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ORIGINAL ARTICLE

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The effect of cytostatic drug treatment on intestine-specific transcription factors Cdx2, GATA-4 and HNF-1α in mice

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Abstract Chemotherapy-induced intestinal damage is a very important dose-limiting side effect for which there is no definitive prophylaxis or treatment. This is in part due to the lack of understanding of its pathophysiology and impact on intestinal differentiation. The objective of this study was to investigate the gene expression of the small intestinal transcription factors HNF-1α, Cdx2, GATA-4 in an experimental model of methotrexate (MTX)-induced intestinal damage, and to correlate these alterations with histological damage, epithelial proliferation and differentiation. HNF-1α, Cdx2 and GATA-4 are critical transcription factors in epithelial differentiation, and in combination they act as promoting factors of the sucrase–isomaltase (SI) gene, an enterocyte-specific differentiation marker which is distinctly downregulated after MTX treatment. Mice received two doses of MTX i.v. on two consecutive days and were sacrificed 1, 3 and 7 or 9 days after final injection. Segments of the jejunum were taken for morphological, immunohistochemical and quantitative analyses. Intestinal damage was most severe at day 3 and was associated with decreased expression of the transcriptional factors HNF-1α, Cdx2 and GATA-4, which correlated well with decreased expression of SI, and seemed inversely correlated with enhanced proliferation of epithelial crypt cells. During severe damage, the epithelium was preferentially concerned with proliferation rather than differentiation, most likely in order to restore the small intestinal barrier function rather than maintaining its absorptive function. Since HNF-1α, Cdx2 and GATA-4 are critical for intestine-specific gene expression and therefore crucial in epithelial differentiation, these results may explain, at least in part, why intestinal differentiation is compromised during MTX treatment.

Keywords Mucositis · Epithelium · Sucrase–isomaltase · Differentiation · Proliferation

Abbreviations Cdx2: Caudal-related homeobox transcription factor · HNF-1α: Hepatocyte nuclear factor-1 alpha · IHC: Immunohistochemistry · MTX: Methotrexate · SI: Sucrase–isomaltase

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Introduction

One of the most severe side effects of chemotherapy is damage of the gastrointestinal tract often referred to as mucositis. Especially, the epithelium in the small intestine is extremely sensitive to cytostatic drug treatment [18], since it is proliferating rapidly. The loss of intestinal epithelial integrity causes symptoms of malabsorption, like pain and diarrhea, and an enhanced risk on septic bacteraemia. Mucositis is therefore a serious side effect that is often dose-limiting [24], and influences the quality of life. It is estimated that this disorder affects around 500,000 patients worldwide annually [6]. Despite a great

amount of research, there is currently no definitive prophylaxis or treatment for this chemotherapy-induced side effect. This is in part due to the lack of understanding of its pathophysiology.

Methotrexate (MTX) is frequently used in the treatment of leukemia, osteosarcoma and other malignancies. Especially in childhood, cancer patients receiving high dose treatments, MTX is known to cause this severe side effect [17]. MTX is a folate antagonist and a strong inhibitor of dihydrofolate reductase (DHFR), which is a key enzyme in the thymidylate cycle. Because of an arrest in thymidylate biosynthesis, the replication of DNA will be inhibited. Therefore, in the small intestine MTX primarily targets the proliferating cells in the crypts of Lieberkühn [12].

In animal models, the effects of MTX on the small intestinal epithelium in time are well described and characterized by an inhibition of proliferation, loss of crypts, flattening of the epithelium and atrophy of the villi [18, 26, 33]. We along with others demonstrated a down-regulation of enterocyte-specific gene expression crucial for degradation and absorption of nutrients [16, 26, 33, 34]. Goblet and Paneth cells seem to be selectively spared [33]. Furthermore, the epithelium surrounding the Peyers' patches also seems to be spared from MTX-induced damage, whereas isolation stress seems to aggravate the intestinal damage [32].

At present, the molecular mechanisms underlying the dynamic processes of intestine-specific gene expression, cell fate determination, cellular differentiation and intestinal development are poorly understood. However, the influence of specific transcription factors is beginning to be unraveled. The epithelial specific transcription factors Cdx2, GATA-4 and HNF-1α are important for intestinal development, differentiation and gene expression. Cdx2, a member of a homeobox gene family related to Drosophila caudal, is expressed in all epithelial cells in the small and large intestine of the adult mouse [5, 9]. Cdx2 modulates proliferation, apoptosis, cell-adhesion and columnar morphology [1, 20, 25]. Cdx2 is also necessary for the expression of a number of intestine-specific genes [1, 2, 14, 29]. By targeting these processes and genes, Cdx2 promotes the appearance of a mature intestinal cell phenotype. GATA-4, a member of the GATA gene family, is expressed in heart, ovary, testis, lung, liver and the small intestine, but not the colon [15]. GATA-4 is important for development since GATA-4 knockout mice die at embryonic day 9.5 lacking a primitive heart and foregut. Hepatocyte nuclear factor-1 alpha (HNF-1α) is a homeodomain-containing transcription factor that plays an important role in regulation of gene expression in pancreatic beta-cells, intestine, kidney and liver [22]. In combination Cdx2, GATA-4 and HNF-1α have been shown to be crucial for developmentally regulated expression of the sucrase–isomaltase (SI) gene [2] as several other enterocyte-specific markers [2, 5, 7, 8].

Sucrase–isomaltase is a brush border enzyme with an important function in degradation of disaccharides [30] and is specifically expressed by enterocytes in a differentiation-specific pattern and therefore is a widely used marker for intestinal differentiation [21]. SI is almost undetectable at birth and increases to adult levels during the suckling–weaning transition. Its expression is characterized by a strong expression at the crypt–villus junction and mid-villus, and a decreased intensity towards the tips of the villi [21]. The expression of SI is affected by MTX-induced intestinal damage [19, 34].

