Loss of enterocyte mass is accompanied by diminished turnover of enterocytes after myeloablative therapy in haematopoietic stem-cell transplant recipients

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Background: Intestinal mucosal barrier injury (MBI), resulting from myeloablative conditioning for haematopoietic stem-cell transplantation (HSCT), is an important cause of morbidity. Despite its frequency, recognition presents a challenge, while the aetiology needs still to be unravelled. The relationship between enterocyte mass and enterocyte loss was explored by examining citrulline serum levels and by assessing circulating intestinal fatty acid-binding protein (I-FABP) and ileal bile acid-binding protein (I-BABP), proteins released by dying mature enterocytes.

Patients and methods: Thirty-four adult patients with haematological malignancy received allogeneic HSCT (HSCT day 0) 12 days after being given idarubicin, cyclophosphamide and total body irradiation as myeloablative conditioning, a regimen known to induce oral and intestinal MBI. Serum levels of citrulline, I-FABP and I-BABP were measured on HSCT days \(-12, -6, 0, +7, +14\) and \(+21\).

Results: Myeloablative conditioning resulted in a significant decrease in serum citrulline with the nadir on HSCT day \(+7\); thereafter, levels rose gradually. Simultaneously, a significant decrease in I-FABP and I-BABP levels occurred from the day of transplant until day \(+14\).

Conclusions: Simultaneous reduction and subsequent increase of citrulline and I-FABP and I-BABP levels following cytotoxic treatment show that enterocyte mass corresponds to lower rate of dying enterocytes, indicating reduced turnover of enterocytes. Assessment of enterocyte turnover and mass offers opportunities for evaluation of new MBI therapies.

Key words: enterocyte mass, enterocyte turnover, mucositis, myeloablative therapy

introduction

Mucosal barrier injury (MBI) of the digestive tract is an inevitable and frequent side-effect of the intensive myeloablative conditioning employed to prepare for haematopoietic stem-cell transplantation (HSCT). MBI presents with oral mucositis and corresponding gastrointestinal signs and symptoms of mucositis, like abdominal pain, diarrhoea, vomiting and weight loss [1, 2]. MBI together with profound neutropenia predispose patients to local invasion by microorganisms and, subsequently, to systemic life-threatening infections [2, 3].

MBI has been attributed to the high physiological proliferation and turnover of intestinal epithelial cells, which are highly susceptible to cytotoxic therapy [2, 4–6]. Evidence for the loss of small bowel epithelial cell mass has been provided by declined serum levels of citrulline in HSCT recipients suffering from severe oral and GI mucositis following intensive myeloablative therapy [7, 8]. The levels of circulating citrulline, an amino acid not incorporated into proteins, reflect functional enterocyte mass. Differentiated small intestinal enterocytes specifically produce citrulline from glutamine and are responsible for the major part of the total amount of serum citrulline [9, 10].

The origin of the reduced functional intestinal epithelial cell mass is hitherto unclarified. Both an increased cell death of enterocytes and a reduced turnover might explain the reduced functional enterocyte mass. Measurement of endogenous cytosolic enterocyte proteins, like fatty acid-binding proteins (FABP), has been shown to be useful to estimate enterocyte loss, e.g. after episodes of acute intestinal ischaemia and...
inflammation, including necrotising enterocolitis [11–13].
FABP comprise a class of low-molecular mass (14–15 kDa)
cytosolic proteins found in high concentrations in tissues
involved in the uptake and consumption of fatty acids [14].
Intestinal fatty acid-binding protein (I-FABP) is primarily
limited to mature enterocytes of small and large intestine.
It circulates in low amounts in the blood stream of healthy
individuals [11]. Ileal bile acid-binding protein (I-BABP) is
exclusively present in mature enterocytes of the jejunum and ileum [15, 16]. Liver fatty acid-binding protein (L-FABP) is
localised in small amounts in the mature enterocytes of the
small and large intestine but in abundance in the liver [14].

The expression of L-FABP and I-FABP messenger RNA and proteins was barely affected during the phase of crypt and villous damage in rats treated with the cytostatic drug methotrexate (MTX) unlike what was found for other enterocyte markers, including glucose transporter 5, carnitine palmitoyltransferase synthase, sucrase-isomaltase, sodium glucose cotransporter 1 and lactase. This was underscored by immunohistochemistry [17].

