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Biochemical and Biophysical Research Communications 314 (2004) 138–142
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9-Hydroxystearic acid upregulates p21^{WAF1} in HT29 cancer cells

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Received 11 December 2003

Abstract

Growing evidence supports the critical role of lipid peroxidation products in the control of cell proliferation. In previous studies we demonstrated the efficient restriction of the proliferation rate in several cell lines resulting from the in vitro treatment with endogenous lipid polar components of cell membranes. Among these, 9-hydroxystearic acid (9-HSA), a primary intermediate of lipid peroxidation, induced a significant arrest in G0/G1 in HT29 colon cancer cells. In response to 9-HSA treatment of HT29 we observed cell growth arrest and increase in p21^{WAF1} expression both at the transcriptional and the translational levels. Growth of p21^{WAF1}-deleted HCT116 human colon carcinoma cells was not inhibited by 9-HSA. We present evidence that p21^{WAF1} is required for 9-HSA mediated growth arrest in human colon carcinoma cells.

Keywords: Endogenous lipid peroxidation products; 9-HSA; p21^{WAF1}; Colon cancer; PCR

Lipid peroxidation products have been extensively studied as modulators of DNA synthesis as well as of cell proliferation. In particular, a growing number of experimental data concerning the involvement of hydroperoxy- and hydroxy-derivatives of polyunsaturated fatty acids in the control of cell proliferation and other cellular responses have been published [1]. Lipid peroxidation in normal and tumor cells has been investigated in our laboratory [2,3]. In Morris hepatomas with different growth rates it was found that the content of polyunsaturated fatty acids, and therefore, the content of lipid peroxidation short and long chain derivatives, as well as antioxidant enzymatic and nonenzymatic defences, decreased with the tumor growth rate. Three unusual hydroxyderivatives of octadecadienoic (HODE$_s$) and eicosatetraenoic (HETE$_s$) acids (18:2 and 20:4) were identified in normal rat liver and in rat hepatomas. Interestingly, their endogenous content decreased in tumors as compared to normal cells [4]. Furthermore, two monohydroxy fatty acids, 9- and 10-hydroxystearic acids (9- and 10-HSA), were isolated [5,6]. Their total endogenous content was inversely related to the cellular density. In previous studies we found that the treatment with the synthetic mixture of 9- and 10-HSA (20–100 μM) inhibited cell proliferation of different cell lines [7–10]. In HT29 colon cancer cells the inhibitory effect was associated with significant changes in cell distribution in the cycle phases and arrest in G0/G1 [9]. Progression through the cell cycle is mediated by a family of protein kinases named cyclin dependent kinases (CDKs). CDKs are composed of a catalytic subunit and a regulatory subunit called cyclin [11]. The CDK activities that govern cell cycle transition involve phosphorylation/dephosphorylation of the catalytic subunits, degradation of the cyclin subunits, and binding of CDK inhibitory proteins (CKIs) [12]. Superimposed checkpoint control following exposure to DNA-damaging agents or growth arrest signals is often mediated by CKI induction [13,14]. p21^{WAF1} belongs to the Cip/Kip class of CKIs, including also p27^{Kip1} and p57^{Kip2} [15–17], and is a powerful inhibitor of CDK-cyclin complexes, including cyclin CDK2/A, cyclin CDK1/B, cyclin CDK2/E, and cyclin CDK4/D. Its role is prom-

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doi:10.1016/j.bbrc.2003.12.066

inently played in the early and late controls of the G1 checkpoint under both p53-dependent and p53-independent conditions, but it also contributes to regulate the transition from G2 to M [18–21]. In addition, $p21^{WAF1}$ inhibits the DNA elongation in vitro by binding the proliferating cell nuclear antigen (PCNA) and interfering with the ability of the latter to activate polymerase δ, affecting, as a consequence, both DNA replication and DNA repair [22]. The mechanisms by which 9-HSA interferes with and possibly regulates cell proliferation are still unclear. In this paper we present evidence that p21 expression is induced by 9-HSA and that p21 is required for 9-HSA mediated growth arrest in colon carcinoma cells.