Cells respond to external stimuli by changes in gene expression, which are largely dependent on transcription factors. In this study we investigated the role of the SI gene transcriptional activators, Cdx2, GATA-4 and HNF-1α, during MTX-induced intestinal damage and repair.

Materials and methods

Animals

Animal experiments were performed with permission of the Animal Ethics Committee of the Erasmus MC-Sophia. Upon arrival at our institute, 10-week-old male BALB/c mice (Charles River, Les Oncins, France) were housed individually in micro-isolator cages under specific pathogen-free conditions with free access to a standard palletized diet (Hope farms, Woerden, The Netherlands) and water. After 1 week of adjustment to the new environment, MTX [Emthrexate (PF) Pharma Chemie, Haarlem, The Netherlands] was injected intravenously. To determine the optimal MTX dose to induce severe intestinal damage and allow regeneration within 7–9 days, a dose–response curve was performed with increasing concentrations of MTX (data not shown), ranging from 20 mg/kg in a single dose (which induced mild intestinal damage) to a final concentration of 120 and 60 mg/kg on two subsequent days. The latter dose was chosen in the studies described here.

At day 1 and 0 mice were injected with 120 and 60 mg/kg, respectively. Controls were given equivalent volumes of 0.9% NaCl. Mice were sacrificed at day 1, 3 and 7 or 9 after final MTX injection. Day 1 represents the early phase of the induced intestinal damage, day 3 the phase of severe intestinal damage, day 7 and 9 both represent the regenerative phase in which day 7 is considered as the middle and day 9 the end of intestinal regeneration. Histological, immunohistochemical and protein data were derived from intestinal segments collected at day 9. The quantification of PCNA, HNF-1α, Cdx2 and GATA-4 were performed on nuclear extracts collected at day 7. Per time-point 4–6 MTX-treated animals and 2 control animals were sacrificed. One hour before sacrifice the mice were injected with 120 μl 10 mg/ml 5-bromo-2′deoxyuridine (BrdU), a uridine analog, to locate the proliferating cells. Segments of mid-jejunum were collected and either processed immediately for histological analysis or snap-frozen in liquid nitrogen for storage at −80°C and subsequent protein isolation.

Histochemistry

Five millimeter segments of mid-jejunum were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated and embedded in Paraplast Plus (Sherwood Medical, Den Bosch, The Netherlands) as previously described [33]. Four micrometer sections were routinely stained with hematoxylin (Vector Laboratories, Burlingame, CA, USA) and eosin (Sigma-Aldrich, Zwijndrecht, The Netherlands) to study morphological alterations of the crypts and villi. Immunohistochemistry (IHC) was performed as described previously [33] with some minor modifications. The sections for BrdU staining required an extra adjustment to this protocol of HCl incubation, washing with borate buffer and pepsin treatment, as described before [19]. In short, sections were blocked as described and incubated overnight with the following antibodies diluted in 1% BSA, 0.1% Triton X-100 in PBS: mouse monoclonal anti-BrdU (1:250, Roche Applied Sciences, Indianapolis, IN, USA), goat polyclonal anti-mouse GATA-4 (1:2,500, sc-1237X, Santa Cruz Biotechnology), goat polyclonal anti-human HNF-1α (1:2,500, sc-6547X, Santa Cruz Biotechnology). For the enterocyte marker SI and transcription factor Cdx2 sections were blocked for 30 min with 1% blocking reagent (Roche, Almere, The Netherlands) in PBS and incubated overnight with rabbit polyclonal anti-rat SI (1:9,000 in PBS, kindly provided by Yeh [35]), or rabbit polyclonal anti-mouse Cdx2 (1:500 in PBS [20]). Immunoreactions were detected using Vectastain ABC Elite Kit (Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, The Netherlands).

Protein dot blotting

The expression of enterocyte markers was detected and quantified as described previously [34]. Briefly, 5 mm segments of the mid-jejunum were homogenized, protein concentration was measured and 50 μg protein of each homogenate was dot-blotted on nitrocellulose (Protran BA83, 0.2 μm; Schleicher & Schuell, Dassel, Germany). Hereafter blots were blocked for 1 h with blocking buffer containing 50 mM Tris, pH 7.8, 5% (wt/vol) nonfat dry milk powder (Campina Melkunie, Eindhoven, The Netherlands), 2 mM $CaCl_2$, 0.05% (vol/vol) Nonidet P40® (BDH, Brunschwig Chemie, Amsterdam, The Netherlands) and 0.01% Antifoam B (Sigma-Aldrich, Zwijndrecht, The Netherlands). Blots were incubated overnight at 4°C with rabbit polyclonal anti-rat SI (1:1,000 [35]) diluted in blocking buffer. After washing with blocking buffer blots were incubated with ^{125}I-labeled protein A (specific activity 30 mCi/mg, Amersham Biosciences, Roosendaal, The Netherlands) for 2 h at room temperature. Specific binding of ^{125}I-labeled protein A to the enterocyte marker antibodies was measured using PhosphorImager detection. The elicited signal was quantified by ImageQuant software (Molecular Dynamics, B&L systems, Zoetermeer, The Netherlands) and the expression of the enterocyte markers was expressed per microgram protein.