In the present study, circulating levels of FABP were determined longitudinally along with citrulline serum levels in a cohort of HSCT recipients suffering from severe mucositis after intensive myeloablative conditioning in order to relate the known reduced functional enterocyte mass with enhanced cell death or reduced turnover of enterocytes.

patients and methods

patients
From July 1999 to July 2002, 34 adult patients admitted to the department of Haematology, Radboud University Nijmegen Medical Centre, received a T-cell depleted sibling HSCT on HSCT day 0 to treat haematological malignancy and consented to participate in the study [7, 18]. The preparative myeloablative conditioning regimen consisted of idarubicin starting 12 days before transplant (HSCT day −12), followed by cyclophosphamide on HSCT days −6 and −5 and a total of 9 Gy total body irradiation on HSCT days −2 and −1. All patients received cyclosporin and the same anti-infecrive prophylaxis as well as empirical antibiotic therapy when fever occurred. The local ethics committee approved the study.

citrulline assay
Serum had been collected on HSCT days −12, −6, 0, +7, +14 and +21. In practice, some samples were not taken. The serum was stored in aliquots at −80°C until further analysis. Citrulline concentrations were measured (micro molar) by a standard procedure for determining amino acids [7].

I-FABP assay
Serum I-FABP was determined using a highly specific commercially available enzyme-linked immunosorbent assay (ELISA) that selectively detects human I-FABP (standard: 20–5000 pg/ml) (provided by Hycult Biotechnology, Uden, The Netherlands).

I-BABP assay
The I-15P expression vector pET/Hu I-15P, which contains complementary DNA-encoding human I-BABP, was kindly donated by Dr Fujii [15, 16]. Prokaryotic protein expression was achieved by transformation of pET/Hu I-15P into BL21 cells. After harvesting and lysing of the cells, the recombinant human I-BABP was purified with an ion exchanger (DE-52, Whatman, Kent) and gel filtration (Superdex-75 column, Pharmacia GE Healthcare, Diegem, Belgium). The antisera against recombinant human I-BABP was obtained by immunising of New Zealand white rabbits with I-BABP and adjuvant carried out under a protocol approved by the Institutional Animal Care Committee of the University of Maastricht. The rabbit antisera was purified by affinity chromatography on protein A sepharose.

The following ELISA was developed to measure human I-BABP. Anti-I-BABP immunoglobulin G (IgG) was coated at a concentration of 2.5 μg/ml in phosphate-buffered saline (PBS) 1 h at 37°C on a 96-well plate (ImmuNo-Maxisorp; Nunc, Roskilde, Denmark). Free sites were blocked by 1 h of incubation with 1% bovine serum albumin in PBS at room temperature. Test samples as well as human recombinant I-BABP, used as standard, were incubated for 1 h at room temperature. Then biotinylated anti-I-BABP IgG was added as the detection antibody for 1 h at room temperature. Horseradish peroxidase–streptavidin conjugate (Zymed Laboratories, Inc., San Francisco, CA) was used to develop the signal reaction in combination with 3,3,5,5-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and H2SO4. Colour intensity was measured by determining the absorbance at 450 nm using a micro-ELISA reader. The detection range was 0.32–5 ng/ml. The specificity of the rabbit anti-human I-BABP was tested by incubating human recombinant I-FABP, L-FABP and lysed human ileum and colon together with the standard human recombinant I-BABP. The anti-I-BABP specifically reacted with human I-BABP, human ileum, but not with human colon, I-FABP or L-FABP.

Statistical analysis
Statistical analysis was carried out using Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA). Concentrations were presented as mean ± standard error of mean (SEM). Normality of all data obtained was verified by Kolmogorov–Smirnov test. Distribution of serum citrulline and FABP concentrations did not pass the normality test on HSCT day +7 for citrulline and I-BABP and on HSCT day 0, +7 and +14 for I-FABP. Therefore, Friedman two-way analysis of variance by ranks was used to analyse changes between HSCT days in serum citrulline and FABP levels. A Wilcoxon matched pairs test was used to calculate significant differences of serum citrulline and FABP with respect to baseline (HSCT day –12). Correlations were calculated using Spearman correlation coefficient. A P value <0.05 was considered to be statistically significant.

