

# Chemotherapy-Induced Mucositis Is Associated with Changes in Proteolytic Pathways

JONATHAN LEBLOND,\* FLORENCE LE PESSOT,† AURÉLIE HUBERT-BURON,\* CÉLIA DUCLOS,†  
JACQUES VUICHOD,‡ MAGALI FAURE,‡ DENIS BREUILLÉ,‡ PIERRE DÉCHELOTTE,\*  
AND MOÏSE COEFFIER\*,<sup>1</sup>

\*Appareil Digestif Environnement Nutrition (EA3234), Institut Hospitalo-Universitaire de Recherche Biomédicale and Institut Fédératif de Recherches Multidisciplinaires sur les Peptides (IFRMP23), University of Rouen and Rouen University Hospital, France; †Service d'anatomie pathologique, Rouen University Hospital, France; and the ‡Nestlé Research Center, Nutrition and Health Department, Lausanne, Switzerland

Mucositis, a common toxic side effect of chemotherapy, is characterized by an arrest of cell proliferation and a loss of gut barrier function, which may cause treatment reduction or withdrawal. Gut integrity depends on nutritional and metabolic factors, including the balance between protein synthesis and proteolysis. The effects of methotrexate (MTX; a frequently used chemotherapeutic agent) on intestinal proteolysis and gut barrier function were investigated in rats. Male Sprague-Dawley rats received 2.5 mg/kg of MTX subcutaneously during 3 days and were euthanized at Day 4 (D4) or Day 7 (D7). We observed at D4 that MTX induced mucosal damage and increased intestinal permeability (7-fold) and the mucosal concentration of interleukin (IL)-1 $\beta$  and IL-6 (4- to 6-fold). In addition, villus height and glutathione content significantly decreased. Intestinal proteolysis was also affected by MTX as cathepsin D activity increased at D4, whereas chymotrypsin-like proteasome activity decreased and calpain activities remained unaffected. At D7, cathepsin D activity was restored to control levels, but proteasome activity remained reduced. This disruption of proteolysis pathways strongly contributed to mucositis and requires further study. Lysosomal proteolytic activity may be considered the main proteolytic pathway responsible for alteration of mucosal integrity and intestinal permeability during mucositis, as cathepsin D activity was found to be correlated

with mucosal atrophy and intestinal permeability. Proteasome regulation could possibly be an adaptive process for survival. Future investigation is warranted to target proteolytic pathways with protective nutritional or pharmacological therapies during mucositis. *Exp Biol Med* 233:219–228, 2008

**Key words:** chemotherapy; mucositis; gut barrier; proteolysis; methotrexate

## Introduction

Treatment of cancer patients frequently consists of a combination of radiation therapy and chemotherapy. This often induces toxic side effects that are painful and sometimes long lasting. Aside from the bone marrow, a major dose-limiting organ in anticancer treatments is the intestine (1). Due to a major cellular renewal (2) and fast protein turnover in the mucosa (3), the gut is highly sensitive to anticancer drugs. These drugs frequently induce mucositis, which refers to erosive and ulcerative lesions on the mucosa from the mouth to the anus. Mucositis is therefore associated with gut barrier disruption (4, 5), and is related to an imbalance between proliferation and apoptosis of gut epithelial cells. The incidence of mucositis symptoms (i.e., pain, bloating, and diarrhea) ranges from 40% to 76% of patients according to protocol of chemotherapy (1). Despite recent studies (1, 4, 6, 7), the underlying mechanisms of gastrointestinal damage induced by cancer chemotherapy remain only partly understood. However, two dysfunctions have been clearly demonstrated to be involved: a decreased intestinal cell proliferation and an increased apoptosis in intestinal crypt cells (7). Within a few hours after the beginning treatment, chemotherapy drugs stimulated apoptosis (8) and limited cell division by inhibiting DNA and RNA synthesis (9, 10). Previous animal studies

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<sup>1</sup>To whom correspondence should be addressed at ADEN (EA3234, IFRMP23), 22 boulevard Gambetta, 76183 Rouen cedex 1, France. E-mail: moise.coeffier@univ-rouen.fr or moise.coeffier@chu-rouen.fr

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have reported that chemotherapy may cause intestinal apoptosis and crypt hypoplasia (11–13). Drug effective concentrations during anticancer treatments have led to a reduction in the number of intestinal epithelial cells within days, and result in diarrhea as a main clinical characteristic. The gut barrier integrity and function are rapidly altered during chemotherapy treatment of various cancers, as shown by the increase in intestinal permeability measured with probe sugars (14, 15). Furthermore, chemotherapy-induced mucositis is associated with changes in the flora of the stomach, jejunum, colon, and feces (6). Consequently, small intestine injuries may contribute to increased morbidity and mortality in cancer patients and a reduction in therapeutic efficacy.

Methotrexate (MTX) is a folate antagonist widely used in the treatment of many cancers. Due to its activity as an inhibitor of dihydrofolate reductase, MTX blocks DNA synthesis (16), which leads to cell cycle arrest and apoptosis in various cell types, both in the tumor and in sensitive tissues with high proliferation rates (i.e., the gut). Hypoproliferation is often followed by impaired epithelial integrity. In addition, other mechanisms could also be involved in deleterious effects of MTX, such as alteration of glutathione (GSH) metabolism (17, 18), nuclear factor (NF)- $\kappa$ B-induced proinflammatory mediator production (19), and alterations of protein metabolism (1). However, the alterations of gut proteolysis pathways during MTX treatment remain unknown. It could be hypothesized that MTX may alter gut barrier function by exacerbating proteolysis.

As such, the aim of the present study performed in rats was to evaluate the effects on intestinal proteolysis of mucositis induced by MTX treatment.

