Transmission of Apoptosis in Human Colorectal Tumor Cells Exposed to Capecitabine, Xeloda, Is Mediated via Fas

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Abstract
We developed an original in vitro model dedicated to the exploration of molecular pharmacology of the new oral fluoropyrimidine capcitabine (Xeloda). More specifically, in this report, we investigated whether apoptosis induced by capcitabine was mediated by the Fas/FasL system. To achieve this goal, a specific in vitro coculture model mixing hepatoma and human colorectal cell line was used. A bystander effect was observed between HepG2 and LS174T cells treated with capcitabine. Besides this, Xeloda showed a 7-fold higher cytotoxicity and markedly stronger apoptotic potential in thymidine phosphorylase (TP)-transfected LS174T-c2 cells. The striking enhancement of thymidylate synthase inhibition that we observed in cells with high TP activity was most probably at the origin of the potentiation of capcitabine antiproliferative efficacy. In addition, this increase of sensitivity was accompanied by a strong overexpression of the CD95-Fas receptor on the cell surface. Both Fas and FasL mRNA expression were triggered after exposing TP+ cells to the drug. This implication of Fas in Xeloda-induced apoptosis was next confirmed by using antagonistic anti-Fas and anti-FasL antibodies that proved to reverse capcitabine antiproliferative activity, thus highlighting the key role that Fas could play in the optimization of an antitumor response to fluoropyrimidine drugs. Our data, therefore, show that TP plays a key role in the capcitabine activity and that the Fas/FasL system could be considered as a new determinant for Xeloda efficacy.

Introduction
Because cancer now is considered more as a deficiency of apoptosis rather than a mere proliferation issue (1), new anticancer drugs are evaluated by their ability to induce or restore apoptosis. Yet, little is known on the way that capcitabine, the novel oral FUra3-prodrug carbamate, acts as an apoptotic agent.

Because of the double hepatic + tumoral activation pattern of capcitabine, little in vitro data are available thus far to provide mechanistic basis for understanding the apoptotic potential of this drug. Recent studies suggested that fluoropyrimidine-induced apoptosis could be, at least partially, mediated via the Fas system (2, 3).

In the present work, after developing a coculture model designed for the in vitro study of capcitabine, we investigated whether a Fas component could be found, therefore, in the transmission of apoptosis in human colorectal tumor cells exposed to Xeloda.

Materials and Methods

Cell Lines and Chemicals. Human colorectal LS174T and TP-transfected LS174T-c2 cells were obtained and fully characterized as described previously (4, 5). Human HepG2 hepatoma and colorectal tumor cells were cultivated in the same plates using Nunc tissue culture inserts (Polylabo, Strasbourg, France). Tritiated dUMP (16 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Capcitabine was a kind gift from Dr. H. Ishitsuka, Nippon-Roche, Kamakura, Japan. Anti-Fas CH11 MoAB was purchased from Immunotech (Marseille, France), anti-Fas ZB4, anti-FasL BR17 antagonistic MoAbs, and Annexin V kit came from Euromedex (Souffelweyersheim, France).

Antiproliferative Studies. HepG2 and either LS174T WT or LS174T-c2 cells were seeded, respectively, in the top and bottom chambers of 8-well strip membranes in 96-well plates. Exponentially growing cells were next exposed to increasing concentrations of capcitabine. The medium was supplemented with 750 ng/ml ZB4 MoAB or 100 ng/ml BR17 MoAB when the latter were used in the experiments. After 72 h of continuous exposure, LS174T viability was assessed using the classic colorimetric MTT test (6).

TS Inhibition Studies. Exponentially growing LS174T WT and LS174T-c2 cells, cultivated in 6-well plates with HepG2 using culture inserts, were exposed to 150 µm capcitabine for 24 h. After removal of the drug, cells were allowed to grow in capcitabine-free medium for 48 h more. TS activity was assessed at 8 and 24 h. Restoration of TS activity was next
monitored at 24 and 48 h after removal of the drug. TS activity was evaluated following a modified Roberts method as described previously (2, 5, 7).

Fas Receptor Cell Surface Expression. HepG2 and either LS174T WT or LS174T-c2 cells were seeded, respectively, in the top and bottom chambers of culture inserts in 6-well plates. Cells in exponential phase were next exposed to 300 μM capcitabine for 48 h. After 48 h of continuous exposure, LS174T cells were harvested and cell surface expression of CD95 Fas receptor was measured by flow cytometry analysis as described previously (2, 5).

