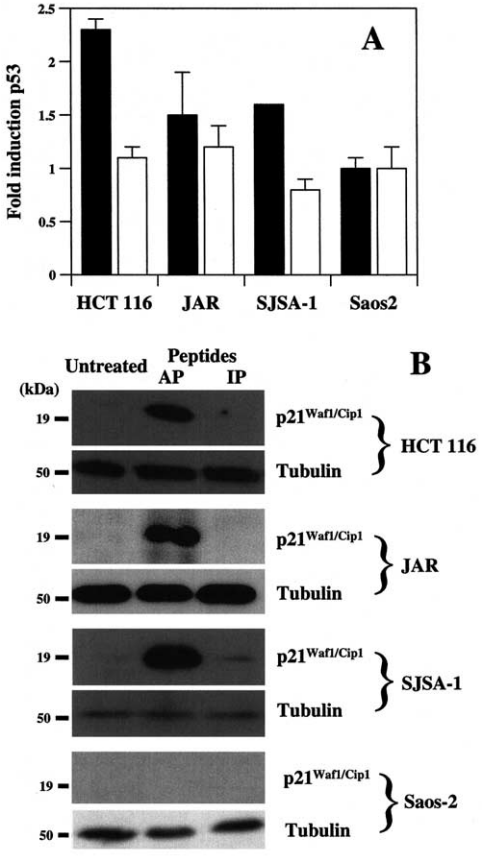

Fig. 2. Induction of the p53 and $p21^{Waf1/Cip1}$ proteins by AP peptide. A: After 24 h treatment with AP peptide (black bars) or IP peptide (white bars), the accumulation of the p53 protein was determined by quantitative ELISA. The induction of p53 was normalized to the amount of the p53 protein present in the corresponding untreated cells (arbitrarily taken as 1). Fold induction p53 corresponds to the ratio between the amount of p53 present in the treated cells and that in the untreated cells. The amount of p53 detected in the p53-negative cells, Saos-2, corresponds to the background value of the assay. B: The $p21^{Waf1/Cip1}$ protein present in the untreated cells or in the cells treated for 24 h with 500 μM AP or IP peptide was detected by Western blot. The tubulin content of the different extracts was also detected by Western blot. The molecular weights are indicated.

3.4. Analysis of the p53-dependent apoptotic pathway in HCT 116 cells

The absence of caspase-3 activity in the HCT 116 cells after AP peptide treatment could be explained by the fact that when the $p21^{Waf1/Cip1}$ protein binds to procaspase-3 [19] it prevents caspase-3 activation. Alternatively, the conversion of procaspase-3 into its active form by caspase-9, a mediator of p53-dependent apoptosis [20], could be prevented. We therefore determined whether procaspase-9 is processed after AP peptide treatment (Fig. 4A). The procaspase-9 protein present in HCT 116 cells is not processed upon AP peptide treatment, while it is processed in SJSA-1 cells in which caspase-3 is activated (Fig. 3B). Therefore, no caspase-3 activity is detected in the HCT 116 cells because its activating enzyme, caspase-9, is not activated. During apoptosis, cytochrome-*c* is released from the mitochondria into the cytoplasm, where it initiates dATP-dependent oligomerization of Apaf-1. Procaspase-9 is then recruited and converted into its active form, caspase-9 [21]. Since p53 induces the release of cytochrome-*c* from the mitochondria [22], we investigated whether the AP peptide stimulates this release. Our results show that the cytochrome-*c* protein remains in the mitochondrial fractions of untreated cells or HCT 116 cells treated with AP and IP peptide (Fig. 4B). Therefore, the AP peptide-mediated activation of the p53 protein does not lead to the release of cytochrome-*c* from the mitochondria in HCT 116 cells.

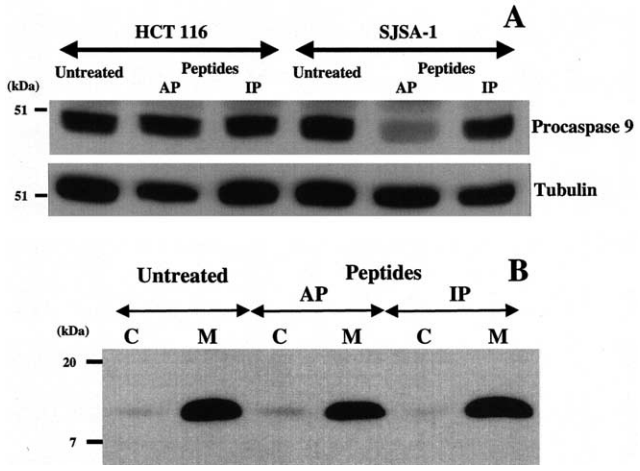

Fig. 4. Analysis of the p53-dependent apoptotic pathway in HCT 116 cells. A: The HCT 116 and the SJSA-1 cells were incubated for 48 h in the absence (untreated) or presence of AP or IP peptide. The content of procaspase-9 and tubulin in the different cells was measured by Western blot. B: The HCT 116 cells were incubated for 48 h in the absence (untreated) or presence of AP or IP peptide. Cytosolic (C) and mitochondrial (M) extracts were prepared and the cytochrome-*c* was detected by Western blot.