## Materials and Methods

**Animals, Housing, and Diet.** Animal care and experimentation complied with both French regulations and European Community regulations. During 1 week, male Sprague-Dawley rats (Charles River Laboratories, L'Arbresle, France) were acclimatized at 25°C with a 12:12-hr light:dark cycle. In order to adapt to their new environment, 200–250 g animals were housed in individual metabolic cages at least 2 days prior to the study. Rats were given *ad libitum* access to water and standard chow.

**Experimental Protocol. MTX Injections.** Rats were subcutaneously (sc) injected during the first 3 days (D0, D1, and D2) with MTX (Teva Pharma, Courbevoie, France) or 0.9% NaCl solution as a control. In a preliminary experiment (data not shown), three MTX doses were compared (1, 2.5, and 5 mg/kg), and 2.5 mg/kg of MTX was chosen, because it was a good compromise between the lack of mucositis at 1 mg/kg/day evaluated by body weight loss, anorexia, and intestinal damage and the major early mortality at 5 mg/kg/day. In addition, a previous study reported intestinal absorption alterations using three sc injections of 2.5 mg/kg of MTX (4). Rats were euthanized at

D4 or D7. The numbers of rats were 18 for the MTX group (9 for D4 and 9 for D7) and 12 for the control group (6 for D4 and 6 for D7).

**Daily Clinical State Monitoring.** From D1 to sacrifice day, body weight and food intake were monitored at 24 hr intervals.

**Sacrifice and Tissue Sampling Collection.** Animals were decapitated after carbon dioxide inhalation, after which the jejunum was obtained and rinsed with ice-cold PBS (140 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>). For histological and immunohistochemistry assessments, two proximal samples (1 cm long each) were fixed in formalin (10%). Three consecutive pieces (3 cm long each) were removed from each sample and mucosa was scraped for determination of mRNA expressions (proximal jejunum), proteolytic activities (proximal jejunum), and cytokine concentrations (mid-point). Then middle pieces (1 cm long) were removed for assessment of GSH content. Samples were immediately frozen in liquid nitrogen and stored at –80°C. For mRNA, TRIZOL Reagent (Invitrogen, Cergy-Pontoise, France) was previously added to collecting tubes.

**Histological Parameters.** Fixed intestinal tissues were embedded in paraffin wax blocks and 5- $\mu$ m sections were stained with hematoxylin-eosin. Sections were scored by the same pathologist blinded to the treatment allocation (NaCl or MTX). Epithelial necrosis, inflammatory cell infiltration, and exocytosis were assessed using semi-quantitative scores that ranged from 0 (no damage) to 3 (severe damage) for each parameter (villus atrophy, necrosis, inflammation, and exocytosis) (4). Villus height was measured on 10 well-oriented villi from each rodent using the analysis software Leica QWin (Leica Microsystems, Bensheim, Germany).

**Cell Proliferation.** The cell proliferation rate was evaluated by the incorporation of 60 mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, Saint-Quentin-Fallavier, France) injected intraperitoneally 1 hr before sacrifice. Immunohistochemistry was performed on fixed and paraffin-embedded tissue with BrdU monoclonal antibody (dilution 0.1, M0744; DakoCytomation, Trappes, France) according to a standard immunohistochemical method using a streptavidine-biotin/horseradish peroxidase detection system (5001, DakoCytomation). All samples were processed on a Techmate 500 automated immunostainer (DakoCytomation). BrdU detection was then scored from 0 (no labeling) to 3 (high level of labeling) by the same pathologist blinded to treatment.

**Evaluation of Intestinal Permeability.** One day before sacrifice, a bolus dose of 5 ml/kg/day Iodixanol (320 mg iodine/ml Visipaque; Amersham Health, Velizy, France) was administered by oral gavage to evaluate the intestinal permeability, as previously described (20). Urine samples were collected over the following 24 hrs and frozen at –20°C. Iodixanol was assessed in urine by high-performance liquid chromatography (20), and results were

**Table 1.** Sequences of Primers Used for Quantitative Polymerase Chain Reaction

| Genes       | Abbreviations | Entrez gene ID | Product size | Sequences (5' → 3')  |                      |
|-------------|---------------|----------------|--------------|----------------------|----------------------|
|             |               |                |              | Forward primers      | Reverse primers      |
| β-Actin     | ACTB          | 60             | 314 bp       | GCCCCTCTGAACCCCTAAG  | ATGTCACGCACGATTTC    |
| Cathepsin D | CSTD          | 1509           | 218 bp       | GTCTTGCTGCTCATTCTCGG | TGGGCATCCAGGTAGTTTTT |
| Calpain 1   | CAPN1         | 823            | 253 bp       | GCCCAACAAAGAGGGTGAC  | GCTGAAGCGTTAGTGCG    |
| Calpain 2   | CAPN2         | 824            | 187 bp       | CGAGACACTGCGGAACGAG  | GCGGGTAGCACCTCCAAT   |
| Ubiquitin C | UBC           | 7316           | 150 bp       | GAAGACCCTGACAGGCAAGA | ACACCTCCCCATCAAACCC  |

expressed as total amount of iodixanol excreted in urine per day.

**Intestinal Cytokine Concentrations.** Proteins were extracted from scraped jejunal mucosa by crushing in ice-cold PBS (400 µl/100 mg of tissue) containing 0.1% protease inhibitor cocktail (P8340; Sigma-Aldrich) and followed by 15 mins of centrifugation at 4°C and 3200 g. Supernatants were then collected and stored at -80°C. Intestinal cytokine concentrations were evaluated by specific ELISA assay (Quantikine; R&D Systems, Abington, UK) for interleukin (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α according to manufacturer's instructions. Assays in samples and standards were performed simultaneously. The optical density was read at 450 nm in a Σ960 photometer (Metertech, Taipei, Taiwan). Concentrations were expressed as picograms per milligram of total protein. Total protein concentrations were previously determined using Bradford assay (21).

