Ascorbate up-regulates MLH1 (Mut L homologue-1) and p73: implications for the cellular response to DNA damage

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We have found previously that ascorbic acid (vitamin C), as well as acting as a radical scavenger, may modulate the expression of several genes [i.e. *fra-1*, glutathione S-transferase Pi (*GSTpi*) and Mut L homologue-1 (*MLH1*)] in human keratinocytes. In the present paper, we demonstrate that *MLH1*, as well as its downstream target *p73*, can be positively modulated by this antioxidant vitamin, indeed, up-regulation of the two mRNAs was observed after just 2 h, and increased further up to 16 h of treatment. Modulation of *MLH1* and *p73* gene expression improves cellular susceptibility to apoptosis triggered by the DNA-damaging agent cisplatin. Indeed, in ascorbate-supplemented cells, increased cisplatin-induced apoptosis was seen,

involving activation of the *MLH1*/c-Abl/*p73* signalling cascade. Our results were further confirmed by studies performed on genetically defined mutants, i.e. mouse embryo fibroblasts derived from knock-out animals for c-Abl or p53, as well as human colon carcinoma cell lines deficient in *MLH1*. The increased sensitivity to cisplatin observed in ascorbate-loaded cells appeared to be dependent exclusively on *MLH1* and c-Abl expression, and independent of p53. These data suggest a potential mechanism accounting for the anti-carcinogenic and anti-cancer activities of vitamin C.

Key words: c-Abl, cell death, cisplatin, vitamin C.

INTRODUCTION

Clinical and epidemiological studies have indicated that certain dietary factors may be effective in cancer prevention. However, the mechanisms whereby specific antioxidant nutrients may play a role in the prevention of cancer are controversial and not fully elucidated. Recent studies have demonstrated that antioxidants can modulate several cellular signalling pathways, thus controlling gene expression, and that these nutrient–gene interactions may have important therapeutic implications. By using a cDNA microarray approach, we have previously identified specific genes [*fra-1*, glutathione S-transferase Pi (*GST-pi*) and Mut L homologue-1 (*MLH1*)] whose expression was differentially regulated after ascorbate (vitamin C) supplementation [1]. Modulation of the *MLH1* gene is characterized further in the present paper.

MLH1 is a component of the DNA mismatch repair machinery, which allows the removal of base–base mismatches and insertion–deletion loops that arise during DNA replication, genetic recombination and DNA damage [2]. Defects in this gene lead to the accumulation of replication errors and predispose to hereditary non-polyposis colorectal cancer [3]. The cellular response to DNA damage requires activation of *MLH1*, which can co-operate with the tumour-suppressor *p53* gene to promote cell cycle arrest and cell death [4–6]. Some DNA-damaging agents (such as cisplatin, ionizing radiation and γ -radiation) can lead to an alternative apoptotic pathway. In this case, *MLH1*

engagement activates the c-Abl tyrosine kinase, which can either stabilize or phosphorylate the p53 homologue *p73*, depending on the DNA damage inducer used and the cell type analysed [7–9]. Unlike death primed by p53, *p73*-induced apoptosis appears to be tightly associated with the mismatch repair system: *MLH1*-deficient cells are resistant to killing by cisplatin, and this phenomenon is correlated with an inability to activate the c-Abl/*p73* transduction pathway [7].

A link between vitamin C and DNA repair has been suggested. Intracellular ascorbate can protect human lymphocytes against oxidative DNA damage in a dose-dependent manner [10,11]. Furthermore, DNA levels of 8-oxodG, a biological marker of oxidative DNA damage *in vivo*, decrease significantly after ascorbate supplementation, and this decrease is strongly correlated with increases in the plasma ascorbate concentration [12]. These pieces of evidence suggest that antioxidant defences, together with DNA repair, may contribute to give a steady-state level of damage, which represents a minimal risk for the cell. Although interesting, the exact correlation between ascorbate and the DNA repair machinery has not been extensively investigated.

Here we demonstrate that ascorbate up-regulated *MLH1* and *p73* transcripts, thus potentiating the mismatch-repair-dependent, p53-independent cell death pathway induced by DNA damage. Our results suggest that induction of these ascorbate-sensitive transcripts increases cellular susceptibility to the pharmacological action of the chemotherapeutic agent cisplatin.