results

patient characteristics
Of the original 34 patients, five patients were excluded from this study because blood was collected on less than three study days. The remaining 29 patients (11 females, 18 male) had a mean age of 49 years (range 25–65 years). Nine patients had acute myeloid leukaemia, six had non-Hodgkin’s lymphoma, five had acute lymphoblastic leukaemia, five had myelodysplastic syndrome, two had chronic myeloid leukaemia and one patient each had myeloproliferative disease, chronic lymphocytic leukaemia and myelofibrosis. All had received a T-cell-depleted sibling HSCT to treat their haematological malignancy.

transplant-related complications. All patients suffered from severe oral and intestinal mucositis [18]. All except one patient were treated empirically for fever during neutropenia with a cephalosporin. Nine patients had bacteraemia due to oral viridans streptococci occurring between HSCT days +1 and +8.
Acinetobacter baumanii was recovered from a single aerobic blood culture from one patient on day +1 after transplant but no other Gram-negative bacilli were recovered from blood cultures or surveillance specimens of any other patient during the study period.

There were eight serious adverse events resulting in death. Four patients died because of an infectious event: one on HSCT day +9 because of sepsis syndrome, another on HSCT day +12 because of sepsis syndrome after Streptococcus mitis bacteraemia, one on HSCT +13 due to invasive Candida infection and a fourth on HSCT day +21 because of invasive pulmonary aspergillosis. Another four patients died because of a noninfectious event due to multiorgan failure on HSCT days +13 and +43 and one because of grade IV acute GvHD on HSCT day +55 and the other due to cerebral haemorrhage after primary graft rejection on HSCT day +56. Although the mortality rate is high, it is not markedly higher than what has been reported [19]. This particular intensive preparative myeloablative conditioning regimen consisted of idarubicin, cyclophosphamide and total body irradiation and was used to counterbalance the increased graft rejection and increased relapse rate associated with the T-cell depletion used at that time.

**serum citrulline concentrations after myeloablative conditioning regimen for HSCT**

The mean (SEM) initial citrulline serum concentration on HSCT day −12 was 31.6 (1.6) μM, which was comparable to baseline values in previous study [7]. Following HSCT, a significant decrease in serum citrulline concentrations was observed (Friedman test: P < 0.0001) with the nadir observed on HSCT day +7 [10.5 (1.5) μM, Figure 1, see Figure 2A for seven individual patients]. At the last study day (HSCT day +21), the mean serum citrulline value [15.1 (1.4) μM] had increased, but was still significantly lower than baseline (P = 0.0005). Since serum citrulline concentration had been shown to be a reliable marker of functional enterocyte mass, our data indicate that myeloablative therapy, accompanied by symptoms and signs of severe mucosal damage, results in clear transient reduction in the small intestinal epithelial cell mass [7].

**serum I-FABP and I-BABP concentrations after myeloablative conditioning regimen for HSCT**

In the untreated situation, substantial variability in I-FABP serum concentrations was noted, ranging from 20 to 336 pg/ml

![Figure 1. Serum citrulline concentrations after myeloablative conditioning regimen for haematopoietic stem-cell transplantation (HSCT). Serum citrulline concentrations declined significantly at HSCT day −6 and remain decreased until HSCT day +21, indicating rapid and continuous loss of small bowel enterocyte mass following intensive myeloablative therapy.](image1)

![Figure 2. Serum citrulline, intestinal fatty acid-binding protein (I-FABP) and ileal bile acid-binding protein (I-BABP) concentrations for seven individual patients after myeloablative conditioning regimen for haematopoietic stem-cell transplantation. Serum citrulline (A), I-FABP (B) and I-BABP (C) concentrations show that almost all patients follow the same pattern.](image2)
Following myeloablative conditioning regimen for HSCT, the Friedman test indicated a significant decrease in serum I-FABP values during the study period (\( P = 0.0009 \)). Serum I-FABP levels remained unchanged at HSCT day \(-6 \) [67.9 (13.6) pg/ml, \( P = 0.416 \)]. From HSCT day 0, a significant decrease of I-FABP levels [27.6 (4.2) pg/ml, \( P = 0.001 \)] compared with day \(-12 \) was found, which proceeded until HSCT day +14. The decrease in serum I-FABP concentration between HSCT days \(-6 \) and \( 0 \) occurred in all but one patient. The lowest mean I-FABP concentration measured was found on HSCT day +7 [21.1 (1.1) pg/ml], similar to the nadir of serum citrulline values.