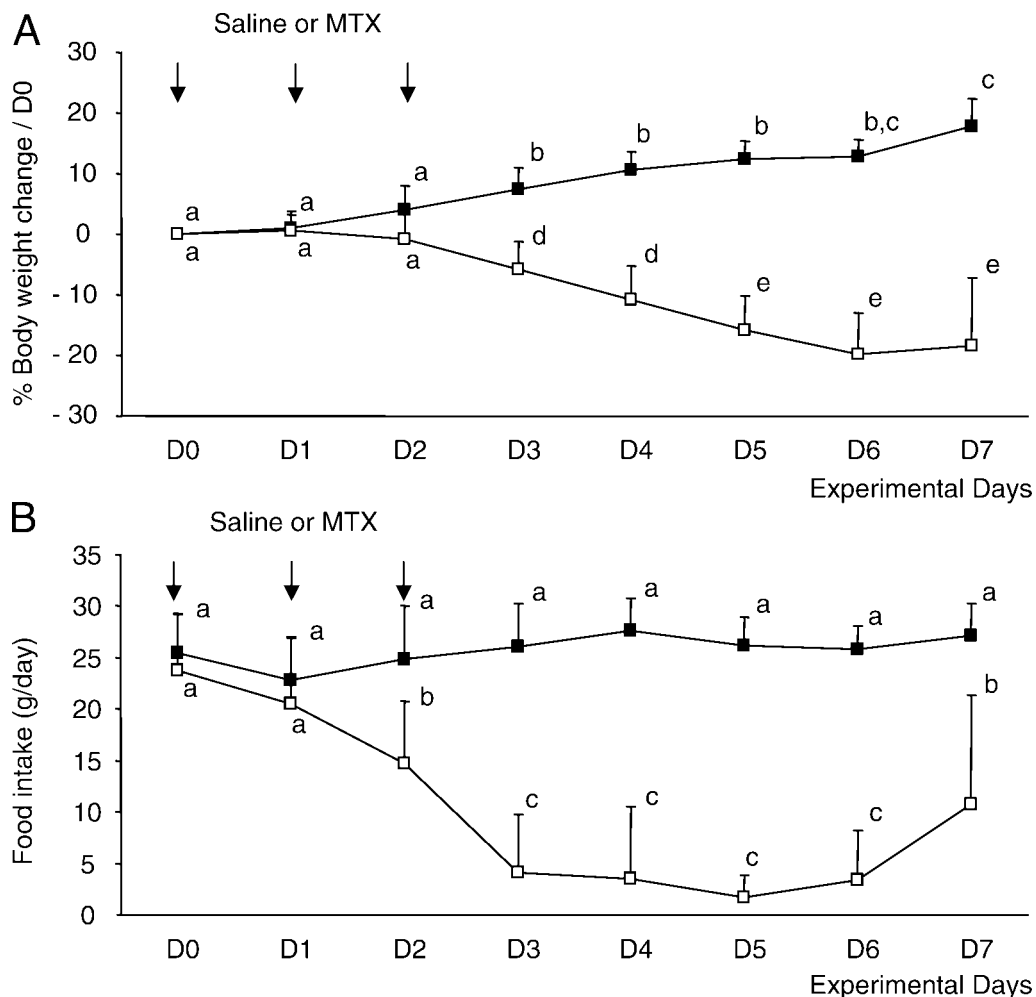
**GSH Content.** To evaluate oxidative stress, GSH content was assessed in the jejunum by a standardized spectrophotometric assay (22). Jejunal samples were crushed in ice-cold 5% perchloric acid solution (300 µl/100 mg tissue), permitting proteins to precipitate. After 15 min of centrifugation at 4°C and 1500 g, supernatants were collected and stored at -80°C. Fifty microliters of samples or standards (reduced GSH; Sigma-Aldrich) were placed in a 96-well microplate, and then 100 µl of reaction buffer (0.4 mM dinitrobenzoic acid, 0.4 mM reduced nicotinamide adenine dinucleotide phosphate, and 1.5 U/ml of GSH reductase) were added to each well. The microplate was placed in the Σ960 photometer microtiter plate reader and assessment was performed at 405 nm every 2 mins over a 20-min period. GSH content was expressed as micromoles per gram of tissue wet weight.

**Evaluation of Gene Expressions for Proteolytic Pathway Components by Real-Time Quantitative Polymerase Chain Reaction (qPCR) on LightCycler.** Proteolytic pathways were analyzed as previously described (23). Briefly, for each sample, total RNA from jejunal mucosa was extracted in cold TRIZOL Reagent. After reverse transcription of 1.5 µg total RNA into cDNA by using 200 U of SuperScript II Reverse Transcriptase (Invitrogen), qPCR was performed by SYBR Green technology on LightCycler automate (Roche Molecular

Biochemical, Mannheim, Germany) in duplicate for each sample. One microliter of template DNA (sample cDNA or purified PCR products) were amplified in a reaction volume of 10 µl by using transcript-specific primers (0.5 µM each) according to manufacturer's instructions (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemical). Specific primers (Table 1) were designed, checked by National Center for Biotechnology Information BLAST search, and synthesized (Invitrogen). β-Actin was the endogenous reference gene. Calpains 1 and 2, cathepsin D, and ubiquitin C were the genes of interest. The size of PCR products was checked on agarose gels. For relative quantification, previously purified and quantified PCR products for the different genes were used as external standards. Dilutions in series were used to generate standard curves. The copy numbers of mRNA for amplified genes were determinate from the standard curves with LightCycler software version 3.5 (24). To minimize variability between samples in both RNA quality and quantity, quantitative results are presented as the ratio between the absolute copy number for the different target genes and the absolute copy number of β-actin (25).