Materials and methods

Cell culture and treatments. The colon cancer cell line HT29 was purchased from American Type Culture Collection (ATCC, Manassas, VA). HCT116, p21−/− cells were kindly provided by B. Vogelstein (John's Hopkins University School of Medicine, Baltimore, MD) [23]. HT29 were maintained in RPMI 1640 medium (Labtek Eurobio, Milan, Italy), HCT116, p21−/− in Mac Coy 5A (Labtek Eurobio, Milan, Italy), supplemented with 10% FCS (Euroclone, Milan, Italy) and 2 mM L-glutamine (Sigma–Aldrich, St. Louis, MO) at 37 °C and 5% CO_2. HT29 were seeded at 2×10^4 cells/cm^2 in a plastic well (60 cm^2) and allowed to grow for 1 day before being exposed to 100 μM 9-HSA. Some cells were treated concomitantly with the protein synthesis inhibitor cycloheximide (Sigma–Aldrich, St. Louis, MO) (10 μg/mL). 9-HSA was synthesized in our laboratory. The HCT116, p21−/− were treated with 50 μM 9-HSA.

Flow cytometric analysis. HT29 and 9-HSA treated cells were detached with 0.11% trypsin (Sigma–Aldrich, St. Louis, MO) 0.02% EDTA (Sigma–Aldrich, St. Louis, MO), washed in PBS, and centrifuged. The pellet was resuspended in 0.01% Nonidet P-40 (Sigma–Aldrich, St. Louis, MO), 10 μg/mL RNase (Sigma–Aldrich, St. Louis, MO), 0.1% sodium citrate (Sigma–Aldrich, St. Louis, MO), and 50 μg/mL propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO), for 30 min at room temperature in the dark. Propidium iodide fluorescence was analyzed by using a flow cytometer Epics Elite (Coulter) and cell cycle analysis was performed using the M Cycle (Verity) and MODFIT 5.0 softwares.

Immunoprecipitation and immunoblotting. Cells were harvested as indicated previously, washed with ice-cold PBS, and lysed according to Yamashita et al. [24]. Lysed cells were sonicated and then centrifuged at 12,000*g* for 20 min at 4 °C. The protein concentration of cell extracts was determined by using the Bio-Rad protein assay kit. A sample containing 500 μg protein was incubated overnight at 4 °C with 10 μg of anti-$p21^{WAF1}$ monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California). Fifty microliters of protein A–Sepharose (50% v/v) (Amersham, Uppsala, Sweden) was added to each sample, incubated for 1 h and then centrifuged at 12,000*g* for 20 min at 4 °C, and washed four times with 0.1 M potassium phosphate buffer, pH 8.0. Immunoprecipitated $p21^{WAF1}$ was extracted from the complex with 30 μL denaturing buffer (6 mM Tris–HCl, pH 7.5, 65 mM β-mercaptoethanol, 1% SDS, and 10% glycerol) by heating at 100 °C for 5 min. The proteins were resolved by SDS–PAGE and immunoblotted with anti-$p21^{WAF1}$ antibody. Detection of immunoreactive bands was performed with a secondary antibody conjugated with horseradish peroxidase and developed with the enhanced chemiluminescence system (ECL) (Amersham, Uppsala, Sweden).

Isolation of RNA. Total RNA was isolated with RNeasy Tissue kit (Qiagen, Hilden, Germany) and according to the manufacturer's instructions. The exact amount of extracted RNA was measured by optical density at 260 nm and its integrity was assessed by electrophoresis on agarose gel.

Preparation of the RNA competitor for quantitative polymerase chain reaction. An application of recombinant polymerase chain reaction (PCR) methodology was used to generate a deletion in cloned $p21^{WAF1}$ cDNA plasmid pZLWAF1, a gift from B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). For the detection of $p21^{WAF1}$ cDNA, two PCR primers were selected using a dedicated software (Oligo 5.0, from National Biosciences, Plymouth, MN): CHS21: 5′CCTAAGAGTGCTGGGCATTTT3′ and CHS22: 5′TGAATTTCATAACCGCCTGTG3′. Amplification of pZLWAF1 on cDNA generated by reverse transcription polymerase chain reaction (RT-PCR) from cell extracts produces a DNA fragment of 200 bp in length (template fragment). The 143L39 primer (5′TGAATTTCATAACCGCCTGTGACACCCCTCCAGAGGGTA 3′) shares the 5′ sequence with CHS22 and includes a 18 bp sequence corresponding to the downstream sequence in WAF1 coding sequence at the 3′ end, designated to generate the competitor DNA molecule. When the primer 143L39 was used together with the primer CHS21, the amplified fragment (Δ_{19}hWAF1 competitor fragment) carried a deletion of 19 bp compared to the wild type amplified fragment. Δ_{19}hWAF1 was 181 bp in size and thereby 1 ng equals 5.06×10^9 molecules.