Real-Time PCR Study of Fas and Fas-L mRNA Expression. Exponentially growing LS174T WT and LS174T-c2 cells, cultured in 6-well plates with HepG2 using culture inserts, were exposed to 150 μM capcitabine for 24 h. After removal of the drug, cells were allowed to grow in capcitabine-free medium for 48 h more. Fas and FasL expression was measured at 8, 24, and 48 h by real-time quantitative PCR.

Total RNA (2 μg) was reverse-transcribed into cDNA using 1-μg hexamers (Pharmacia Biotech, Orsay, France) and M-MLV reverse transcriptase as described by the manufacturer (Invitrogen-Life Technologies, Inc., France). Human Fas, Fas-L mRNA, and 18S rRNA were amplified (Fas: forward primer, 5′-TTGTTGAACCCGCTCAAG-3′; and reverse primer, 5′-AATCTAGCAAAGCTGAAGACCACT-3′; Fas-L: forward primer, 5′-TCCCAAGGTCTTGTTGAC-3′; and reverse primer 5′-TCACCACGTGTAAGACCACT-3′; 18S: forward primer, 5′-CTACCAATCAGGAAGGCA-3′, and reverse primer 5′-TTGGTGACCCGCTCAAG-3′), and were detected and quantitated in real time using an ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA) as described elsewhere (8, 9).

The amplification mixture contained 0.2 μM cDNA derived from 50–150 ng total RNA. A two-step PCR was performed for 40 cycles. The reaction yielded a 116-bp PCR product for Fas, 110-bp for Fas-L and 100-bp for 18S. Fas and Fas-L were normalized to their respective 18S RNA levels to minimize the threshold of the signal. To create standard curves for each gene, RNAs were produced by in vitro transcription from linearized templates corresponding to Fas, Fas-L, and 18S cDNA constructs using T7 or T3 polymerases and M hexamers (Pharmacia Biotech, Orsay, France) and M-MLV reverse transcriptase as described by the manufacturer (Invitrogen-Life Technologies, Inc., France). Human Fas, Fas-L and 18S cDNA constructs using T7 or T3 polymerases and M hexamers (Pharmacia Biotech, Orsay, France) and M-MLV reverse transcriptase as described by the manufacturer (Invitrogen-Life Technologies, Inc., France). Human Fas, Fas-L mRNA, and 18S rRNA were amplified (Fas: forward primer, 5′-TTGTTGAACCCGCTCAAG-3′; and reverse primer, 5′-AATCTAGCAAAGCTGAAGACCACT-3′; Fas-L: forward primer, 5′-TCCCAAGGTCTTGTTGAC-3′; and reverse primer 5′-TCACCACGTGTAAGACCACT-3′; 18S: forward primer, 5′-CTACCAATCAGGAAGGCA-3′, and reverse primer 5′-TTGGTGACCCGCTCAAG-3′), and were detected and quantitated in real time using an ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA) as described elsewhere (8, 9).

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The results were calculated as picograms of Fas or Fas-L per nanograms of 18S, and finally expressed as relative levels (%) compared with untreated cells.

Apoptosis Studies. HepG2 and either LS174T WT or LS174T-c2 cells were seeded, respectively, in the top and bottom chambers of culture inserts in 6-well plates. Cells in exponential phase were next exposed to 300 μM capcitabine. After 48 h of continuous exposure, LS174T cells were harvested and subjected to propidium iodide/Annexin V double staining. Early and late apoptosis were then discriminated as described previously (2, 5) by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson, Le Pont de Claix, France).

Results and Discussion

Results of the cytotoxic studies are summarized in Fig. 1. A and B. Determination of capcitabine IC50. LS174T WT and LS174T-c2 cells were exposed either alone or with HepG2 cells to capcitabine for 72 h. Values are the mean ± SD of three separate experiments. *, significantly different from the capcitabine IC50 in LS174T cultivated alone (P < 0.05, t test).

Results of the cytotoxic studies are summarized in Fig. 1. A and B. Antiproliferative assays showed that both LS174T WT and LS174T-c2 cells were more sensitive to capcitabine when cultivated with the same plates as HepG2 hepatoma. Xeloda IC50 values were 890 ± 48 and 630 ± 14 μM in LS174T WT alone and cultivated with HepG2, respectively. Similarly, in LS174T-C2 subline, the IC50 fell from 330 ± 4 down to 89 ± 6 μM when cultivated in the same plates as hepatoma cells. This bystander effect is most probably caused by the diffusion of the first metabolites of Xeloda from liver to colorectal cells. This hypothesis is fully consistent with the design of the drug that requires hepatic carboxyl esterase to ensure proper activation toward cytotoxic metabolites (10). Because LS174T cells display little carboxyl esterase activity compared with HepG2 (data not shown), developing a coculture model was necessary, therefore, to overcome the lack of this critical activating enzyme.