**Evaluation of Proteolytic Pathway Activities.** Scrapings of jejunal mucosa were extracted by crushing in ice-cold lysis buffer containing 30 mM Tris-HCl (pH 7.2), 1 mM dithiothreitol, and 1% Triton X-100, placed on ice for 15 mins and then centrifuged for 15 mins at 4°C and 6000 g. Lysosomal cathepsin D activity was quantified with InnoZyme Cathepsin D Immunocapture Activity Fluorogenic Assay Kit (Calbiochem, San Diego, CA), as previously described (24). Evaluation of calpains 1 and 2 and ubiquitin-proteasome system (chymotrypsin-like) activities were performed by spectrofluorimetry on a Mithras LB 940 microtiter plate fluorometer (Berthold Technologies, Bad Wildbad, Germany) using fluorogenic proteasome substrate, Suc-LLVY-MCA (PSIII; Calbiochem) in the presence or absence of specific inhibitors, as previously described (23). Activity values were expressed in relative fluorescence units (RFU)/µg of total proteins for cathepsin D activity and in arbitrary units for calpain and chymotrypsin-like proteasome activity.

**Calculation Methods and Statistical Analysis.** Statistical analysis was performed using StatView 5.0 software (SAS Institute, Cary, NC) and consisted of a two-way analysis of variance (ANOVA), with treatment (control or



**Figure 1.** Body weight changes and food intake of MTX-treated rats. (A) Variation of body weight and (B) food intake in control (closed squares) and in MTX-treated rats (open squares). Values are means  $\pm$  SD from 12 control and 18 MTX-treated rats for D0–D4 and 6 control and 9 MTX-treated rats for D5–D7. *P* values: (A) treatment,  $P < 0.0001$ ; day, not significant (NS); interaction,  $P < 0.0001$ ; (B) treatment,  $P < 0.0001$ ; day,  $P < 0.0001$ ; interaction,  $P < 0.0001$ . Values without a common letter differ (Bonferroni post tests).

MTX) and day (D4 or D7) as variables, followed by Bonferroni *post hoc* tests when interaction between treatment and day was significant. For daily monitored parameters, repeated measures two-way ANOVA was performed. For all tests,  $P < 0.05$  was considered significant.

## Results

**Body Weight and Food Intake.** In preliminary experiments, significant weight variations were observed for MTX-treated rats, reflecting a dose-dependent relationship (data not shown). In rats injected with 2.5 mg/kg (Fig. 1A), weight loss started on the third experimental day and was maximal on D6 (about  $-20\%$ ). In contrast, the weight of control rats increased (about  $+20\%$ ).

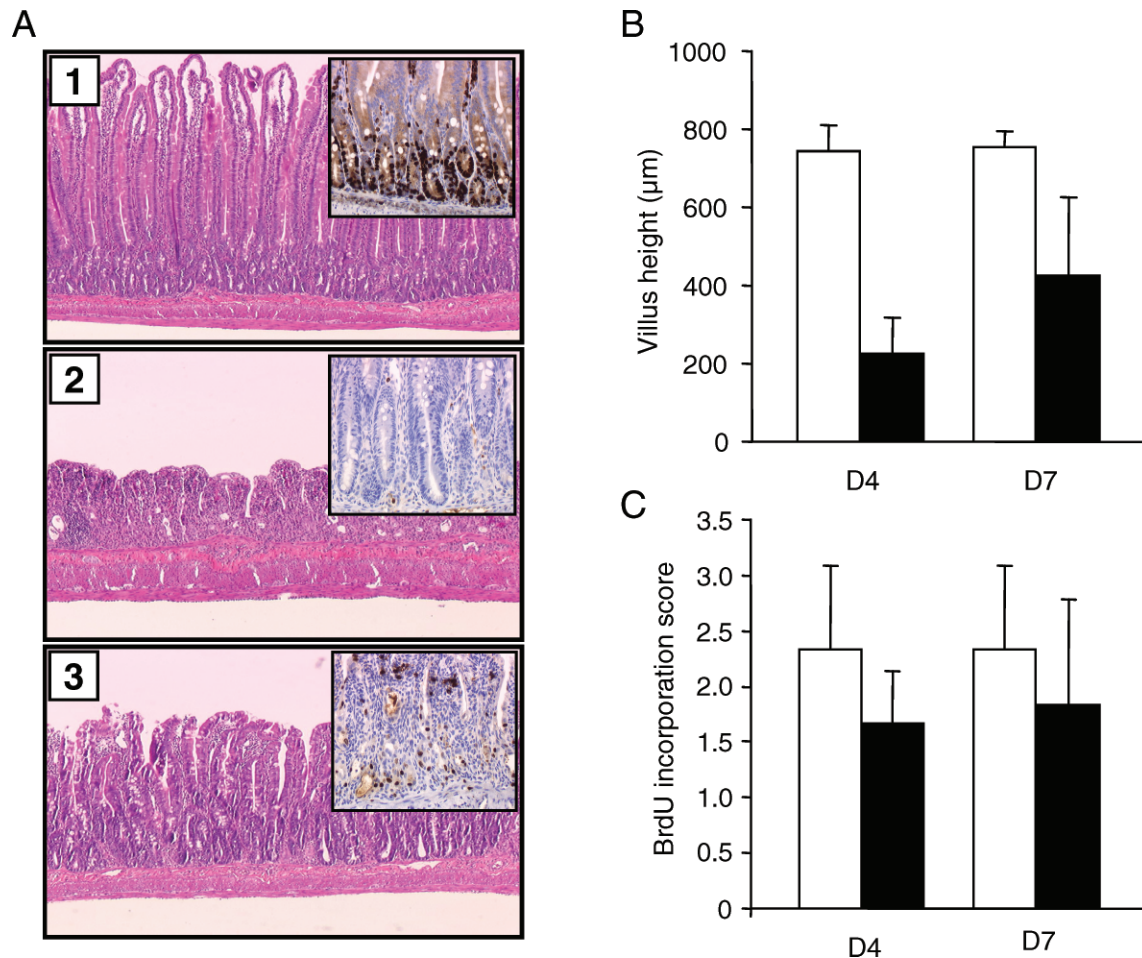
Food intake was similar between control and MTX-treated rats at D1 (Fig. 1B). In MTX-treated rats, a significant reduction of food intake was observed from D2 to D7 compared with control rats ( $P < 0.001$ ). At D7, food

intake of MTX-treated rats increased compared with D6 ( $P < 0.01$ ), but did not return to the level of control rats.