Quantitative RT-PCR assay. Reverse transcription was performed according to [25]. Competitive PCRs were carried out by addition of scalar amounts of specific competitor fragments (7.32×10^{-19}, 1.17×10^{-18}, 1.46×10^{-18}, 2.20×10^{-18}, and 2.93×10^{-18} mol, respectively) to a fixed quantity (2 μL) of the cDNA, followed by PCR amplification. After completion of the PCR, 28 μL aliquots of the reaction mixtures were analyzed on a 2% ethidium-stained agarose gel. The intensity of the bands was determined by densitometry, and a calibration curve was developed by plotting the ratio of the band intensities of the PCR products against the amount of moles of competitor added.

Statistical analysis. Student's *t* test was used for repeated measurement values. A *p* value below 0.05 was considered significant.

Results

In vitro effects of 9-HSA on HT29 cell proliferation

The treatment with 100 μM 9-HSA resulted in a significant inhibition of cell proliferation after 24 h. The distribution of HT29 cells in different phases of the cell cycle is shown In Table 1. Treatment with 100 μM 9-HSA decreased S-phase activity by 50.2% compared with untreated controls, and the growth inhibition was associated with a strong arrest in G0/G1. Because the cell cycle inhibitor, $p21^{WAF1}$, is known to induce G1 arrest [26] we assayed the effect of 9-HSA on the expression of $p21^{WAF1}$ protein. We investigated whether 9-HSA affects the expression of $p21^{WAF1}$ in HT29 cells, which lack functional p53, by using a $p21^{WAF1}$ monoclonal antibody. HT29 cells were treated with 100 μM 9-HSA for 24 h, harvested, and the levels of $p21^{WAF1}$ in the total cellular proteins were examined by immunoprecipitation. As shown in Fig. 1 the level of $p21^{WAF1}$ was increased in cells treated with 100 μM 9-HSA. 9-HSA did not induce expression of two other cell cycle inhibitors, p16 and p27 (data not shown).

Table 1
Cell cycle distribution of HT29 cells treated with 100 μM 9-HSA

HT29	% G0/G1	% S	% G2/M
Control	59.0 ± 1.0	24.1 ± 5.6	12.5 ± 3.0
Treated with 9-HSA	80.8 ± 0.8	12.0 ± 1.6	7.0 ± 0.8

Data are expressed as means of triplicate experiments ± SD.

Competitive RT-PCR

We have developed a competitive RT-PCR method that coamplifies a 200 bp sequence of $p21^{WAF1}$ and a 181 bp sequence of competitor. A fixed amount of HT29 cDNA was RT-PCR amplified with different concentrations of competitor for $p21^{WAF1}$ (7.32×10^{-19}, 1.17×10^{-18}, 1.46×10^{-18}, 2.20×10^{-18}, and 2.93×10^{-18} mol, respectively) corresponding to 0.5×10^7, 0.8×10^7, 1.0×10^7, 1.5×10^7, and 2.0×10^7 molecules. The ratio of PCR products was graphed as a function of added RNA competitor after separation of the template and the competitor by electrophoresis (Fig. 2). The results obtained showed a regression line of $y = 1.0314 \times -0.0063$ ($r = 0.99$). The concentration of $p21^{WAF1}$ RNA was then determined as the concentration of DNA competitor that would have given the same intensity as the $p21^{WAF1}$ cDNA analyzed. Due to the size difference between competitor and sample templates (181 bp and 200 bp for $p21^{WAF1}$) the number of competitor molecules at the equivalence point was multiplied by 0.9 for $p21^{WAF1}$ (these values correspond to the ratio in size between competitor and sample template PCR products). These numbers were then converted to absolute numbers of single gene molecules/μg of total RNA. Each quantification was repeated three times on cDNAs from at least three separate experiments.

9-HSA induction of $p21^{WAF1}$ RNA expression

9-HT29 cells were treated with 100 μM 9-HSA for 24 h. RNAs were extracted and subjected to competitive RT-PCR analysis. 9-HSA treatment increased $p21^{WAF1}$ transcript levels. Concomitant treatment of HT29 cells with 9-HSA and 10 μg/mL of the protein synthesis inhibitor cycloheximide (CHX) revealed that $p21^{WAF1}$ induction by 9-HSA is not blocked (Fig. 3).