However, in contrast to serum citrulline levels, I-FABP returned to baseline levels on HSCT day +21 [86.3 (28.6) pg/ml, \( P = 0.922 \)]. Similar to the I-FABP levels, also for I-BABP serum concentration substantial variation was present before treatment with a myeloablative conditioning regimen for HSCT, ranging from 0.6 to 8.5 ng/ml (Figure 3B, see Figure 2C for seven individual patients). Following preparative regimen, the Friedman test indicated significant reduction in serum I-BABP levels during the study period (\( P < 0.0001 \)). Serum I-BABP levels showed from HSCT day \(-6 \) onwards a significant decrease [2.1 (0.6) ng/ml, \( P = 0.004 \)] compared with HSCT day \(-12 \). This diminution continued until HSCT day +14. The decrease in serum I-BABP concentration between HSCT days \(-12 \) and \(-6 \) occurred in 26 of 29 patients. The nadir mean I-BABP concentration was found on HSCT day 0 [0.8 (0.1) ng/ml]. In accordance with the normalisation of serum I-FABP levels on HSCT day +21, a return to baseline levels of serum I-BABP on HSCT day +21 was found [3.7 (1.6) ng/ml, \( P = 0.470 \) versus HSCT day \(-12 \)]. Interestingly, all serum I-BABP levels correlated significantly with serum I-FABP levels (Spearman \( r = 0.436, P < 0.0001 \)).

**ratio of serum I-FABP : citrulline and I-BABP : citrulline after myeloablative conditioning regimen for HSCT**

In order to study whether the functional cell mass is directly related to the loss of villous enterocytes, the ratio of serum values of I-FABP and citrulline was determined at each time point. At baseline, the mean ratio I-FABP : citrulline was 27.6 (4.8) (Figure 4A). The ratio remained unchanged during the complete study period (Friedman test \( P = 0.600 \)), indicating that a lower mass of functional small bowel enterocytes is accompanied by loss of enterocytes. Only on HSCT day +21, a higher mean ratio was found [66.5 (24.2)] compared with all preceding study days, caused by a relative elevation of I-FABP rather than citrulline levels. However, this increased ratio was not significant (\( P = 0.424 \)) compared with HSCT day \(-12 \).

The specificity of I-BABP for mature enterocytes of jejunum and ileum allows us to relate the loss of enterocytes of the jejunum and ileum with functional cell mass. Before treatment, the mean ratio I-BABP : citrulline was 11.6 (1.9) (Figure 4B). The ratio remained unaltered during the study period (Friedman test \( P = 0.460 \)), indicating that the mass of small bowel enterocytes is related with the loss of jejunal and ileal enterocytes.

**discussion**

Human histological studies on intestinal mucositis are nearly impossible due to the severity of mucositis and increased bleeding tendency. Therefore, circulating levels of citrulline and enterocyte cytosolic proteins I-FABP and I-BABP are investigated longitudinally in a cohort of patients receiving the same intensive myeloablative conditioning for HSCT. A substantial variation in circulating levels of both FABP before treatment is observed, which is comparable with data of healthy individuals [20]. The expression of both I-FABP and I-BABP is

Figure 3. Serum intestinal fatty acid-binding protein (I-FABP) and ileal bile acid-binding protein (I-BABP) concentrations after myeloablative conditioning regimen for haematopoietic stem-cell transplantation (HSCT). Serum I-FABP (A) and I-BABP (B) concentrations decreased significantly at HSCT day 0 and returned to baseline levels at HSCT day +21, indicating loss of turnover of intestinal epithelial cells followed by a recovery after 3 weeks in patients undergoing intensive myeloablative therapy.
limited to differentiated enterocytes present on the upper one-third part of the villus in the small intestine [11, 16]. These FABP-containing cells are known to detach from the villus or die at the end of their life span [21]. Taking into consideration the very short half-life of these proteins (~11 min), we consider that the baseline I-FABP and I-BABP circulating levels represent the continuous release of FABP originating from mature detaching or dying enterocytes. Some of the baseline FABP (and citrulline) serum levels might also relate to previous chemotherapy. Since the main loss of enterocytes in the healthy intestine consists of senescent enterocytes which detach into the gut lumen at the end of their life span, the steady-state circulating levels of FABP represent the loss of enterocytes and thus also the production of enterocytes or in other words the turnover of enterocytes.