Western blot analysis nuclear extracts

Nuclear extracts were prepared as described by Van Seuningen et al. [31] with minor modifications. Immediately after isolation of the intestine, the epithelial cell layer was scraped off from an 8 cm segment of the proximal jejunum and suspended in freshly prepared lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μM sodium molybdate, 10 mM β-glycerophosphate, 10 mM Na-fluoride, 100 μM Na-orthovanadate, 13.84 mg/ml *p*-nitro-phenylphosphate. After centrifugation for 1 min at 9,000 rpm at 4°C the supernatant was discarded and the pellet resuspended in 150 μl lysis buffer plus 0.1% NP-40 and gently homogenized on ice, vortexed and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant containing the cytosol fraction was diluted with 225 μl dilution buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF and stored at −80°C. The obtained pellet was resuspended in 125 μl extraction buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μM sodium molybdate, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 100 μM sodium orthovanadate, 13.84 mg/ml *p*-nitro-phenylphosphate. The suspension was gently homogenized, vortexed and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant containing the nuclear fraction was diluted with 187.5 μl dilution buffer, centrifuged for 5 min at 14,000 rpm at 4°C, transferred to a new tube and stored at −80°C until further processing. Protein concentration was measured using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) following the manufacturer's protocol.

Twenty microgram of nuclear extract was loaded per lane and run on a 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Protran BA83, 0.2 μm; Schleicher & Schuell) and blocked for 1 h at room temperature in blocking buffer containing 50 mM Tris, pH 7.8, 5% (wt/vol) nonfat dry milk powder (Campina Melkunie, Eindhoven, The Netherlands), 2 mM $CaCl_2$, 0.01% Antifoam B (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.05% Triton X-100. Blots were incubated overnight at 4°C with primary antibodies diluted in blocking buffer: goat polyclonal anti-mouse GATA-4 (1:5,000, sc-1237X, Santa Cruz Biotechnology), goat polyclonal anti-human HNF-1α (1:5,000, sc-6547X, Santa Cruz Biotechnology), mouse monoclonal anti-human Cdx2 (1:1,000, BioGenex, San Ramon, CA, USA), or mouse monoclonal anti-human PCNA, clone PC10 (1:250, Novo Castra

Laboratories, Newcastle upon Tyne, UK). After washing with PBS, 0.2% Tween-20 blots bound antibodies were revealed using horseradish peroxidase (HRP) conjugated goat anti-mouse or rabbit anti-goat (1:1,000) and SuperSignal® West Femto Luminol Enhancer kit (Pierce, Rockford, IL, USA). The signal was detected and quantified by the ChemiGenius gel documentation system (Syngene, Cambridge, UK). If necessary blots were stripped by incubating twice for 30 min with 0.2 M glycine, pH 2.2, 0.1% SDS, 1% Tween-20. TCF4, [mouse monoclonal anti-human TCF4, clone 6H5-3 (1:250, Upstate, Waltham, MA, USA)] was used as internal control in the Western blot analysis since the expression of TCF4 was preserved during MTX treatment.

Statistical analysis

Changes in protein expression levels during damage and regeneration were statistically analyzed using the Kruskal–Wallis *H* test followed by the Mann–Whitney *U* test. A $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

Results

MTX damages the epithelial structure and function of the small intestinal epithelium

To ascertain the effect of cytostatic drugs on small intestinal morphology, epithelial proliferation, differentiation and function, we developed a mouse model. The mice were injected i.v. at day 1 with 120 mg/kg MTX and at day 0 with 60 mg/kg MTX, creating a window in the MTX administration similar to the previously described rat MTX model [32, 33]. In this model we examined the MTX-induced intestinal damage in an early phase at day 1 and in a more severe phase at day 3. The subsequent regeneration of the intestinal epithelium was studied at day 7 or 9. Days 7 and 9 represent the regenerative phase in which day 7 is considered as mid-regenerative phase and day 9 as the end of the regenerative phase. The histological, immunohistochemical and protein data were collected at day 9. Data derived from nuclear extracts were obtained at day 7. Histological evaluation of the jejunum revealed progressive loss of structure characterized by flattening of villus and crypt epithelial cells, progressive villus atrophy, crypt loss and crypt abscesses, starting at day 1 (Fig. 1). Intestinal damage was most severe at day 3, thereafter the jejunal epithelium regenerated and was virtually normal at day 9.

To gain insight in the expression of an intestine-specific brush border enzyme after MTX-treatment, a SI immunohistochemical staining was performed. At day 1 SI was expressed at the brush border along the whole villi, which was comparable with the expression of SI in the control epithelium (Fig. 2a, b). SI expression was virtually absent at day 3, when morphological damage was most severe (Fig. 2c). Only weak staining was still detectable on small parts of the villi where the epithelium was less affected. During the regenerative phase at day 9 the expression of SI was back to the control level again (Fig. 2d).

To quantify these observed histological changes, protein dot blotting analyses were performed using an enterocyte-specific SI antibody (Fig. 3). This revealed in essence the same results as the immunohistological data. The SI protein expression at day 1 was similar to the control situation. The expression decreased significantly at day 3, concomitant with the most severe epithelial damage and returned to control level at day 9.