More interestingly, TP-transfected cells proved to be up to 7-fold more sensitive to Xeloda, thus highlighting the role TP plays as a determinant to capcitabine efficacy. TP has indeed a double action in the Xeloda tumoral activation process: first in the cleavage of doxifluridine into FUra (11, 12), and second in the direct conversion of FUra to anti-TS FdUMP via the DNA pathway (2, 4, 5, 13).

This hypothesis of an increased formation of anti-TS FdUMP in the LS174T-c2 subline that displayed higher TP expression was strongly reinforced after studying the inhibition, then the restoration, of TS activity in cells exposed to Xeloda.
Indeed, stronger and longer TS inhibition was observed in cells with high TP yield and treated with capecitabine (Fig. 2). Eight h after exposing the cells to capecitabine, a near-total (97%) TS inhibition was reached in the LS174T-c2 subclone, whereas no decrease in activity was yet observed in the parental line. Besides, in TP-transfected cells, TS activity remained significantly decreased by 63 and 35% at 48 and 72 h, respectively, long after the drug had been removed. Interestingly, in WT cells a slight induction of TS activity was observed at 48 h, an up-regulation frequently described after exposing tumor cells to fluoropyrimidines and usually associated with a loss of drug efficacy (14, 15).

Conversely, no such induction was found in TP-transfected cells, thus suggesting that in these cells, the bioactivation rate of Xeloda to anti-TS FdUMP was high enough to inhibit durably the activity of the up-regulated TS protein. High-performance liquid chromatography studies, indeed, showed quicker clearance of parent-drug capecitabine in LS174T-c2 cells compared with the WT ones, thus confirming that a more efficient metabolization of the drug had occurred (data not shown).

The critical role TP plays in capecitabine efficacy was next confirmed by comparing the apoptosis induction in WT and TP-transfected cells. Results showed that, whereas little cell death occurred in LS174T cells exposed to Xeloda, both early and late apoptosis were increased by 244 and 262%, respectively, in the LS174T-c2 subclone (Fig. 3).

We next assessed to what extent exposing the cells to capecitabine would induce an overexpression of the Fas receptor. First, flow cytometry analyses revealed a 200% overexpression of cell-surface Fas receptor in Xelodasensitive LS174-c2 cells, whereas no significant increase was found in resistant LS174T WT cells (Fig. 4).
The triggering of the Fas system was further confirmed by studying the genetic expression of both Fas and FasL, which were found to be strikingly increased by the treatment in TP-overexpressing tumor cells by 1000 and 4000%, respectively (Fig. 5, A and B).

Finally, this critical implication of Fas was next confirmed by using antagonistic anti-Fas and anti-FasL MoABs, both of which strongly reversed capecitabine antiproliferative action (Fig. 6).

Recent studies have underlined the potential role that Fas could play in the transmission of apoptosis by fluoropyrimidine drugs (2, 3). Thymineless death induced by these drugs is probably the key mechanism by which the Fas/FasL system is triggered (16, 17), a hypothesis consistent with our data on TS inhibition in LS174T-c2 cells. Consequently, one can speculate that tumor cells that are resistant to Fas-mediated apoptosis would be resistant to fluoropyrimidine-based chemotherapies as well. Indeed, we previously showed that improving FUra efficacy using biochemical modulator could fail in the CaCo2 adenocarcinoma because of the lack of Fas receptor on cell surface (2).

Similarly, the implication of Fas in Xeloda-induced apoptosis, as shown in our study, strongly suggests that Fas functionality and expression level could be considered as a new determinant in the response to capecitabine, especially because we demonstrated that blocking Fas resulted in a striking desensitization of our model.

Complementary studies carried out in nude mice bearing xenografted LS174T WT and -c2 sublines will be performed to confirm in vivo the role of TP and Fas in Xeloda efficacy.

Determining the factors involved in drug response is a new challenge in modern chemotherapy (18). Various enzymes such as TP, dihydropyrimidine dehydrogenase, or TS levels have already been presented as response factors with Xeloda (19–21). Clinical studies will be necessary to determine whether Fas can be considered as a new determinant in response to FUra prodrugs as well, e.g., by comparing Fas expression in patient biopsies with the treatment outcome.

In this report, we presented a new experimental model suitable for the in vitro study of FUra prodrug capecitabine. Using an original real-time quantitative reverse transcription-PCR method, we showed that Xeloda induced apoptosis in a Fas-dependent manner, and that this implication of Fas was dependent on the level and duration of TS inhibition. The recent sudden increase of oral FUra alternatives gives to our work some clinical relevance because it provides experimental basis for understanding and predicting the role of Fas/FasL in the outcome of fluoropyrimidine-based chemotherapies.

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