**Intestinal Morphometry, Cell Proliferation and Intestinal Permeability.** The 2.5 mg/kg MTX dose induced severe intestinal damage (Fig. 2A). MTX increased the histological score (in arbitrary units) for villus atrophy ( $1.9 \pm 0.9$  vs.  $0 \pm 0$ ), necrosis ( $1.4 \pm 1.1$  vs.  $0 \pm 0$ ), inflammation ( $1.7 \pm 0.5$  vs.  $0.2 \pm 0.4$ ) and exocytosis ( $1.1 \pm 0.7$  vs.  $0 \pm 0$ ). The resulting total histological score at D4 was  $6.2 \pm 3.0$  in MTX-treated rats versus  $0.2 \pm 0.4$  in control rats. At D7, the mucosa was partially restored to control level ( $3.3 \pm 2.7$ ). Each score was significantly affected by MTX ( $P < 0.01$  for treatment, not significant for day and interaction). Villus height (Fig. 2B) significantly decreased in MTX-treated rats ( $P < 0.001$ ) compared with control animals.

BrdU incorporation (Fig. 2) was lower in crypt cells of MTX-treated rats than in control rats at D4 and D7, but the difference only approached statistical significance ( $P = 0.06$ ).





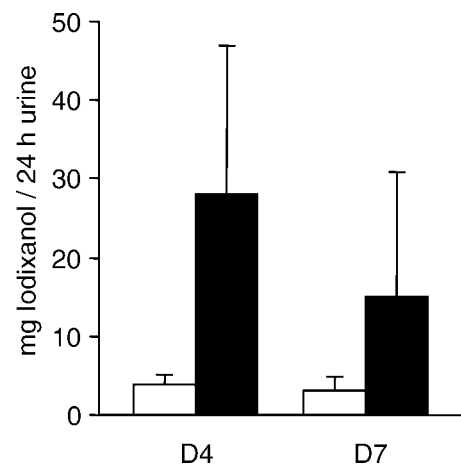
**Figure 2.** Histology, villus height, and cell proliferation in the jejunum of MTX-treated rats. (A) Jejunal mucosa (large picture, magnification:  $\times 5$ ) and BrdU incorporation (small picture in right corner, magnification:  $\times 20$ ) in control rats (1) and in MTX-treated rats at D4 (2) and at D7 (3). (B) Villus height of control (open bars) and MTX-treated rats (closed bars) at D4 and D7. *P* values: treatment,  $P < 0.0001$ ; day,  $P < 0.05$ ; interaction, NS. (C) BrdU incorporation score in crypt cells of control (open bars) and MTX-treated rats (closed bars) at D4 and D7. *P* values: treatment, NS; day, NS; interaction, NS. Values are means  $\pm$  SD.

Intestinal permeability was significantly increased in MTX-treated rats (7.4-fold at D4 and 4.8-fold at D7;  $P < 0.01$ ; Fig. 3).

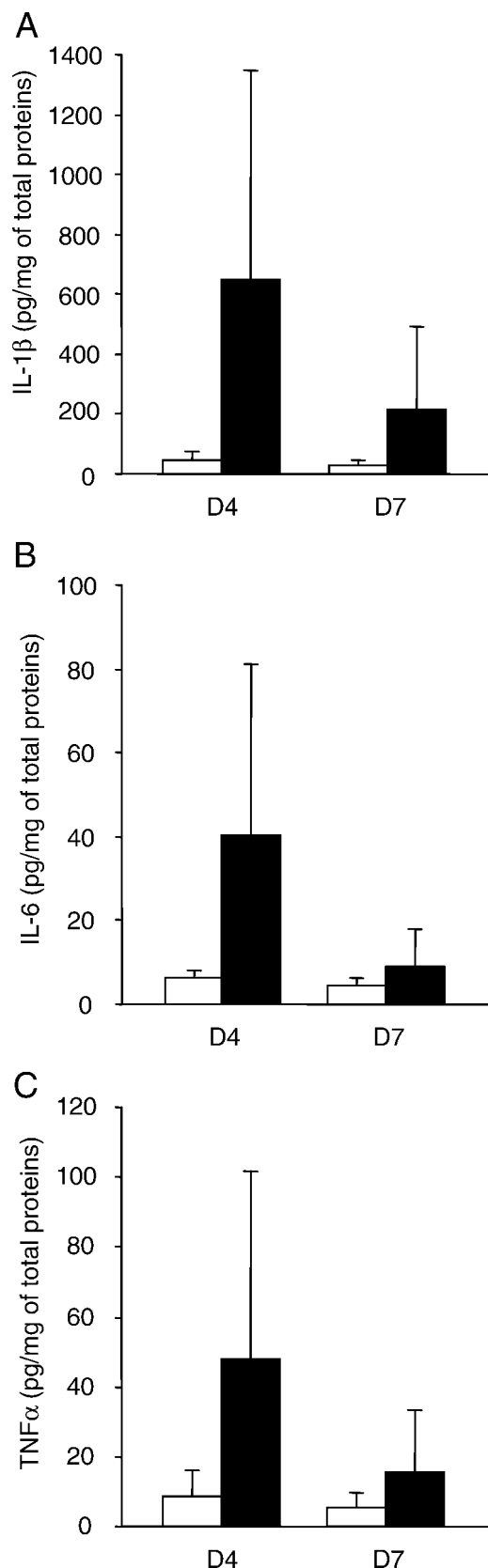
**Intestinal Cytokine Concentrations.** IL-1 $\beta$  and IL-6 concentrations (Fig. 4) increased in the jejunal mucosa of MTX-treated rats compared with control rats ( $P < 0.05$ ). Alteration of TNF- $\alpha$  concentration did not reach significance ( $P = 0.07$ ). IL-4, IL-10, and IFN- $\gamma$  remained undetectable in most jejunal samples (data not shown).