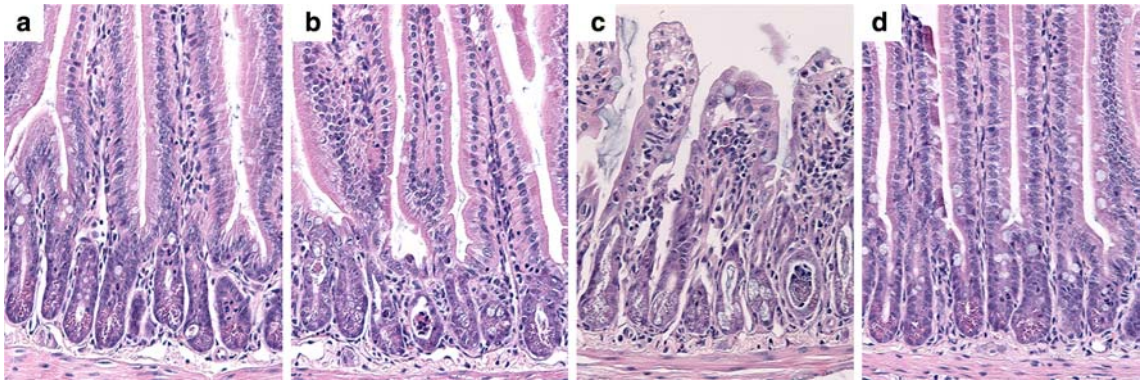

Fig. 1 Morphology of the murine small intestine after MTX treatment. BALB/c mice were treated with 120 and 60 mg/kg MTX i.v. at day 1 and 0. Segments of the small intestine were collected at day 1, representing early damage; at day 3, representing severe intestinal damage; and at day 7 or 9, representing the regenerative phase. The intestinal segments were stained with hematoxilin and eosin for morphological evaluation. Morphology of the jejunum of control mouse (**a**), at day 1 (**b**), day 3 (**c**) and day 9 (**d**). The morphology was mildly affected at day 1 (**b**), characterized by mild flattening of crypt epithelium and development of a few crypt-abcesses. At day 3 (**c**) the morphology was severely affected: extensive flattening of crypt epithelium, villus atrophy and progressive deterioration of the lamina propria. At day 9 (**d**) the morphology was virtually normal again. These photographs are representative examples of a group of four to six animals per time-point

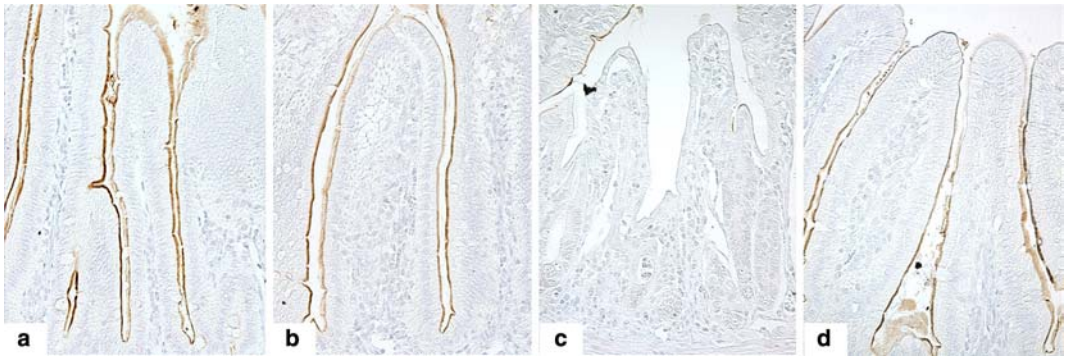

Fig. 2 Effect of MTX treatment on the enterocyte-specific sucrase–isomaltase (*SI*) expression in the small intestinal epithelium. SI expression in the jejunum of a control mouse (**a**) and mice at day 1 (**b**), 3 (**c**) and 9 (**d**), detected by immunohistochemistry. Brush border staining of SI was readily detectable in the control mouse (**a**) at day 1 (**b**), was virtually absent at day 3 (**c**) and was normal again at day 9 (**d**)

Increase of epithelial proliferation during severe MTX-induced damage

Loss of epithelial structure and SI expression were most pronounced at day 3. To determine the rate of epithelial proliferation during the period of MTX-induced damage and subsequent regeneration, two proliferation assays were performed. Proliferation was localized by detection of incorporated BrdU, injected 1 h before the mice were sacrificed, and quantified by determining the expression of the proliferation cell nuclear antigen (PCNA) in jejunal epithelium at several time-points similar as described previously [3, 28]. At day 1, BrdU incorporation was localized to the lower part of the crypts, virtually the same as in the control situation (Fig. 4a). The epithelial proliferation zone increased dramatically at day 3 and was localized almost along the entire length of the crypts. During the regenerative phase at day 9 proliferation decreased again, but was still slightly higher than control.

The quantitative PCNA data showed similar changes in the intestinal proliferation after MTX treatment (Fig. 4b). PCNA expression decreased to 65% of control level at day 1, but this decrease did not reach statistical significance. PCNA expression increased significantly (almost sixfold) at day 3, a time-point at which morphological damage was most severe. During regeneration at day 7 epithelial PCNA expression decreased, but was still elevated in comparison to the PCNA expression in the control situation. In Fig. 4c, a representative Western blot demonstrating the expression of PCNA and the internal control TCF-4 are shown.

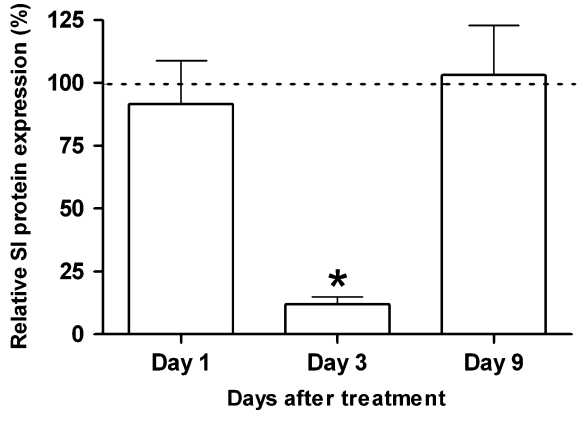

Fig. 3 The relative SI protein expression of the small intestine. Protein expression was quantified by protein dot blotting analysis using a specific polyclonal SI antiserum. The SI protein expression of the control mice was set at 100%. SI expression remained almost stable at day 1, but decreased significantly at day 3. The SI expression returned to normal levels at day 9. $*P < 0.05$ day 3 versus control