Abbreviations used: AA-2P, magnesium L-ascorbate 2-phosphate; CTD, C-terminal repeat domain of RNA polymerase II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; MEF, mouse embryo fibroblast; *MLH1*, Mut L homologue-1; RT-PCR, reverse transcription-PCR.

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EXPERIMENTAL

Cell cultures

Non-tumorigenic human HaCaT cells [13] were kindly provided by Professor N. E. Fusenig (Division of Differentiation and Carcinogenesis In Vitro, Institute of Biochemistry, German Cancer Research Center, Heidelberg, Germany). p53^{-/-} and c-Abl^{-/-} mouse embryo fibroblasts (MEFs) were obtained by mating the appropriate heterozygous mutant mice [14,15]. c-Abl expression in Abl-deficient cells was reconstituted by retrovirus-mediated gene transfer (c-Abl^{+/+} cells) [16].

All cells employed were grown in a 1:1 (v/v) mixture of minimal essential medium and Ham's F-12 medium (Gibco, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (HyClone, Oud-Beijerland, Holland), 1.2 g/litre sodium bicarbonate, 1% (v/v) non-essential amino acids and 15 mM Hepes, at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were split 1:6 twice weekly, and were fed 24 h before each experiment.

For ascorbate treatments, magnesium L-ascorbate 2-phosphate (AA-2P; Wako Pure Chemical Industries, Neuss, Germany) was used. We decided to use the phosphorylated derivative of ascorbate as it was stable in the culture medium, thus avoiding the generation of secondary products, derived from ascorbate oxidation, which could lead to a mistaken interpretation of results. Indeed, exogenously provided AA-2P, after enzymic cleavage by membrane phosphatases [17], accumulated as ascorbic acid inside HaCaT keratinocytes, with a system saturable at 1 mM AA-2P and able to reach 40-fold intracellular accumulation of ascorbate after 5 h [18]. In order to avoid confusion from the significant differences existing between the amount of AA-2P provided exogenously and the intracellular ascorbate concentration, we will always refer to the exogenous concentrations of AA-2P.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was purified from cells by a guanidinium isothiocyanate method (Trizol; Gibco). A 1 µg portion of total RNA was employed for cDNA synthesis using the Superscript Preamplification System and an oligo(dT) primer (Gibco); 10% of the first-strand reaction was then used for semi-quantitative PCR. Before proceeding to first-strand cDNA synthesis, any residual genomic DNA was removed from the samples by incubation with RNase-free DNase I; control reactions were performed in order to ensure complete removal of DNA. For PCR amplification, conditions were examined carefully so that the reaction was terminated during the exponential phase of amplification of each gene. Linear amplification was observed after 20 cycles, except for p73 amplification, which required an additional 15 cycles. The PCR reaction was carried out in a volume of 50 µl in the presence of 3 µCi of [α -³²P]dCTP. The amplification parameters were as follows: 94 °C for 30 s, 60 °C for 30 s and 68 °C for 2 min. Portions of 20 µl of the reaction were electrophoresed on a 6% (w/v) polyacrylamide gel, which was then dried and subjected to autoradiography.

The primers used were as follows. MLH1: (+), 5'-AGCCT-CTGAGCAAACCCCTGTC-3'; (-), 5'-CCATCTTCCTCTG-TCCAGCCAC-3'; c-Abl: (+), 5'-AGTGGGAGATGGAAC-GCACG-3'; (-), 5'-AGGCAGTTTCGGGCAGCAAG-3'; p53: (+), 5'-TGCTTTCCACGACGGTGACACG-3'; (-), 5'-GACTGCTTGTAGATGGCCATGG-3'; p73: (+), 5'-ACTT-TGAGATCCTGATGAAG-3'; (-), 5'-CAGATGGTCATGC-GGTACTG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): (+), 5'-TGAAGGTCGGAGTCAACGGATTG-3';

(-), 5'-GCCATGGAATTTGCCATGCCATGGGTGG-3'. Products were validated by size determination and sequencing.