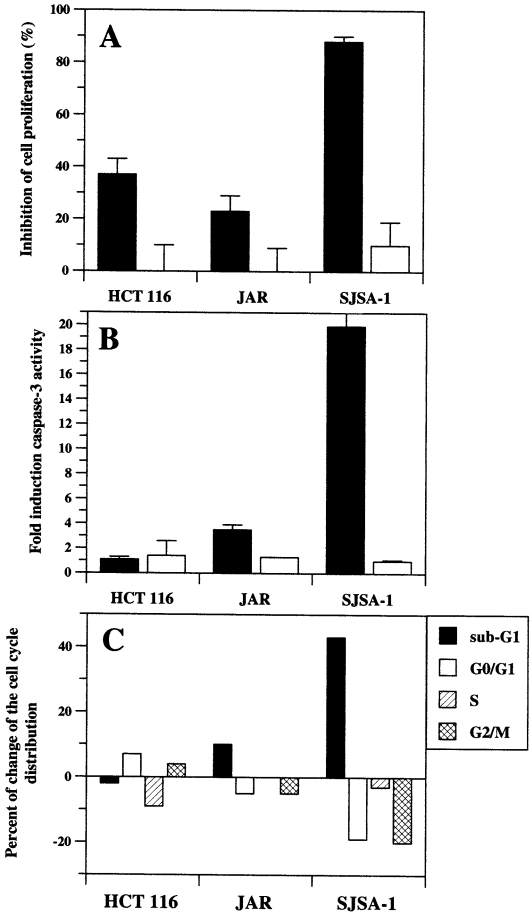

Fig. 3. Effect of AP peptide on cell proliferation and viability. A: The cells were treated for 72 h with AP peptide (black bars) or IP peptide (white bars), and the number of viable cells measured. The data were normalized to the number of cells present in the corresponding untreated samples. B: The cells were treated for 72 h with the AP peptide (black bars) or IP peptide (white bars), and the caspase-3 activity present in the cellular lysates was determined. Fold induction caspase-3 activity corresponds to the ratio between the caspase-3 activity present in the treated cells and that detected in the untreated cells (arbitrarily taken as 1). C: The cells were treated for 72 h with the AP peptide stained with propidium iodide, and their DNA content analyzed by FACS. The figure represents the percent of change in cell cycle distribution after AP peptide treatment.

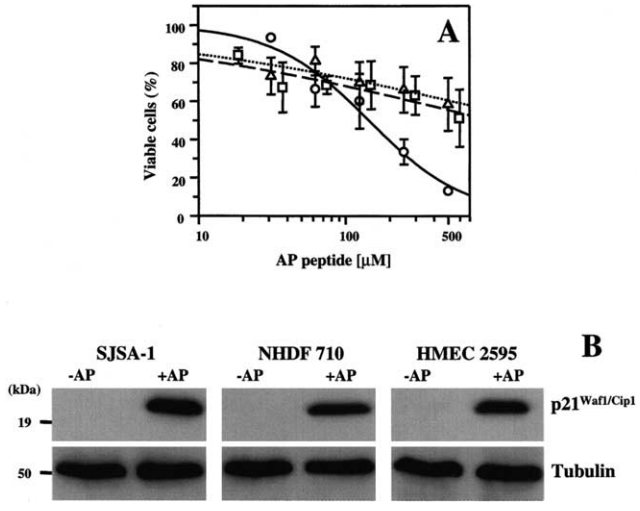

Fig. 5. The AP peptide is more toxic to tumor cells than to non-tumor cells. A: The tumor cells SJSA-1 (open circles) and non-tumor cells NHDF 710 (open triangles) and HMEC 2595 (open squares) were treated for 72 h with different concentrations of AP peptide and the number of viable cells was measured. B: The $p21^{Waf1/Cip1}$ protein present in the extracts of untreated cells (−AP) or cells treated for 24 h with AP peptide (+AP) was detected by Western blot. The tubulin content of the different extracts was also determined.