Down-regulation of the intestine-specific transcription factors

The intestine-specific transcription factors Cdx2, GATA-4 and HNF-1α are important in intestinal development, differentiation and morphogenesis. Moreover, the combination of these three transcription factors specifically activate the transcription of SI [2]. Therefore, the current study was extended to determine whether the down-regulation of intestinal differentiation illustrated by the decrease in SI expression was preceded by alterations in expression of these three transcription factors. To study the effect of MTX on the localization and expression of these three specific transcription factors, immunohistochemical staining was performed on sections of the collected intestine. All three transcription factors were detectable in the nuclei of jejunal epithelial cells along the crypt–villus axes (Fig. 5). Staining was not detectable in the Paneth cells at the bottom of the crypts. At day 1 the expression of Cdx2, GATA-4 and HNF-1α was similar to the control situation. At day 3 the expression of these transcription factors was virtually absent in the crypt cells and the number of villus cells expressing them was drastically decreased (Fig. 5). The nuclear expression of Cdx2 completely disappeared,

however, there was some cytoplasmatic staining of Cdx2 visible at the base of the villi. At day 9 expression and localization of all three transcription factors were similar to control (data not shown).

The expression of the intestine-specific transcription factors Cdx2, GATA-4 and HNF-1α before and after MTX treatment was quantified by Western blot analyses of nuclear extracts derived from the jejunal segments. All measured transcription factors showed a similar pattern of expression after MTX treatment (Fig. 6). Strikingly, at day 1 all three transcription factors showed (a trend towards) increased expression; Cdx2 expression was 25-fold higher, HNF-1α 1.6-fold higher, and GATA-4 was twofold higher than in controls. At day 3 Cdx2 expression decreased to virtually nondetectable levels, HNF-1α decreased to 50% and GATA-4 to 20% of control level. The regenerative phase (day 7) was characterized by a significant increase in expression of all three transcription factors relative to day 3, Cdx2 and HNF-1α were also significantly increased in