Western blotting

Cells subjected to different treatments were resuspended in lysis buffer (25 mM Tris/HCl, pH 8.0, 1 mM EDTA and 0.5% SDS). After sonication and protein determination, 40 µg (200 µg in the case of p73 immunoblotting) of total protein was subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel and then electroblotted on to a PVDF membrane. The blots were blocked with 5% (w/v) non-fat dry milk (Bio-Rad, Hercules, CA, U.S.A.) and then incubated with the specific primary antibody. The following antibodies were used: anti-p53 (SC6243 clone FL393; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-c-Abl (K-12; Santa Cruz), anti-p73 (H79; Pharmingen, San Diego, CA, U.S.A.) and anti-MLH1 (Ab-1; Oncogene Research Products, Cambridge, MA, U.S.A.). After washing and incubation with the relevant horseradish peroxidase-conjugated secondary antibodies, detection was carried out by ECL⁺ (Amersham).

c-Abl tyrosine kinase assay

c-Abl tyrosine kinase activity was measured in an immunocomplex kinase assay, as described previously [7,19]. A fusion protein (GST-CTD-Crk) containing a modified C-terminal repeat domain of RNA polymerase II (CTD) and the c-Abl phosphorylation site in the adapter protein Crk (J. Gong and J. Y. J. Wang, unpublished work) was used as a specific substrate to assay c-Abl kinase activity. Briefly, keratinocytes were lysed in RIPA buffer with the addition of protease inhibitors and 1 mM dithiothreitol, 20 mM β -glycerophosphate, 10 mM NaF and 1 mM sodium orthovanadate. A 500 µg aliquot of cellular extract from each sample was incubated for 2 h at 4 °C with 2 µg of anti-Abl (24-11) monoclonal antibody (Santa Cruz). After addition of Protein G-agarose (Boehringer Mannheim), samples were incubated for an additional 30 min. The immunoprecipitates were then washed three times with RIPA buffer and once with kinase reaction buffer (50 mM Tris/HCl, 10 mM MgCl₂, 1 mM dithiothreitol, 20 mM β -glycerophosphate, 10 mM NaF and 1 mM sodium orthovanadate), and resuspended in 20 µl of kinase reaction buffer containing 6 µg of GST-CTD-Crk, 25 µM ATP and 10 µCi of [γ -³²P]ATP. Following a 30 min incubation at 30 °C, the reaction was terminated with 10 µl of 3 × SDS sample buffer. Samples were resolved on SDS/10%-PAGE, blotted on to a PVDF membrane and analysed by autoradiography. The amount of immunoprecipitated c-Abl was detected with anti-c-Abl (K-12; Santa Cruz).

Determination of cell death

The sensitivity of p53^{-/-} [14], c-Abl^{-/-} [15] and c-Abl^{+/+} [16] MEFs and HaCaT cells to cisplatin was determined by counting the total number of cells using Crystal Violet staining. Briefly, cells were seeded at 8 × 10⁴/well into eight-well plates. The next day, cisplatin and/or AA-2P was added at the indicated concentrations and the cells were incubated for 48 h. Cells were then fixed and stained with 0.1% Crystal Violet. After washings with PBS, Crystal Violet was extracted with 10% (v/v) acetic acid and the amount of dye recovered was determined from its absorbance at 600 nm. The absorbance (units) in treated wells (in triplicate) was normalized to that in untreated wells (also in triplicate) collected at the same time point. The death of HaCaT cells, as well as of HCT116 cells (mismatch-repair-defective colon carcinoma cell line) and HCT116-3(6) cells (with a

functional *MLH1* gene, as a result of transfer of chromosome 3) [20], was determined by DNA fragmentation. Cells subjected to different treatments were collected by centrifugation at 200 *g* for 10 min and fixed with 1:1 (v/v) PBS and methanol/acetone (4:1, v/v) solution at -20°C . The cell cycle was evaluated by flow cytometry using propidium iodide (50 $\mu\text{g}/\text{ml}$) staining (Sigma, St. Louis, MO, U.S.A.) [21,22], after prior incubation with 13 k-units/ml RNase A (Sigma) (20 min incubation at 37°C), on a FACS-Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were excited at 488 nm, using a 15 mW argon laser, and fluorescence was monitored at 585/42 nm, at a rate of 200 events/s. A total of 10000 events was evaluated using the Cell Quest Programme (Becton-Dickinson). Electronic gating FL2A/FL2W was used, when appropriate, to eliminate cell aggregates.

