THYMIDYLATE SYNTHASE INHIBITION INDUCES P53 DEPENDENT AND INDEPENDENT CELL DEATH

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1. INTRODUCTION

Thymidylate synthase (TS) is an important target for chemotherapy in colon cancer. It is the rate limiting de novo enzyme for synthesis of thymine nucleotides, one of the precursors for DNA synthesis. TS can be inhibited by several clinically active compounds\(^1,2\) such as 5-fluorouracil (5-FU) and the antifolates (AG337 (Thymitaq, Nolatrexed), ZD1694 (Tomudex, Raltitrexed), LY231514 (MTA or pemetrexed). This will lead to inhibition of DNA synthesis and imbalance in dUTP/dTTP pools which will result in DNA damage. DNA damage can trigger downstream events such as p53\(^3\), bax\(^4\), Fas receptor and caspase-3 to initiate apoptosis\(^5\). It is however not completely known which mechanisms are involved in the onset of apoptosis after TS inhibition. Therefore, we studied the induction of downstream events after exposure of colon cancer cell lines to 5-FU and antifolates and the role that p53 plays during the process of apoptosis initiation.

2. METHODS

The human colon carcinoma cell lines Lovo, LS174T (wild-type (wt) p53), WiDr and SW948 (mutant (mt) p53) were cultured at 37°C in a 5%
CO₂ humidified atmosphere in DMEM supplemented with 5% FCS. Exponentially growing cells were treated with IC50 and 10 x IC50 concentrations of 5-FU, AG337, MTA, and ZD1694. After 24, 48, 72 and 96 hr of drug exposure both floating and adherent cells were harvested. Cells were counted and cytopsins (for May Grunwald Giemsa (MGG) staining and immunocytochemistry), cell pellets (for western blotting) and cell suspensions (for FADU (Fluorometric Analysis of DNA Unwinding) analysis) were prepared. All experiments were done at least three times.

For the determination of the protein expression of TS, p53, bcl-2 and bax western blotting was performed as described previously. The changes in expression are given as: - for < 0.3 fold compared to untreated cells; +/- for 0.3-0.7; = for 0.7-1.3; +/- for 1.3-2.0; + for 2-5 and ++ for > 5 fold compared to untreated cells.

For immunocytochemistry standard methods were applied. Cytospins of WiDr and Lovo cells were fixed in 100 % acetone and incubated for 1 hr with Fas receptor (1:25; clone DX2, Oncogene), Fas ligand (1:400; clone 33, Transduction) and caspase-3 (1:500; clone 19, Immunotech). After addition of the secondary antibody for 30 min, staining was developed by incubation with sABC-HRP (1 : 200; 1 hr) followed by DAB (10 mg/ml) with 0.025 % H₂O₂ for 3 min. For Fas ligand and caspase-3 an additional amplification step was added the sABC-HRP step to get more intense staining. Two individuals scored independently the intensity of the staining from low (+) to high (++++).

3. RESULTS

TS inhibition resulted in DNA damage in all wt and mt p53 cell lines. Induction of DNA damage was not cell line or p53 dependent but drug dependent. Exposure of Lovo and WiDr cells to AG337 (10 x IC₅₀) resulted in 10-20 % more DNA damage than after MTA or 5-FU treatment for 72 hrs. The induction of DNA damage triggered changes in the expression of downstream events. The protein expression of the target enzyme TS increased (2-5 fold) in all cell lines after 5-FU and ZD1694 treatment (Table 1), independent of the status (wt or mt) p53. The antifolates, AG337 and MTA also induced TS expression in Lovo and LS174T but was not induced in the mt p53 WiDr and SW948 cells (Table 1) after exposure to 5-FU and antifolates. Similar results were found for the bax expression. The expression of bcl-2 hardly changed after 24 hrs treatment (Table 1) in Lovo and LS174T whereas in WiDr and SW948 cells no bcl-2 expression was detectable before of after drug exposure. Exposure of AG337 to Lovo and WiDr cells using