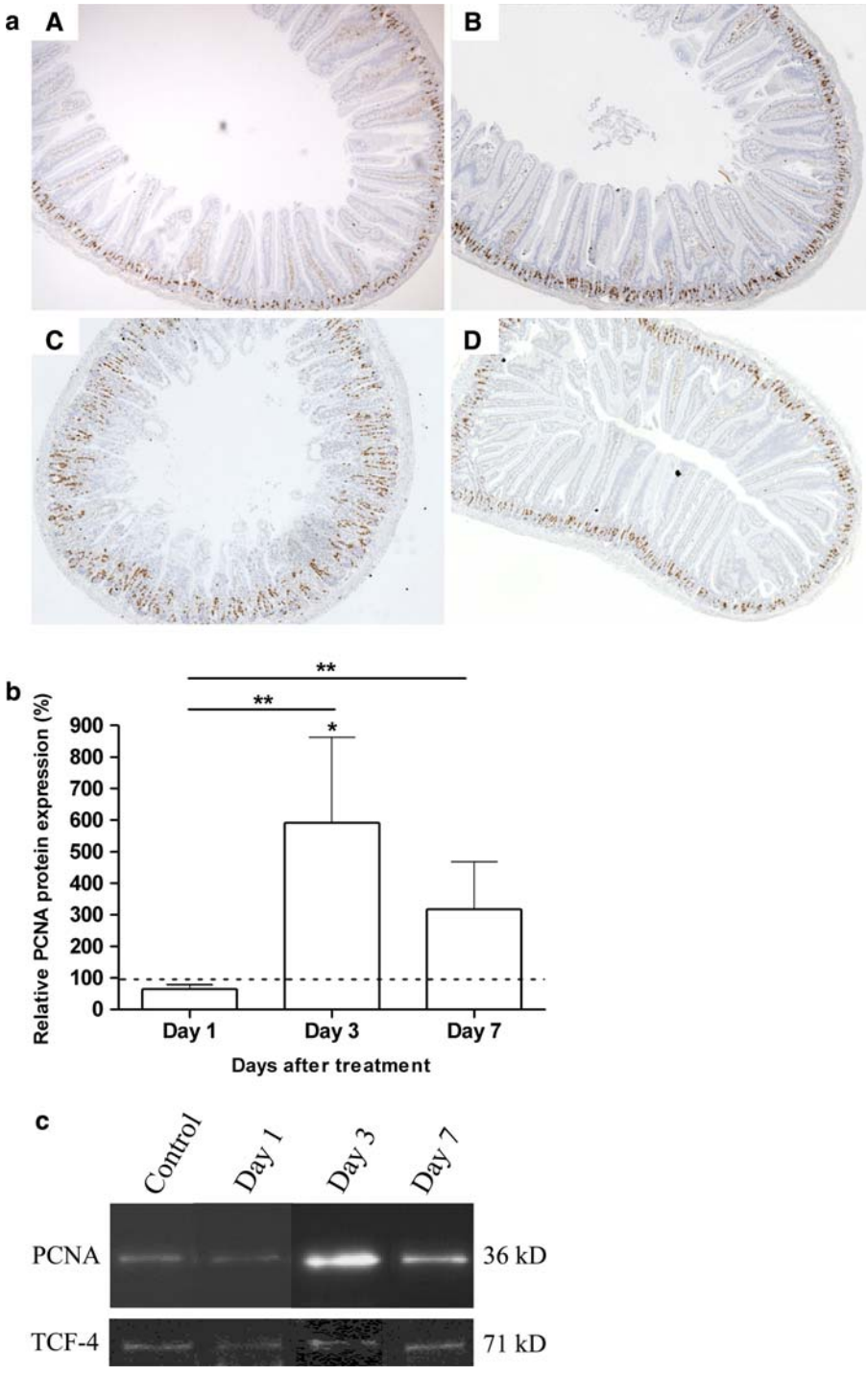

Fig. 4 Effect of MTX treatment on epithelial proliferation. Localization of epithelial proliferation was visualized by detection of BrdU incorporation (**a**). BrdU incorporation of the jejunum of a control mouse (*A*) and mice at day 1 (*B*), day 3 (*C*) and day 9 (*D*). At day 1 (*B*), BrdU incorporation was still localized to the lower part of the crypts matching the control situation. At day 3 (*C*), epithelial proliferation increased and was localized almost along the entire length of the crypts. At day 9 (*D*) the crypts showed still some hyper-proliferation compared to control. PCNA expression was quantified by Western blot analysis using a PCNA-specific monoclonal antibody (**b**). The PCNA protein expression of the control mice was set at 100%. PCNA protein expression was unchanged at day 1, and increased significantly at day 3 in comparison to control situation and expression at day 1. Thereafter the expression decreased during regenerative phase at day 7, but was still relatively elevated to the control situation and even significantly increased in comparison to day 1. $*P < 0.05$ day 3 versus control. $**P < 0.05$ day 1 versus day 3 and day 1 versus day 7. Representative Western blot demonstrating PCNA and TCF4 expression which was used as internal control (**c**). All results are shown for one mouse at each time point. *To the right* the size of the proteins are given

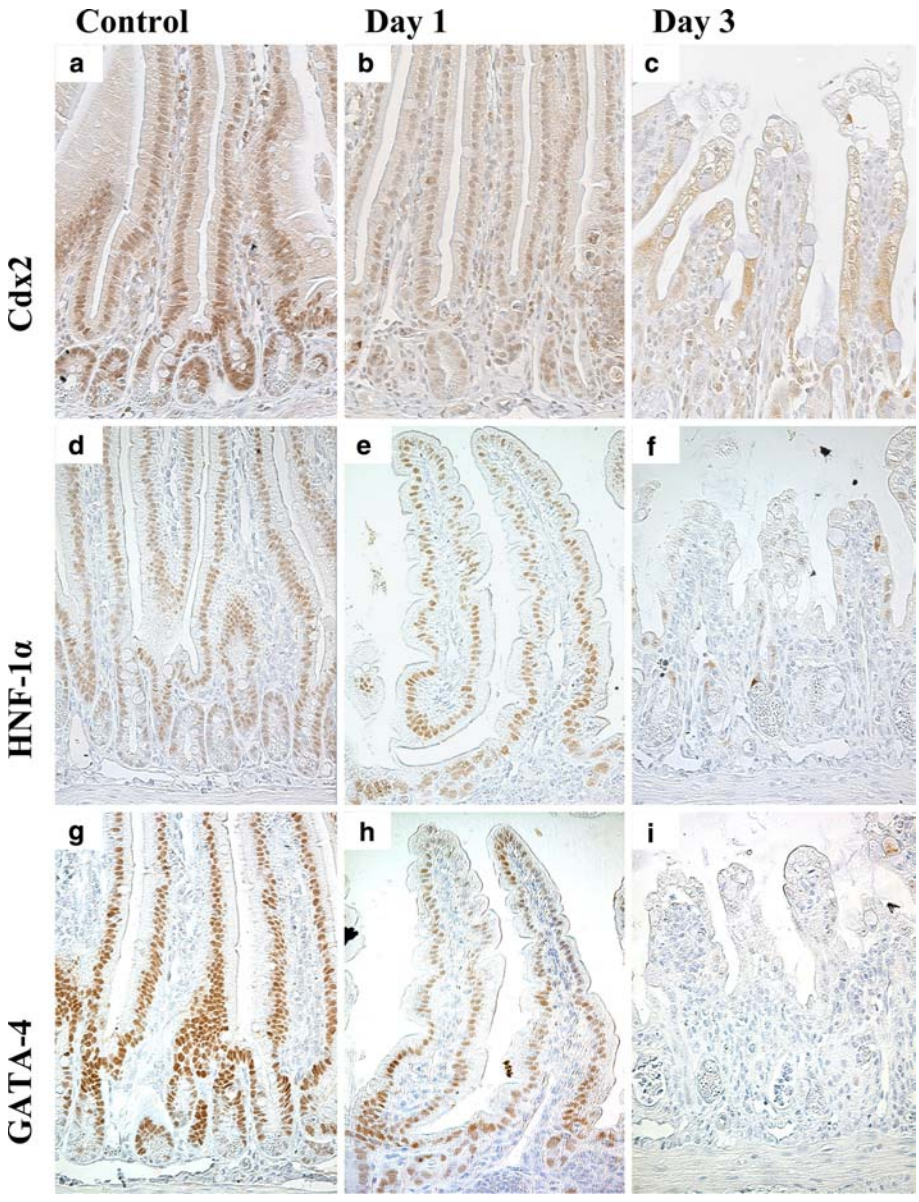

Fig. 5 Effect of MTX treatment on the enterocyte-specific transcription factors in the small intestinal epithelium. Immunohistochemical staining of Cdx2 (**a**), HNF-1α (**d**) and GATA-4 expression (**g**) in control mice. At day 1, Cdx2 (**b**), HNF-1α (**e**) and GATA-4 (**h**) showed no changes in expression, but at day 3, the expression of Cdx2 (**c**), HNF-1α (**f**), GATA-4 (**i**) was down regulated. At day 7 (data not shown) the same pattern of expression was seen as in control mice

comparison to control situation. Cdx2 expression was 14-fold higher, HNF-1α 3.8-fold and GATA-4 threefold higher than control levels (Fig. 6).