Relevance of $p21^{WAF1}$ in 9-HSA-mediated cell growth inhibition

We next focused on the requirement of $p21^{WAF1}$ gene for the cell growth inhibition by 9-HSA. For these studies, we used the parent human colon carcinoma cell line, HCT116, p21 −/−. The treatment with 50 μM 9-HSA did not inhibit growth in HCT116 −/− cells, as shown in Fig. 4. These data suggest that $p21^{WAF1}$ gene expression is crucial for the growth inhibition induced by 9-HSA.

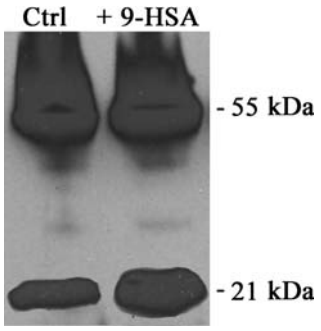

Fig. 1. $p21^{WAF1}$ overexpression inhibits HT29 cells growth. Immunoblot analysis of $p21^{WAF1}$ in HT29 control and treated with 100 μM 9-HSA for 24 h. Cell lysates were immunoprecipitated with $p21^{WAF1}$ antibody and then immunoblotted with anti-$p21^{WAF1}$ antibody. Detection of immunoreactive bands was performed with a secondary antibody conjugated with horseradish peroxidase and developed with the ECL. Fifty five kDa is the position of the heavy chain of the immunoglobulin used in the immunoprecipitation.

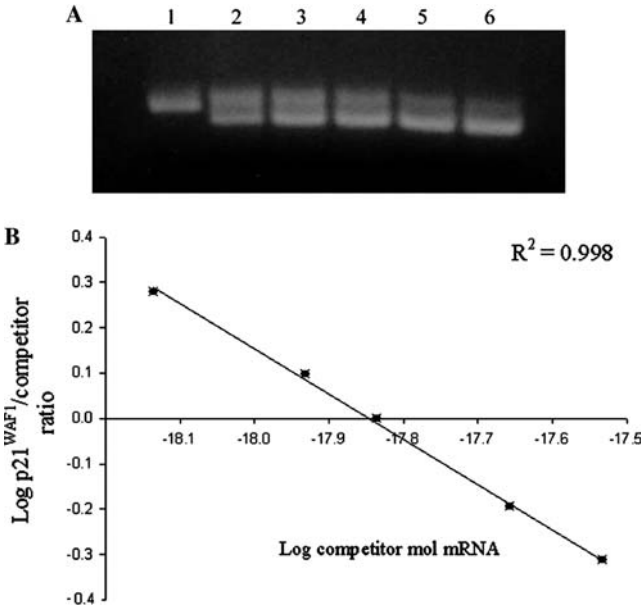

Fig. 2. Competitive RT-PCR. (A) A fixed amount of HT29 cDNA (2 μg) was amplified with different concentrations of competitor. The amounts of competitor for $p21^{WAF1}$ were 0 mol (lane 1), 7.32×10^{-19} mol (lane 2), 1.17×10^{-18} mol (lane 3), 1.46×10^{-18} mol (lane 4), 2.20×10^{-18} mol (lane 5), and 2.93×10^{-18} mol (lane 6). The lower band is PCR-amplified from the competitor (181 bp), whereas the upper band originates from $p21^{WAF1}$ (200 bp). (B) The log of the ratio of amplified template to competitor product is graphed as a function of the log of a known amount of competitor added to the PCR. When the molar ratio of the template and the competitor is equal to 1, the log of the ratio is equal to 0 and the concentration of the sample can be read on the *x*-axis.