**Jejunal GSH Content.** At D4, GSH content markedly decreased in the jejunum of MTX-treated rats as compared with control rats:  $0.762 \pm 0.745$  versus  $2.25 \pm 0.708$   $\mu\text{mol/g}$  tissue ( $P < 0.05$ ). At D7, the depletion of jejunal GSH content was no longer considered significant ( $2.77 \pm 1.465$  vs.  $2.11 \pm 0.657$   $\mu\text{mol/g}$  tissue). GSH content in the liver was not affected by MTX treatment (data not shown).

**Jejunal Mucosa Protein Content.** The jejunal mucosa protein content decreased markedly with MTX ( $P$



**Figure 3.** Intestinal permeability of MTX-treated rats. Iodixanol urinary excretion over 24 hrs in control (open bars) and MTX-treated (closed bars) rats at D4 and D7 (means  $\pm$  SD). *P* values: treatment,  $P = 0.0025$ ; day, NS; interaction, NS.



**Figure 4.** Proinflammatory cytokine concentrations in jejunal mucosa of MTX-treated rats. (A) IL-1 $\beta$ , (B) IL-6, and (C) TNF- $\alpha$  jejunal concentrations in control (open bars) and MTX-treated rats (closed bars) at D4 and D7 (means  $\pm$  SD). *P* values: (A) treatment, *P* =

< 0.01) at D4 ( $54 \pm 5$  vs.  $105 \pm 8$  mg/g mucosa) and at D7 ( $84 \pm 10$  vs.  $115 \pm 12$  mg/g mucosa).

#### **Influence of MTX on Proteolytic Pathways.**

**Messenger RNA Levels of Proteolytic Pathway Components.** The mRNA level of cathepsin D, a component of the lysosomal pathway (Fig. 5A), was increased by MTX treatment (*P* < 0.05). In regard to the Ca<sup>2+</sup>-activated pathway, calpain 1 and calpain 2 mRNA levels markedly increased in MTX-treated rats (*P* < 0.01; Fig. 5B and C). For the proteasome system (Fig. 5D), the expression of ubiquitin C gene increased in MTX-treated rats at D4 (*P* < 0.01); however, calpain 2 and ubiquitin C mRNA levels of MTX-treated rats were not different from those of control rats at D7.

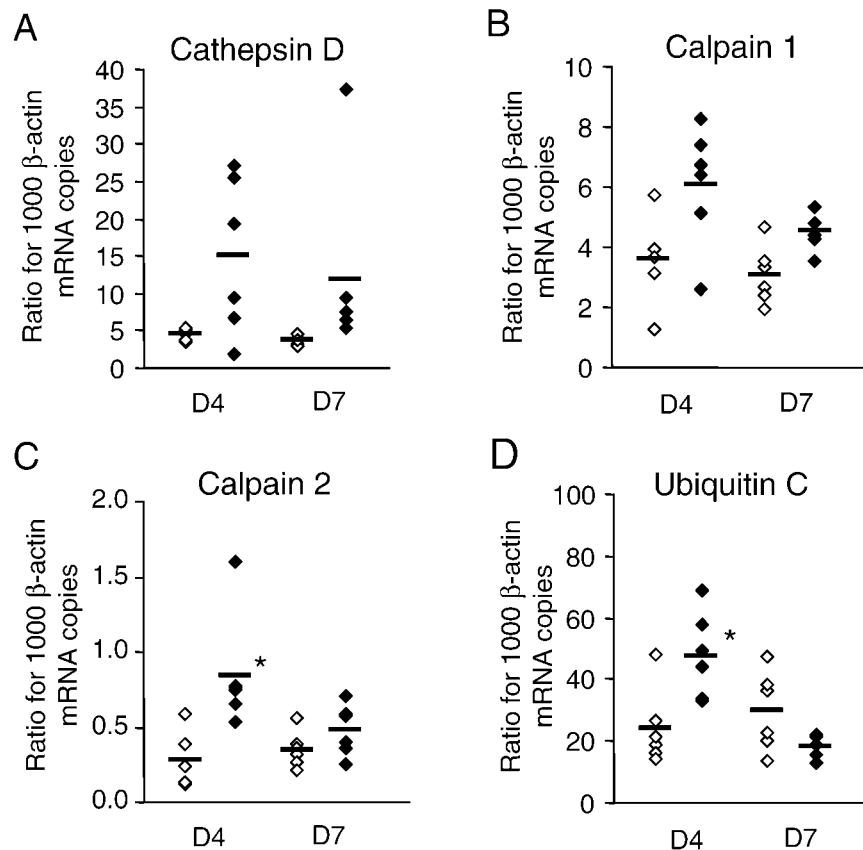
**Proteolytic Pathway Activity.** Cathepsin D-specific activity (Fig. 6A) markedly increased in MTX-treated rats at D4 as compared with control rats (*P* < 0.001), and returned to basal levels at D7. Specific calpain activities were not significantly affected in MTX-treated rats compared with control rats (Fig. 6B). The proteasome activity (Fig. 6C) markedly decreased in MTX-treated rats both at D4 and at D7 (*P* < 0.0001). In addition, cathepsin D activity was correlated with intestinal permeability (Pearson *r* = 0.53; *P* < 0.01) and inversely correlated with villus height (*r* = -0.73; *P* < 0.0001), taking into account the control and MTX groups. Cathepsin D activity was also inversely correlated with villus height in MTX-treated rats (*r* = -0.63; *P* < 0.01; Fig. 7).