Interestingly, a minor increase in the amount of serum FABP was found only in three patients after initiation of the myeloablative conditioning regimen. This indicates that no direct (small) bowel epithelial cell damage occurred during this preparative regimen for HSCT at this time. However, following the preparative regimen, levels of both I-BABP and I-FABP declined in virtually every case. These data show that less cytosolic content of enterocytes is released into the circulation than under normal, healthy circumstances, indicating a decreased rate of dying enterocytes. This, in turn, suggests a reduced turnover of enterocytes after myeloablative conditioning.

This is the first study to report on specific changes in I-BABP levels, representing specific jejunal and ileal enterocyte loss, using a newly developed ELISA for measurement of serum and plasma I-BABP. Our study shows a very rapid decrease in I-BABP circulating levels, a representative of diminished small intestinal turnover of enterocytes following onset of therapy. This occurred 1 week earlier than the marked reduction in I-FABP which is present in enterocytes of the whole intestine. This is consistent with the enhanced susceptibility of small bowel enterocytes to myeloablative regimen and is clearly shown by the rapid alteration of the circulating marker I-BABP. Significantly lower serum citrulline concentrations, an indicator for the functional small bowel epithelial cell mass, occur 2 weeks after the start of myeloablative therapy lagging 1 week behind diminished I-BABP levels and correspond with the decreased I-FABP circulating levels. Importantly, it took ~3 weeks before levels of circulating FABP increased towards normal values, indicating an elevated turnover of enterocytes, which most likely reflects recovery by proliferation. Similarly, rising serum citrulline concentrations indicate an initiation of the recovery of functional enterocyte mass on HSCT day +21. The simultaneous alterations of serum levels of I-BABP, I-FABP (representing loss of cells present in the jejunum–ileum and total intestine) and serum concentrations of citrulline (representing total small intestinal enterocyte mass) indicate that turnover and mass of intestinal epithelium are directly related. This is further supported by the ratios of serum I-FABP : citrulline and I-BABP : citrulline, which clearly indicate that the turnover of (small) intestinal epithelial cells corresponds to the small bowel epithelial cell mass throughout the entire study period.

From these data, we conclude that the reported reduction in enterocyte mass is directly related to a reduced turnover of enterocytes. This lower turnover could be caused by a reduced number of enterocyte stem cells, which is in line with the kinetics of injured gut epithelium after radiotherapy and chemotherapy [4–6, 22]. Human duodenal biopsies of patients receiving non-intensive chemotherapy showed induction of apoptosis in the intestinal crypts, using TUNEL technique and electron microscopy, which occurred before hypoplastic villous atrophy, loss of enterocyte cell height and decreased mitotic count [4]. Presumably, apoptosis reduced the net functional stem-cell population. The authors concluded that the chemotherapy-induced apoptosis of intestinal crypts in patients was not accompanied with mucosal destruction, but rather with morphometric reduction in crypt size and proliferation [4]. Also the recently defined five-phase process of the pathobiology of mucositis described that radiation or chemotherapy leads to direct and indirect injury to epithelial stem cells, resulting in a loss of renewal capacity in the early...
phase of ‘primary damage response and signal amplification’ [6]. Rats treated with MTX showed a similar inhibition of epithelial proliferation, decreased crypt depth and crypt cell apoptosis in the small intestine [5]. In line with these data, we showed no increase of circulating I-FABP and I-BABP levels, indicating no (small) intestinal mucosal cell damage. Our data are supported by the work of Vigneulle et al. [22] in nonhuman primates who show that following radiation-induced intestinal injury, an ablation of intestinal epithelial stem cells leads to a compensatory reduction in villus length (loss of total villous cell mass), which our work indicates to be accompanied by a reduction in loss of senescent cells (cell turnover). The compensatory regeneration of stem cells and villus epithelial cells took \( \geq3 \) weeks to result in a complete recovery, which is in line with our data on continuous low levels of circulating citrulline after myeloablative conditioning [22]. Our data indicate that the loss of senescent cells prevails the complete recovery period.

Intestinal mucositis is a well-known adverse effect of myeloablative therapy and a risk factor for mortality and relapse in patients undergoing autologous stem-cell transplantation, probably due to necessary reductions in treatment intensity and sometimes cessation of treatment [23]. New therapies to treat this toxicity are being developed, which are aimed at protection and enlargement of the enterocyte mass [24, 25]. Combined measurement of the turnover (serum FABP level) and mass (serum citrulline levels) of enterocytes offers the new modality to evaluate such novel treatments in patients.

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**references**