Discussion

The objective of this study was to investigate the gene expression of the transcription factors Cdx2, GATA-4 and HNF-1α in an experimental mouse model of MTX-induced mucositis, and to correlate these alterations with histological damage, epithelial proliferation and SI expression as a marker of enterocyte differentiation. To the best of our knowledge, this is the first time that the changes of these transcription factors were studied in a damage and regeneration model.

In the present study, we observed severe mucosal damage characterized by crypt loss, crypt abscesses, flattening of epithelial cells and villus atrophy at day 3. At day 9, during the regenerative phase, epithelial morphology was virtually normal again. This mouse model therefore was ideal to study responses during damage and subsequent regeneration.

The data presented here show that the cytostatic drug MTX has a time-dependent effect on the small intestinal epithelial proliferation. The epithelium responded by an initial slight decrease in proliferation at day 1, but at day 3 during severe epithelial damage a subsequent significant increase in the rate of proliferation was observed. In the regenerative phase, at day 7, proliferation decreased again, but was still significantly higher than control levels. It is of note that, although the induced morpho-

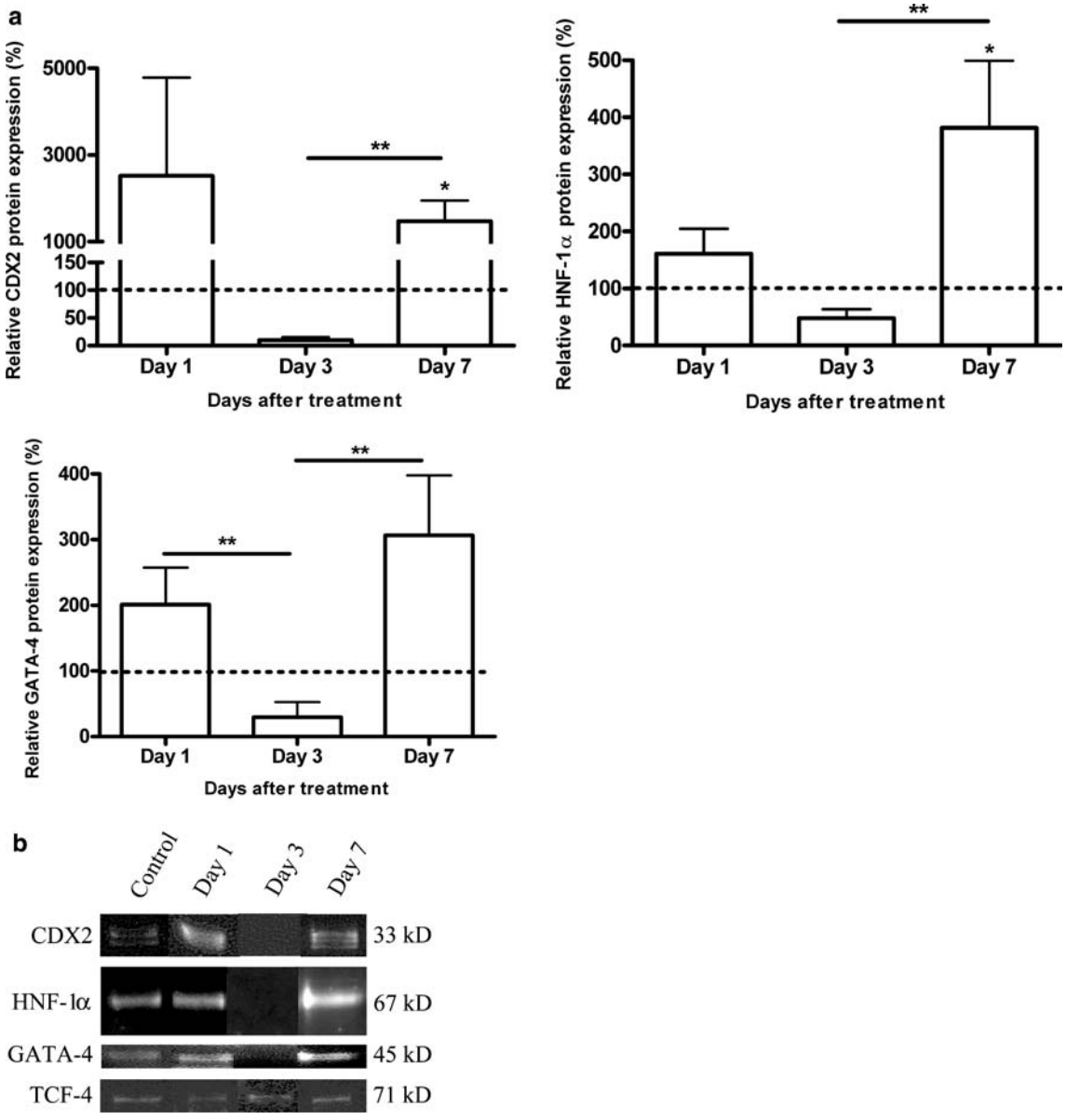

Fig. 6 Expression of the intestine-specific transcription factors Cdx2, HNF-1α, GATA-4. Protein expression of Cdx2, HNF-1α and GATA-4 was determined by Western blot analysis using a Cdx2-specific monoclonal antibody and HNF-1α-specific and GATA-4-specific antisera. Representative Western blots demonstrating the expression of the selected intestine-specific transcription factors (**a**). All results are shown for one mouse at each time-point. *To the right* the size of the proteins are indicated. For quantification the protein expression of the control mice was set at 100% (**b**). The expression of Cdx2, HNF-1α and GATA-4 showed a trend toward an increased expression at day 1, and a decreased expression at day 3. Both increases were not significant. At day 7 the expression of Cdx2 and HNF-1α was significantly increased in comparison to the controls, GATA-4 expression showed a trend toward increased expression. All three transcription factors were significantly increased at day 7 in comparison to the expression at day 3. $*P < 0.05$, day 7 of Cdx2 and HNF-1α versus control, $**P < 0.05$, day 3 of Cdx2, HNF-1α and GATA-4 versus day 7, and GATA-4 day 1 versus day 3

logical damage at day 3 was very severe, the degree of proliferation inhibition at day 1 was very limited. This could suggest that complete inhibition of proliferation did not take place or, with the time-points taken, we might have missed the complete inhibition. However, in the light of recent findings it is perhaps more likely that the induced damage is due to a multi-factorial process and not only depending on inhibition of stem cell proliferation [23]. Other cells in the intestinal mucosa also seem to be involved in the induction of mucositis by producing pro-inflammatory cytokines and reactive oxygen radicals [23]. This might explain why a drastic inhibition of proliferation at day 1 was not observed.

Here, we demonstrated a significant down-regulation of the enterocyte marker SI at day 3 after MTX treatment which is similar to what has been described for the rat model [19, 26, 33, 34]. The clinical consequence of SI down-regulation is dysfunction of degradation and up-

take of sugars by the intestine during villus atrophy. In the rat, other enterocyte markers like lactase, I-FABP, L-FABP, SGLT1 and GLUT5 were also downregulated after MTX treatment similarly to SI, indicating that SI is a good marker for enterocyte gene expression after MTX. At day 3, when morphological damage was most severe and the epithelial SI expression was drastically downregulated, a significant increase in epithelial proliferation was observed compared to control levels. This suggests that during severe intestinal damage, epithelial differentiation was compromised and not epithelial proliferation, as earlier studies also showed [19, 26, 33].