RESULTS

Ascorbate up-regulates *MLH1* expression

Using cDNA microarray hybridization, we have previously identified genes that are differentially expressed after ascorbate loading; indeed, HaCaT keratinocytes incubated with 1 mM AA-2P for 5 h exhibited an expression profile in which several mRNAs were up-regulated by the presence of ascorbate [1]. In the present study, we focused on the *MLH1* gene, a necessary component of the DNA mismatch repair system.

In order to confirm the results obtained with the microarray analysis, we first investigated the time- and dose-dependence of ascorbate-mediated up-regulation of *MLH1* expression by semi-quantitative RT-PCR. With intracellular saturating levels of ascorbate (1 mM AA-2P exogenously) [18], positive modulation of the *MLH1* mRNA level was observed after just 2 h, and increased further up to 16 h of treatment (Figure 1A). Concentrations of AA-2P above 10 μM (ranging from 10 to 100 μM) resulted in the same level of induction (Figure 1A); this indicates that induction is either dose-independent or saturable at low concentrations of ascorbate. Indeed, we found that incubation with 1 μM AA-2P did not change the amount of *MLH1* mRNA, but a positive response was seen with 5 μM AA-2P, although a maximum effect was reached only with concentrations of 10 μM

Figure 1 Ascorbate-mediated regulation of the *MLH1* gene

(A) Dose-dependence and time course of *MLH1* mRNA expression. Keratinocytes were loaded with AA-2P, and then *MLH1* expression was assessed by semi-quantitative RT-PCR. Cells were incubated with 1 mM AA-2P for 2, 5, 8 and 16 h, or with 10, 50 and 100 μM AA-2P for 5 h, as indicated (the left-hand lane contains control cells). GAPDH amplification is also shown as an internal control. Autoradiography is representative of three similar experiments. (B) Western blot analysis of *MLH1*. Keratinocytes were left untreated or were incubated with 50 μM AA-2P, as indicated. After 24 h, cellular extracts were purified and immunoblotted with anti-*MLH1* antibody. The blot is representative of three similar experiments.

Figure 2 Effects of ascorbate on p53, p73 and c-Abl expression

(A) Semi-quantitative RT-PCR analysis of total RNA extracted from HaCaT cells. Purified total RNA was reverse-transcribed and amplified with primers specific for p53 or c-Abl. GAPDH amplification, performed as internal control, is also shown. Lane 1, control cells; lane 2, AA-2P-treated cells (50 μM). Autoradiography is representative of three similar experiments. (B) Western blot analysis of HaCaT cells. Keratinocytes were left untreated (lane 1) or were incubated with 50 μM AA-2P (lane 2). After 24 h, cellular extracts were purified and immunoblotted with either anti-p53 or anti-c-Abl antibodies. The blot is representative of three similar experiments. (C) Dose-dependence and time course of p73 mRNA expression. Keratinocytes were loaded with AA-2P, and then p73 expression was assessed by semi-quantitative RT-PCR. Lane 1, control cells; lanes 2–5, cells incubated with 1 mM AA-2P for 2, 5, 8 and 16 h respectively; lanes 6–8, cells incubated with 10, 50 and 100 μM AA-2P respectively for 5 h. The positions of p73 isoforms are indicated on the right. GAPDH amplification is also shown as an internal control. Autoradiography is representative of three similar experiments. (D) Western blot analysis of p73 α and β isoforms. Keratinocytes were left untreated (lane 1) or were incubated with 50 μM AA-2P (lane 2). After 24 h, cellular extracts were purified and immunoblotted with anti-p73 antibody. The blot is representative of three similar experiments.

AA-2P and above (results not shown). Accordingly, we also found increased expression of *MLH1* protein after incubation with AA-2P (Figure 1B).

