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Induction of apoptosis by R-flurbiprofen in human colon carcinoma cells: involvement of p53

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Abstract

R-Flurbiprofen, a non cyclooxygenase inhibiting non-steroidal anti-inflammatory drug (NSAID), has been found to inhibit tumor growth in various animal models. In vitro experiments have shown that this effect is based on the induction of a cell cycle block and apoptosis. Cell cycle inhibition has been explained by activation of the c-Jun–N-terminal kinase (JNK) and downregulation of cyclin D1 expression. However, the molecular mechanism leading to apoptosis is unknown. Here, we show that treatment of the human colon carcinoma cell line HCT116 with different concentrations of R-flurbiprofen leads to an accumulation of p53 protein which is accompanied by an increase in phosphorylated p53 at serine 15. Mutation of serine 15 to alanine by site directed mutagenesis and overexpression of the mutated p53 gene in HCT116 cells, revealed that these cells are significantly less sensitive to apoptosis induced by R-flurbiprofen than pcDNA control cells, as measured by PARP-cleavage and flow cytometry. By contrast, no difference was detected between HCT116p53ser15ala cells and HCT116 pcDNA cells with respect to induction of a cell cycle block after R-flurbiprofen treatment. Moreover, in nude mice HCT116p53ser15ala overexpressing xenografts were significantly less sensitive to R-flurbiprofen than HCT116 pcDNA control xenografts. In conclusion, we were able to show that induction of apoptosis in HCT116 cells after R-flurbiprofen treatment is at least partly dependent on the tumor suppressor gene p53 and that mutation of p53 at serine 15 impairs the apoptotic potency of R-flurbiprofen.

Keywords: G_1-phase block; Apoptosis; p53; Colon cancer; Flurbiprofen

1. Introduction

More than 20 years ago, it has been shown for the first time that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk for colon cancer [1,2]. For a long time, this effect of NSAIDs was exclusively ascribed to their COX-inhibiting potency. Over the last years, however, a substantial number of publications pointed towards COX-independent mechanisms for the cancer protective effects of NSAIDs [3–13], with each NSAID having a different mode of action. Nevertheless, the molecular COX-independent mechanisms of the anticarcinogenic properties of NSAIDs are not fully understood. R-Flurbiprofen, a 2-arylpropionic acid, is one of the most interesting NSAIDs because it neither inhibits COX-1 nor COX-2 at therapeutically relevant concentrations and showed antinociceptive as well as antitumorigenic effects in vitro and in vivo in different mouse models [14–19]. Moreover, in men R-flurbiprofen is nearly not inverted into its COX-inhibiting antipode, S-flurbiprofen [16,20]. Thus, R-flurbiprofen is of great interest because it does not have the risk to cause the typically known serious side effects in the gastrointestinal tract or the kidney as the classical COX-1 and/or COX-2 inhibiting drugs [21,22].

Abbreviations: COX-2, cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drugs; FCS, fetal calf serum; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorter; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonylfluoride; DTT, dithiotreitol; SDS–PAGE, sodiumdodecylsulfate–polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; AUC, area under the curve; ANOVA, univariate analysis of variance; PARP, poly(ADP)-ribose polymerase

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Recently, we were able to show that the anticarcinogenic effect of R-flurbiprofen is based on an induction of a G_1-phase block and apoptosis in human colon carcinoma cell lines. It turned out that the cell cycle block is partly dependent on activation of the c-Jun-N-terminal-kinase (JNK) accompanied by an increase in DNA-binding activity of the transcription factor AP-1 and downregulation of cyclin D1 expression. By contrast, R-flurbiprofen-induced apoptosis was largely independent of JNK activation [15].