Quantification of Cdx2, GATA-4 and HNF-1α after MTX treatment resulted in a trend towards increased expression at day 1, and a decreased expression at day 3, during severe morphological damage. Besides stimulating the SI promoter the transcription factors Cdx2, GATA-4 and HNF-1α are all involved in intestinal differentiation [2, 5, 7, 9, 14, 20, 25, 27, 29]. This suggests that the observed alterations in expression of these transcription factors during severe MTX-induced damage may lead to changes in the epithelial differentiation.

Previously, it has been shown that cytostatic drugs induce oxidative stress and activation of NFκB as early events in pathways leading to intestinal damage [23]. NFκB is considered a primary regulator of stress response, and activates a number of transcription factors such as Cdx2 [4, 13]. Therefore, the initial increase of transcription factor expression at day 1 after MTX treatment could be explained by the induction of NFκB by MTX. The subsequent decreased Cdx2, HNF-1α and GATA-4 expression occurred at the same time as the decreased SI expression and significantly increased the proliferation, suggesting that the epithelium is more involved in proliferation rather than maintaining its absorptive function and fully differentiated status. This switch from differentiation to proliferation could be caused by the decrease in Cdx2 expression, because Cdx2 plays a key role in both proliferation and differentiation [25]. On the one hand Cdx2 upregulates P21 (WAF/CIP) [1, 10], a cyclin-dependent kinase inhibitor and an active inhibitor of cell proliferation, for the benefit of cell differentiation and maturation. On the other hand Cdx2 stimulates a number of intestine-specific cell fate and differentiation markers like carbonic anhydrase I, intestinal mucin MUC2, IPAL, lactase–phlorizin hydrolase, Math1, and Notch [27]. Thus, the decrease of Cdx2 observed at day 3 might result in a decrease of differentiation markers directly and/or indirectly via down-regulation of p21 expression. The down-regulation in p21 expression in its turn might lead to stimulation of proliferation and loss of differentiation. The mechanism by which MTX down-regulates Cdx2 in this model has not been elucidated. However, we speculate that, as in colon cancer, Cdx2 expression may be downregulated as a result of defects in its transcriptional activation, decreased mRNA synthesis or by gene silencing via DNA hypermethylation and/or histone modifications [11].

This study provides new insight in the pathophysiology of mucositis. The presumed cytotoxic mechanism of MTX, inhibition of DNA replication, is only partially responsible for the MTX-induced intestinal damage. Especially, loss of differentiation and intestinal function, in particular absorption, seemed at least in part to be caused by an inhibition of Cdx2, HNF-1α and GATA-4 expression during severe MTX-induced intestinal damage. Therefore these transcription factors are possible targets in mucositis therapy.

In summary, we report here that after MTX treatment of mice expression of transcription factors Cdx2, HNF-1α and GATA-4, involved in epithelial differentiation, were temporarily and regionally modulated in a way that correlated well with the intestinal morphology and with the expression of SI, and seemed to be inversely correlated with proliferation of epithelial crypt cells. During severe damage, the epithelium was preferentially involved with proliferation rather than differentiation, most likely in order to restore the small intestinal barrier function rather than maintaining its absorptive function. Since HNF-1α, Cdx2 and GATA-4 are critical for intestine-specific gene expressions and therefore crucial in epithelial differentiation, these results may explain, at least in part, why intestinal differentiation is compromised during MTX treatment.

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