3.5. The AP peptide is more toxic for tumor than for non-tumor cells

The AP peptide-mediated induction of apoptosis in the JAR and SJSA-1 cells suggests that inhibitors of the p53–hdm2 interaction could be useful drugs to treat tumors expressing high hdm2 levels. However, the hdm2 protein is a natural regulator of the p53 protein, and the inhibition of the p53–hdm2 interaction stimulates p53 activity in non-tumor cells [23]. Therefore, such molecules may be toxic inducing cell death also in non-tumor tissues. To address this issue the non-tumor cells NHDF 710 and HMEC 2595 were treated with the AP peptide and their viability measured. For comparison, the AP peptide-sensitive tumor cells, SJSA-1, were included in the experiments. The experimental data show that the AP peptide inhibits the proliferation of tumor cells more effectively than non-tumor cells (Fig. 5A). To verify that the lack of effect of the AP peptide in NHDF 710 and HMEC 2595 cells is not due to a poor penetration of this compound in these cells, the induction of the $p21^{Waf1/Cip1}$ protein was measured (Fig. 5B). The AP peptide induces the accumulation of the $p21^{Waf1/Cip1}$ protein in the three cell lines, demonstrating that it penetrates them in a similar fashion.

4. Discussion

In this report, we study the effect of the activation of the p53 pathway by a synthetic inhibitor of the p53–hdm2 interaction, the AP peptide, in three cell lines expressing different levels of hdm2 protein. The HCT 116, JAR and SJSA-1 cells express low, intermediate and high hdm2 levels, respectively. These cell lines were chosen because we did not find exactly matched tumor cell lines expressing very different levels of hdm2 protein. The HCT 116, JAR and SJSA-1 cells contain similar levels of the $p14^{ARF}$ and pRb proteins, two key mediators of the p53 pathway, and they all express the wild-type p53 protein.

The AP peptide induces a low accumulation of the p53 protein in these cells. This weak effect may be explained by the poor bioavailability of the inhibitor due to its peptidic character and its high polarity. Another possibility is that the accumulated p53 stimulates the expression of the hdm2 protein, which in turn induces its degradation. However, despite this slight accumulation, the accumulated p53 protein induces a large expression of the $p21^{Waf1/Cip1}$ protein. Since the AP peptide has no effect on $p21^{Waf1/Cip1}$ levels in both p53-negative and p53-mutant expressing cells [10], the accumulation observed in the three cell lines studied is not due to a p53-independent mechanism. These data show that even a weak accumulation of the p53 protein can lead to a significant induction of p53-dependent genes such as $p21^{Waf1/Cip1}$. The AP peptide-mediated stimulation of the p53 pathway affects differently the proliferation of the three cell lines. It induces apoptosis in the JAR and SJSA-1 but not in the HCT 116 cells. Very interestingly, the induction of apoptosis correlates with the cellular content of hdm2 protein. The more the cell expresses hdm2 the more it is susceptible to AP peptide-mediated apoptosis. This observation should be further validated with the study of other hdm2 overexpressing cell lines. Previous work has shown that the fate of the HCT 116 cells after p53 activation depends on the presence of the $p21^{Waf1/Cip1}$ protein. In its presence, they undergo a cell cycle arrest; in its absence, however, they die from apoptosis [18]. This indicates that the p53-dependent apoptotic pathway is functional in the HCT 116 cells and that the absence of AP peptide-mediated apoptosis in these cells cannot be explained by an alteration of this pathway. We show that the AP peptide-mediated activation of p53 in the HCT 116 cells does not induce the release of the cytochrome-*c* protein from the mitochondria and, consequently, does not initiate the processing of procaspase-9 and procaspase-3. This suggests that in the presence of the $p21^{Waf1/Cip1}$ protein the biochemical events leading to the release of cytochrome-*c* from the mitochondria are prevented during p53-mediated apoptosis. The effect of the AP peptide on two other tumor cell lines, U-2 OS and MCF7, expressing low hdm2 levels was also investigated. The peptide activates p53 in these cells without inducing apoptosis (data not shown). In the course of this work we also tried to obtain stable HCT 116 clones expressing high levels of hdm2 protein but our attempts were unsuccessful, suggesting that high hdm2 levels are not tolerated in these cells.

Our findings indicate that the AP peptide induces preferentially apoptosis in cells overexpressing the hdm2 protein. Therefore, the peptide should affect to a lower extent the viability of non-tumor cells which express very low levels of hdm2 protein, than that of hdm2-overexpressing tumor cells. Indeed, we show that the AP peptide-mediated activation of p53 affects less the proliferation of non-tumor cells than it does with hdm2 overexpressing tumor cells. In agreement with previous data [23], we show that the inhibition of the p53–hdm2 interaction induces p53 activation in normal fibroblasts. However, our results reveal that inhibitors of the p53–hdm2 interaction are more toxic for hdm2 overexpressing tumor cells than for non-tumor cells. Therefore, if this therapeutic window is retained in vivo, inhibitors of the p53–hdm2 interaction will be particularly useful for treating tumors with amplified hdm2. The analysis of hdm2 amplification in about 4000 tumor and xenograft samples shows that hdm2 is amplified in 7% of them [9].

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