One of the main regulators of cell growth and death is the tumour suppressor protein p53. Normal cells express very low levels of p53 protein but various cellular stress stimuli lead to a rapid increase of the p53 protein level which then accounts either for growth arrest or programmed cell death [23]. The increase of p53 protein is mainly dependent on post-translational mechanisms and only to a minor extent due to changes in the transcription rate or an increase in translation of p53 mRNA [24]. Under normal growth conditions, p53 is in complex with the proto-oncoprotein Murine Double Minute-2 (Mdm2) and inactive c-Jun-N-terminal kinase, which are both responsible for the constitutive instability of p53. In response to stress, both proteins are released from the N-terminal region of the p53 protein and p53 is subsequently phosphorylated at several serine and threonine residues leading to its stabilization and accumulation [23,25]. More than 10 different kinases have been described to be involved in phosphorylation of p53 [25]. Several phosphorylation sites have been associated with either stabilization of the protein, modulation of the DNA-binding activity of p53 or activation/inactivation of the sequence specific transactivation domain, which is the binding site of components of the transcriptional machinery and co-activators of transcription [23,25]. Mutations at different phosphorylation sites within the p53 gene gave insight which amino acid residues are necessary for the apoptotic or cell cycle inhibiting activity of p53 [26–29]. Because of the fundamental function of p53 in the induction of apoptosis and cell cycle block, we wanted to analyse whether or not the apoptotic and by that anticarcinogenic effect of R-flurbiprofen depends on modulation of p53. The human colon carcinoma cell line HCT116, which express wild type p53, has been used as model cell line.

2. Materials and methods

2.1. Cells and reagents

The human colon carcinoma cell line HCT116 was purchased from ATCC (American-type culture collection) and cultured in McCOY's 5A medium with L-glutamin, supplemented with 10% FCS (foetal calf serum) at 37 °C in a 5% CO_2 atmosphere.

R-Flurbiprofen was supplied by PAZ Arzneimittelentwicklungsgesellschaft. The optical purity of this enantiomer was >99% (determined by stereoselective HPLC-analysis). The substance was dissolved in phosphate-buffered saline (PBS).

2.2. Cloning and site directed mutagenesis of wt p53

For cloning wildtype (wt) p53 cDNA total RNA from HCT116 cells was prepared by the method of Chomczynski [30]. RT-PCR was performed with the QIAGEN RT-PCR Kit (QIAGEN). Reverse transcription reaction was incubated at 55 °C for 30 min. PCR amplification with p53 specific primers (FW: 5′-TACGAATTCAGTGGGGAACAAGAAGTGGAG-3′, RV: 5′-CAGTGGGGAACAAGAAGTGGAG-3′) was started with an initial activation step at 95 °C for 15 min. The samples were then denatured at 95 °C for 45 s, annealed at 61 °C for 45 s and extended at 72 °C for 1.5 min in 30 repetitive cycles. The PCR-product was loaded onto a 1% agarose gel stained with ethidium-bromid and extracted with a QIAquick gel extraction kit (QIAGEN). Subsequently, the wt p53-cDNA was used to mutate ser15 to ala by PCR-based oligonucleotide-directed mutagenesis. For PCR, we used the p53ser 15ala forward primer (5′-TACGGATCCATGGAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCTCTGGCTCAGGAAAC-3′) and the wt p53 reverse primer (5′-CAGTGGGGAACAAGAAGTGGAG-3′). The resultant PCR product was cloned into the *Bam*HI and *Eco*RI restriction site of the pcDNA-vector (Invitrogen). All PCR-products were verified by sequencing.

2.3. Stable transfection of HCT116 cells with the p53ser15ala cDNA

HCT116 cells were transfected using FuGene-Transfection Kit (Roche Diagonstics GmbH). Two micrograms of each plasmid (pcDNA or p53ser15ala pcDNA) were mixed with 6 μl FuGene reagent and 4 ml McCOY's 5A medium with L-glutamin, the mixture was left at room temperature for 45 min and then added to the cell culture for at least 6 h. Subsequently, medium was resolved and fresh medium was added containing 10% FCS. After 48 h, incubation time cells were splitted 1:8 in medium containing 0.75 mg/ml G418 and after 10 days incubation time-stable transfected cell clones were analysed for p53 expression.

2.4. Western-blot analysis

5×10^5 cells were seeded per 5 cm dish. After 16 h of incubation, cells were treated with either increasing concentrations of R-flurbiprofen (0, 200, 400, 600, 800, 1000 μM) for 20 h or with 800 μM R-flurbiprofen (this concentration showed significant apoptotic activity) for various time periods, washed with PBS, harvested by scraping them in 1 ml PBS and collected by short centrifugation. For whole cell extracts, cell pellets were resuspended in 1 ml lysis-buffer (20 mM Tris–HCl (pH