Ascorbate modulates p73, but not p53

As both p53 and p73 seem to be induced by DNA damage, albeit through distinct mechanisms, we investigated whether ascorbate exerts different effects on targets downstream of MLH1. HaCaT cells were left untreated or were incubated with AA-2P, and gene expression was analysed by semi-quantitative RT-PCR and Western blotting. Ascorbate did not affect either the p53 or the *c-abl* gene, at either the transcriptional or the translational level. Indeed, neither the amounts of p53 and c-Abl transcripts (Figure 2A) nor their protein levels (Figure 2B) were altered after AA-2P supplementation.

In contrast, we observed a time-dependent modulation of the expression of p73 variants, reaching a peak after 8 h of treatment; the α isoform was the most expressed (Figure 2C). This result was confirmed at protein level: increased expression of the α and β p73 isoforms was evident after incubation with AA-2P (Figure 2D).

Ascorbate enhances DNA-damage-induced cell death

The chemotherapeutic agent cisplatin elicits DNA damage and programmed cell death by activating either p53-dependent or MLH1-dependent, p53-independent pathways (more clinically relevant, due to the frequent p53 mutations). In order to investigate if the ascorbate-mediated up-regulation of MLH1 could improve the susceptibility of cells to DNA-damage-induced apoptosis, we treated HaCaT cells with increasing concentrations (1–30 μ M) of cisplatin (II) diammine dichloride (Sigma) for 48 h, and then apoptosis (DNA fragmentation) was assessed by cytofluorimetric analysis; in parallel experiments, cells were co-incubated with cisplatin and 0.05 or 1 mM AA-2P.

After cisplatin treatment, HaCaT cells underwent apoptosis in a dose-dependent manner, as demonstrated by an increase in the percentage of cells showing a hypodiploid DNA content (Figure 3A). Ascorbate alone did not induce cell death (results not shown), but it potentiated cisplatin-induced cell death: indeed, apoptotic events elicited by 1 μ M cisplatin were increased from $11.6 \pm 3.1\%$ to $34.4 \pm 3.8\%$ in the presence of 0.05 mM AA-2P (Figure 3A). Similar results were obtained after incubation of HaCaT cells with 1 mM AA-2P (Figure 3A). With high doses of the chemotherapeutic agent, the effect of ascorbate was less evident: at 30 μ M cisplatin, for example, the percentage of hypodiploid events increased from $52.6 \pm 7.3\%$ to $64.3 \pm 0.8\%$ in the presence of 1 mM AA-2P.

Flow cytometry analysis demonstrated that the synergism between ascorbate and cisplatin did not involve alterations in cell cycle distribution. Cell growth arrest was promoted by the anti-neoplastic drug independently of the presence of the antioxidant vitamin. Indeed, AA-2P-treated keratinocytes showed the same cell cycle distribution as control cells (Figure 3B). Cisplatin significantly modified the distribution of cells into different cell cycle phases, but again the cytofluorimetric profile of keratinocytes treated with cisplatin alone did not differ from that of cells co-incubated with the two compounds (Figure 3C).

Ascorbate enhances cisplatin-mediated activation of the MLH1/c-Abl/p73 pathway

The DNA-damaging compound cisplatin increases and/or stabilizes, through activation of MLH1 and c-Abl, the amount of p73 protein, thus activating the apoptotic pathway downstream of p73 [7]. In order to establish the effects of ascorbate on c-Abl enzymic activity, we performed kinase assays in cells treated with cisplatin alone or in combination with AA-2P. In accordance with expression studies (see Figure 2), c-Abl kinase activity did

Figure 3 Cisplatin-induced cell death in the presence of ascorbate

(A) Dose–response experiments of cisplatin-induced cytotoxicity. HaCaT keratinocytes were incubated with increasing concentrations (1–30 μ M) of cisplatin alone ($\square$) or in combination with 50 μ M ($\blacktriangle$) or 1 mM ($\bullet$) AA-2P for 48 h. After incubation, cells were harvested and cell death was evaluated by cytofluorimetric analysis. Data are means $\pm$ S.D. of duplicate determinations carried out in three different experiments. (B) Cell cycle analysis of AA-2P-loaded keratinocytes. HaCaT cells were left untreated (Ctrl; grey area) or were incubated with 50 μ M AA-2P (black line) for 48 h, then cytofluorimetric analysis was performed. In order to evaluate only the cell cycle profile, cells were gated and sub-G₁ DNA was excluded. Results are means of duplicate determinations carried out in three different experiments. (C) Cell cycle analysis of cisplatin-treated keratinocytes. HaCaT cells were incubated with 1 μ M cisplatin alone (Cisp.; grey area) or in combination with 1 mM AA-2P (black line) for 48 h; the cell cycle distributions were then compared by cytofluorimetric analysis. Results are means of duplicate determinations carried out in three different experiments.