#### **Discussion**

Mucositis is a severe side effect of chemotherapy, which may cause interruption of treatment. Thus, a better understanding of its pathophysiological mechanisms is required to develop adapted therapeutic strategies. We investigated, for the first time to our knowledge, the alterations in the proteolytic pathways during chemotherapy-induced mucositis.

The present work was performed on a well-defined experimental model using the standard antimitotic agent, MTX. The dose of 2.5 mg/kg/day over 3 consecutive days was chosen as a good compromise between the lack of mucositis at 1 mg/kg/day and the major early mortality at 5 mg/kg/day. Using this selected dose, anorexia and body weight loss were maximal between D5 and D6, while severe intestinal damage and barrier dysfunction were observed at D4, with a partial recovery at D7. These histological findings and observed intestinal permeability modifications are in accord with data from previous studies (4, 26). We also observed a trend for a lower crypt cell proliferation in MTX-treated rats than in control rats, both at D4 and at D7. It was previously reported that the preservation of cell proliferation capacity is a key factor in protecting the

$\chi^2$  = 0.0282; day, NS; interaction, NS; (B) treatment, *P* = 0.0125; day, *P* = 0.0353; interaction, NS; (C) treatment, NS; day, NS; interaction, NS.



**Figure 5.** Messenger RNA level for proteolytic pathway components in jejunal mucosa of MTX-treated rats. (A) Cathepsin D, (B) calpain 1, (C) calpain 2, and (D) ubiquitin C mRNA levels in jejunal mucosa of control (open symbols) and MTX-treated (closed symbols) rats at D4 and D7. Values are expressed as ratio of  $\beta$ -actin mRNA level and presented as open and closed symbols for individual data and a bar for mean. *P* values: (A) treatment,  $P = 0.0115$ ; day, NS; interaction, NS; (B) treatment,  $P = 0.002$ ; day, NS; interaction, NS; (C) treatment,  $P = 0.0018$ ; day, NS; interaction,  $P = 0.0376$ . \*  $P < 0.01$  vs. controls using Bonferroni posttests. (D) Treatment, NS; Day,  $P = 0.0185$ ; Interaction,  $P = 0.0013$ . \*  $P < 0.01$  vs. controls using Bonferroni posttests.

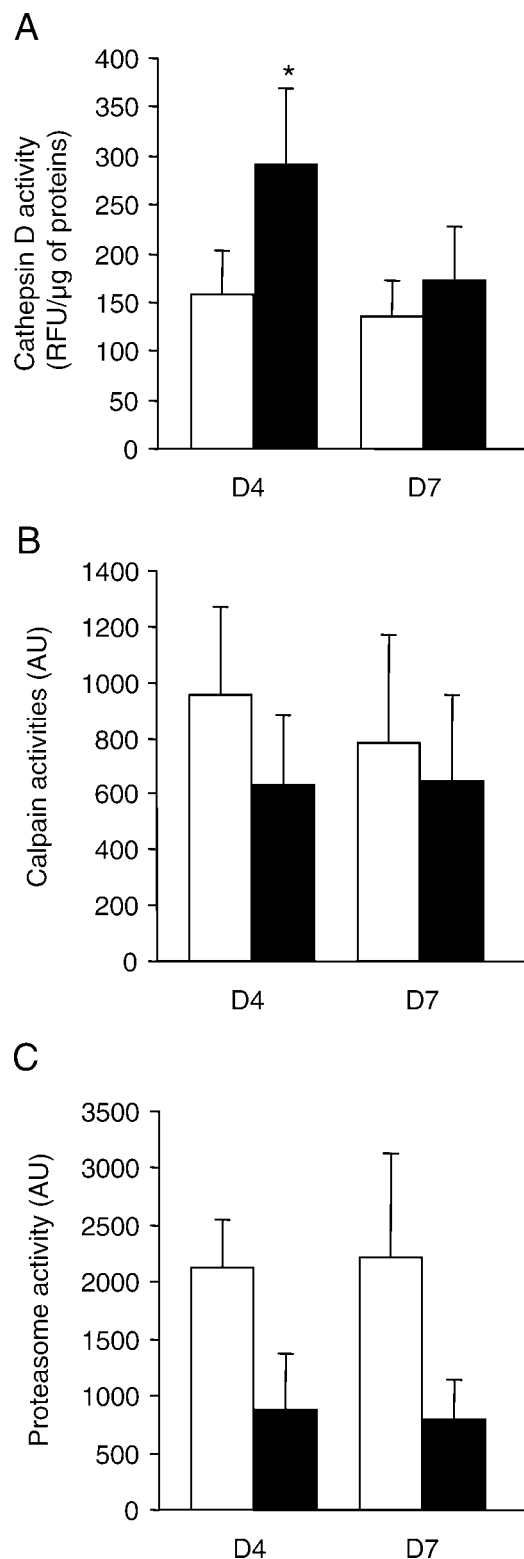
epithelium from the consequences of MTX-induced apoptosis (27). In fact, the Peyer's patches epithelium was reported to be markedly preserved from MTX injury as compared with surrounding epithelium (27). These authors related this protection to the maintenance of goblet cell function (i.e., maintenance of secretion of several trophic factors, such as trefoil factor 3 or the main type of mucins coded by Muc2, Ref. 27). In humans, intestinal hypoproliferation was maximal 3 days after chemotherapy compared with pretreatment values, and was involved in mucositis induction (7); however, other mechanisms may be implicated (i.e., proteolysis).