Discussion

Among lipid peroxidation products, 9-HSA is an endogenous, long chain product identified in epithelial cells, i.e., murine Lewis lung carcinoma (C108), human

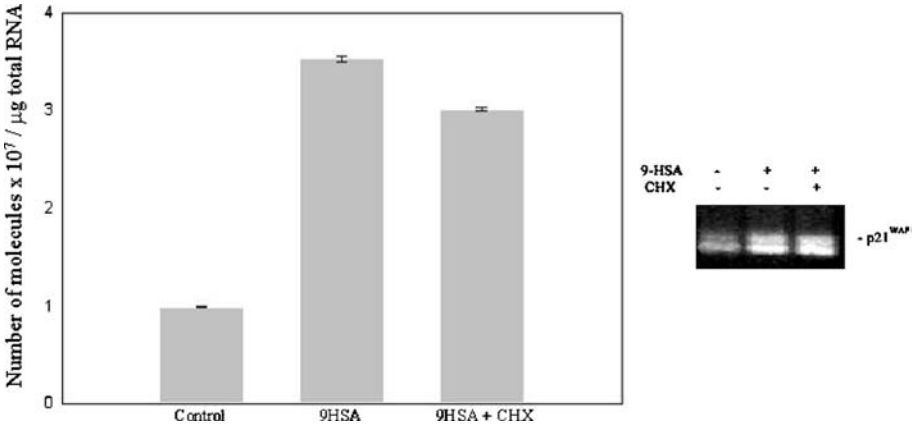

Fig. 3. Induction of $p21^{WAF1}$ transcript by 9-HSA in HT29 cells. (Left) transcript induction was measured by means of competitive PCR 24 h after treatment. It was expressed as an increase of the number of molecules/µg RNA, $(3.5 \pm 0.11) \times 10^7$ in 9-HSA treated versus the control value, $(0.99 \pm 0.006) \times 10^7$. Reported values represent means ± SD of three separate experiments performed in triplicate (right). $p21^{WAF1}$ induction by 9-HSA is not blocked by protein synthesis inhibition. HT29 cells were treated concomitantly with 100 µM 9-HSA and 10 µg/mL CHX for 24 h. $p21^{WAF1}$ mRNA expression was monitored by RT-PCR analysis.

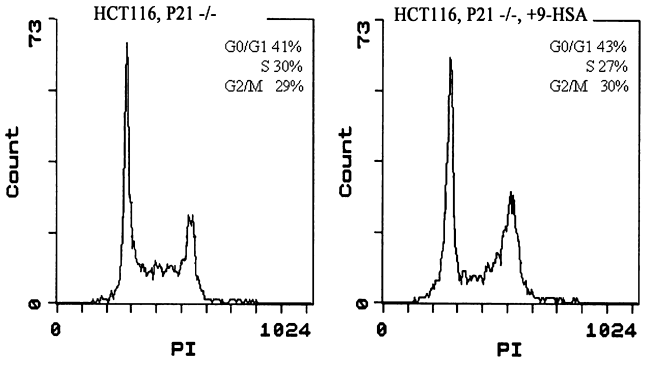

Fig. 4. $p21^{WAF1}$ deletion prevents growth inhibition by 9-HSA. HCT116, p21−/− cells were treated with 9-HSA for 24 h, and the isolated nuclei stained with PI were analyzed by flow cytometry. The treatment with the 9-HSA did not induce any change in the cell cycle distribution.

colon adenocarcinoma (HT29) [5–7], and normal human embryonic intestine cells (I407) [8,9]. When administered to HT29, 9-HSA determines growth inhibition in the G0/G1 cell cycle phase. In search for its targets and mechanisms of action, we find that it transcriptionally upregulates the cell cycle inhibitor $p21^{WAF1}$. Concomitant treatment of HT29 cells with 9-HSA and the protein synthesis inhibitor, cycloheximide, reveals that $p21^{WAF1}$ induction by 9-HSA is not blocked and, in fact, transcript levels are slightly higher, indicating that $p21^{WAF1}$ is induced by 9-HSA as an immediate-early gene. Moreover, $p21^{WAF1}$ appears to be an absolute requirement for cell growth arrest by 9-HSA: in fact, HCT116, p21−/− cell growth is not inhibited by 9-HSA.

The knowledge of a defective control of proliferation in tumor cells and the recent discovery of new growth-inhibitors provide new experimental opportunities in cancer therapy, in the search for drugs specifically acting on cell cycle regulators. This study shows that an endogenous product of lipoperoxidation acts in HT29 as a growth inhibitor through a $p21^{WAF1}$ induction in an immediate-early fashion, independent of p53, and that $p21^{WAF1}$ is required for 9-HSA mediated growth arrest in colon carcinoma cells.

Acknowledgments

We tank Dr. Bert Vogelstein for the HCT116, p21 −/− cell lines. This work was supported by a grant from MURST 60% 2002, COFIN 2002.

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