not show any increase in cells incubated with AA-2P alone in comparison with control cells (Figure 4). As reported previously [7,23,24], cisplatin treatment led to activation of c-Abl kinase activity, resulting in increased levels of phosphorylation of the

Figure 4 Effect of ascorbate on c-Abl tyrosine kinase activity

Cellular extracts collected after a 24 h incubation were analysed for c-Abl enzymic activity, by measuring phosphorylation of CTD/Crk. Keratinocytes were incubated with or without AA-2P and/or cisplatin, as indicated. In the lower panel is shown the amount of immunoprecipitated c-Abl on the same filter after re-probing with antibodies to c-Abl. Autoradiography is representative of three similar experiments.

Figure 5 Co-operative action between ascorbate and cisplatin correlated with the mismatch repair system

HCT116 and HCT116-3(6) cells were incubated with 30 μ M cisplatin alone (dotted bars) or in combination with 50 μ M AA-2P (hatched bars) for 48 h. After incubation, cells were harvested and cell death was evaluated by cytofluorimetric analysis. Untreated (white bars) and ascorbate-treated (grey bars) cells are also shown as controls. Data are means $\pm$ S.D. of duplicate determinations carried out in three different experiments. Significance of differences: * $P < 0.001$ compared with control cell death; # $P < 0.05$ compared with cell death produced by cisplatin alone (one-way ANOVA followed by Tukey–Kramer multiple-comparison test).

specific substrate CTD–Crk (Figure 4). Interestingly, in samples co-incubated with AA-2P and the chemotherapeutic agent, the levels of c-Abl-induced phosphorylation of the substrate were increased further (Figure 4). Since activation of c-Abl by cisplatin is dependent upon the presence of MLH1, it seems clear that

ascorbate, by increasing the amount of MLH1 protein, led to increased activation of c-Abl in response to cisplatin.

The role of the ascorbate-mediated modulation of MLH1 was further confirmed in MLH1-defective cells. The colon carcinoma cell line HCT116 lacks the *MLH1* gene, and this is associated with an increased resistance to several drugs currently used in cancer chemotherapy. Only high cisplatin concentrations were able to significantly increase apoptotic rates: indeed, with concentrations ranging from 1 to 10 μ M, the percentage cell death did not change in comparison with untreated cells (results not shown); with 30 μ M cisplatin the percentage of apoptotic events increased to $28.7 \pm 1.8\%$ (Figure 5). However, in cells co-incubated with cisplatin and 50 μ M AA-2P, we did not find any potentiating effect (Figure 5). In HCT116-3(6) cells, which have a functional *MLH1* gene as a result of transfer of chromosome 3 [20], we observed a partial restoration of co-operative effects between the chemotherapeutic drug and ascorbate: at 30 μ M cisplatin, the percentage of apoptotic events increased from $38 \pm 1.9\%$ to $52 \pm 0.8\%$ in the presence of AA-2P (Figure 5).

Ascorbate-mediated enhancement of DNA-damage-dependent growth arrest is independent of p53

The studies described above were performed using, as an experimental model, the non-tumorigenic HaCaT cell line, which carries two different mutant p53 alleles [25]. Thus the increased sensitivity to DNA-damaging agents observed in ascorbate-supplemented cells proceeded via the MLH1/c-Abl/p73 pathway and appeared to be independent of p53.