In most cells, proteolysis results from the activities of three main enzymatic systems: the lysosomal,  $\text{Ca}^{2+}$ -activated, and proteasome pathways. Data concerning gut proteolysis still remain very limited (23), with the exception of studies in tumorous tissues (28). In the present work, several alterations of the proteolytic pathways were observed in response to MTX treatment. Although calpain mRNA expression increased, its activity remained roughly unaffected, or even tended to decrease (treatment effect:  $P = 0.0707$ ). This may reflect a transient decline of intra-

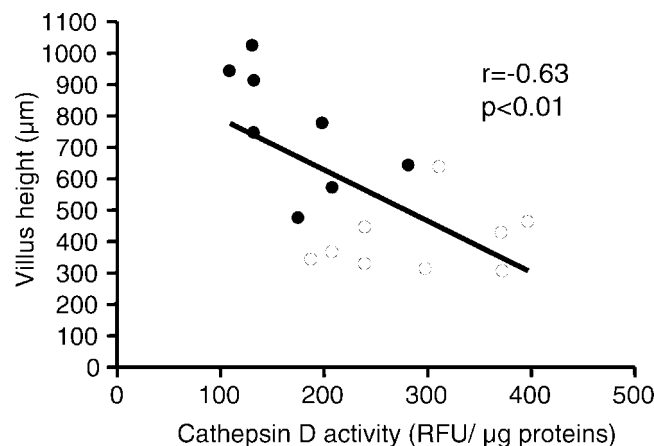
cytoplasmic ionized calcium concentration, inhibiting the activity, and a secondary adaptative increase of gene expression.

MTX treatment increased the mRNA expression and the activity of cathepsin D, both of which were restored to control levels at D7. This marked increase of the cathepsin D activity may reflect an enhanced activation of other lysosomal cathepsins, since cathepsin D activates other cathepsins (e.g., cathepsins B or L) (29). As we reported here, mucosal atrophy and intestinal permeability were correlated to cathepsin D activity, indicating the potential role of lysosomal pathway in MTX-induced damage. Activation of lysosomal pathway may not be only a direct effect of MTX treatment, but also a consequence of nutrient deprivation, as previously described (30). Extracellular release of cathepsins could play a key role both in the loss of intestinal protein content and in tissue necrosis through the digestion of extracellular matrix (31).

In contrast to cathepsin D activity, chymotrypsin-like proteasome activity was decreased by MTX treatment. In the intestinal epithelial cell line, HCT-8, we observed that IFN- $\gamma$  induced an increase of proteasome activity, which



**Figure 6.** Proteolytic pathway activities in jejunal mucosa of MTX-treated rats. (A) Cathepsin D, (B) Calpain, and (C) chymotrypsin-like proteasome activities in jejunal mucosa of control (open bars) and MTX-treated (closed bars) rats at D4 and D7 (means  $\pm$  SD). *P* values: (A) treatment,  $P = 0.0008$ ; day,  $P = 0.004$ ; interaction,  $P = 0.0353$ . \*  $P < 0.001$  vs. controls using Bonferroni post tests; (B) treatment, NS; day, NS; interaction, NS; (C) treatment,  $P < 0.0001$ ; day, NS; interaction, NS.



**Figure 7.** Correlation between cathepsin D activity and villus height in MTX-treated rats. Villus height ( $\mu\text{m}$ ) is represented as a function of jejunal cathepsin D activity in MTX-treated rats euthanized at D4 (open circles) or D7 (closed circles). Pearson  $r = -0.69$ ;  $P < 0.01$ .

was due to the strong activation of the immunoproteasome (23). In the present study, IFN- $\gamma$  was undetectable, IL-1 $\beta$  increased, and TNF- $\alpha$  tended to increase, but these cytokines did not affect the proteasome activities in our previous study. Thus, other regulatory compounds that remain to be identified may account for the observed inhibition of proteasome activity after MTX treatment. One contributing factor might be a large energy deprivation of the remaining mucosa, since proteolysis through the ubiquitin-proteasome system is highly ATP dependent and food intake was markedly reduced at D4. Moreover, the inflammatory process downregulates the intracellular uptake of enterocyte energy substrates, such as glucose or glutamine (32). In contrast, MTX could directly affect the proteasome activity, as previously reported for other chemotherapeutic drugs (33). Finally, the decrease of proteasome proteolytic activities could also be considered a protective process to restore protein content, but this hypothesis requires further investigation (34).

Therefore, our data suggest that proteolysis alterations are involved in the underlying mechanisms of mucositis, and also suggest that the main proteolytic pathway activated by chemotherapy treatment is lysosomal. In addition, we observed that MTX affected GSH jejunal content. At D4, MTX treatment induced a marked decrease of GSH content, as previously reported (17). This reduction of GSH content has also been reported in some models of chemically induced colitis (35), but not in all models (36). In humans, we previously reported that GSH content was reduced in the colonic mucosa of patients with Crohn's disease, the lower levels of GSH being observed in malnourished patients (37). The decrease of GSH after inflammatory injury may reflect both the increased loss of oxidized GSH, which is lost in the lumen, and a reduced capacity of GSH synthesis due to a reduction in the supply of precursors. The depletion of GSH is also detrimental to intestinal cell preservation or restoration, as GSH content is related to the protection



against apoptosis (38). GSH depletion and resulting oxidative stress also contribute to NF- $\kappa$ B inflammatory pathway activation (39) and intestinal inflammation enhancement.

In conclusion, after MTX injection in rats, we observed a gut barrier injury characterized by histological damage, increased intestinal permeability, glutathione depletion, and high mucosal concentrations of proinflammatory cytokines. Interestingly, we have demonstrated alterations of proteolytic pathways, especially enhanced lysosomal cathepsin D activity and inhibited proteasome activity. Further investigation is warranted to explain which particular mechanisms are involved in these proteolytic alterations. Food deprivation, inflammatory response, or the direct effects of MTX could be implicated. For instance, comparison of MTX-treated to pair-fed control rats might contribute to distinguish the effects of MTX from those of food intake reduction. In addition, these alterations may contribute to the toxic side effect of chemotherapy treatments and, consequently, to treatment reduction or withdrawal, as we observed a correlation between cathepsin D activity and mucosal atrophy. To prevent or limit mucositis, the use of an integrated nutritional and/or drug approach targeting several of these metabolic perturbations should be evaluated.

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