To completely exclude a concomitant modulation of p53 by ascorbate, we assessed sensitivity to cisplatin in cell lines with a

Figure 6 Enhancement of cisplatin-induced growth arrest by ascorbate in fibroblasts with defined mutations

The sensitivity of HaCaT cells (A) and p53^{-/-} (B), c-Abl^{-/-} (C) and c-Abl^{+/-} (D) fibroblasts to cisplatin was determined by Crystal Violet staining. Cells were treated with increasing concentrations of cisplatin alone (■) or in combination with 50 μ M (◆) or 1 mM (□) AA-2P, and viability was assessed after 48 h. Data are expressed as percentage viability, with that in control cells being set arbitrarily to 100%. Results are means $\pm$ S.D. of triplicate determinations carried out in three different experiments.

different genetic background. In HaCaT cells, growth arrest promoted by cisplatin was significantly increased when ascorbate was present (Figure 6A), in agreement with results described above (see Figure 3A). This was further supported by experiments carried out on p53^{-/-} primary MEFs [14] (Figure 6B), thus confirming that p53 was not involved in the synergy between cisplatin and ascorbate. In contrast, fibroblasts lacking c-Abl [15] appeared to be insensitive to the ascorbate-mediated enhancement of cisplatin-induced cytotoxicity: indeed, c-Abl-deficient fibroblasts showed similar sensitivity to increasing concentrations of cisplatin irrespective of the presence of ascorbate (Figure 6C), and the re-introduction of c-Abl [16] restored the sensitivity to ascorbate (Figure 6D).

DISCUSSION

We have found previously, by microarray hybridization, that ascorbate (vitamin C) may up-regulate several genes [1]. In the present study, we further investigated the ascorbate-mediated up-regulation of the *MLH1* gene, as an involvement of ascorbate in the regulation of DNA repair enzymes has been postulated [12]. Our data indicate, for the first time, that this antioxidant vitamin positively regulates the apoptotic cascade primed by MLH1 in response to DNA damage [7–9]. The vitamin not only increases MLH1 transcript levels in a time-dependent manner, but also selectively regulates the expression of some p73 splicing variants [26–30]. Whereas ascorbate alone changed neither the expression nor the activity of c-Abl, nonetheless it was able to up-regulate the DNA-damage-mediated induction of c-Abl kinase activity.

The MLH1 protein seems to be a critical factor for determining if damaged cells enter into either one of the two pathways leading to induction of mutation or to cell death. It can also modulate the effectiveness of certain chemotherapeutic drugs: when the mismatch repair protein recognizes cisplatin adducts in DNA, it activates a signal transduction pathway which leads to activation of c-Abl and an increase in p73 protein levels. Ascorbate, by increasing the cellular content of MLH1, improves the cellular response to DNA damage. Indeed, induction of increased *MLH1* gene expression by DNA damage allows faster c-Abl activation; thereafter, increased p73 activation (which is also regulated by ascorbate) could be achieved.

Several strands of epidemiological evidence suggest that consumption of fruit and vegetables is associated with a decreased risk of cancer. Fruit and vegetables are a good source of vitamin C, and this compound may be responsible for their anti-carcinogenic activity. Nevertheless, biochemical mechanisms accounting for this activity are not fully understood. On the basis of our results, we suggest that both the anti-carcinogenic and anti-cancer activities of ascorbate might be explained by modulation of *MLH1* gene expression. The chemopreventive activity may be attributed to the ability of ascorbate to act as a radical scavenger and also to prime, through induction of *MLH1* and *p73* gene expression, the apoptotic programme in DNA-damaged cells, which otherwise would proceed towards tumorigenic progression. By modulating *MLH1* gene expression, ascorbate can also enhance the anti-neoplastic activity of several drugs: in our experimental model, ascorbate, used in combination with cisplatin, increased the apoptosis of tumour cells. These results are in agreement with data reported by Sarna and Bhola [31], who demonstrated a synergistic anti-tumour effect exerted by cisplatin and ascorbic acid against Dalton's lymphoma in mice.

Since the *p73* gene shows very rare mutations in cancer (e.g. in four out of 909 tumours) [32], activation of the MLH1/c-Abl/p73 pathway by ascorbate could have relevant therapeutical

implications. Indeed, combined therapy with ascorbate and DNA-damaging drugs (such as cisplatin) may allow the same pharmacological effectiveness to be attained with lower doses of the chemotherapeutic agent, with a consequent reduction in collateral effects.

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