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Biomarkers for radiation-induced small bowel epithelial damage: An emerging role for plasma Citrulline

Ludy Lutgens, Philippe Lambin

Ludy Lutgens, Philippe Lambin, Department of Radiation Oncology (Maastro), GROW Research Institute, University of Maastricht, Maastricht 6200 MD, The Netherlands
Correspondence to: Dr. Ludy Lutgens, MD, PhD, Department of Radiation Oncology (Maastro), GROW Research Institute, University of Maastricht, Tanslaan 12, 6202 AZ Maastricht, The Netherlands. ludy.lutgens@maastro.nl
Telephone: +31-88-4455600 Fax: +31-88-4455773
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Abstract

Reduction of cancer treatment-induced mucosal injury has been recognized as an important target for improving the therapeutic ratio as well as reducing the economic burden associated with these treatment related sequellae. Clinical studies addressing this issue are hampered by the fact that specific objective parameters, which enable monitoring of damage in routine clinical practice, are lacking. This review summarizes pros and cons of currently available endpoints for intestinal injury. The metabolic background and characteristics of plasma citrulline, a recently investigated biomarker specifically for small intestinal injury, are discussed in more detail.

Key words: Biomarker; Citrulline; Small bowel; Radiation injury

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INTRODUCTION

An increase in the use of multiple treatment modalities is characteristic for current developments in curative cancer treatment. Whereas this strategy has yielded superior treatment results in a variety of solid tumors, treatment related acute toxicity has increased as well[1-6]. Severe radiation induced intestinal injury occurring during a treatment course has a detrimental effect on treatment outcome in cancer patients due to necessary reductions in treatment intensity and/or treatment interruption. In addition, this acute type of epithelial gut damage has also been suggested as one of several mechanisms contributing to late treatment related sequellae[7]. Cancer treatments related epithelial gastrointestinal toxicity has also been recognized as a significant economic burden[8]. Hence, prevention and/or reduction of epithelial gut damage is expected to have a significant clinical and socio-economic impact. However, clinical studies addressing treatment induced gut damage are hampered by the fact that objective parameters, which enable monitoring of damage in routine clinical practice, are lacking. In case of radiation treatment for pelvic and/or abdominal cancers the small bowel is an important dose-limiting organ with regard to both early and late treatment related morbidity. The clonogenic crypt cell is a central target of intestinal epithelial radiation damage[9-12]. Radiation will result in an impairment or loss of cell production and eventually in the loss of functional cells, becoming manifest within days or weeks following single dose or fractionated radiation[9]. A wide diversity of functional disorders has been observed following ionizing radiation such as changes in trans-epithelial transport processes[13,14], gut barrier function[15], motility dysfunction[16,17], or the absorption of various nutrients such as carbohydrates, amino acids, proteins, vitamins and bile acid[18-26]. Some of these functional changes have been correlated with the epithelial cell mass available for absorption[23-26] suggesting a cellular basis in at least part of radiation induced functional disorders.

BIOMARKERS FOR EPITHELIAL INTESTINAL DAMAGE

Clinical symptoms

Clinical symptoms are most commonly used as a surrogate endpoint during and following treatment. Clinical symptoms of acute radiation enteritis include anorexia, nausea, vomiting, abdominal cramps and diarrhea. These symptoms may occur immediately following the start of treatment, although more usually, radiation sequellae become manifest during the 2nd or 3rd wk of fractionated treatment and lasting 2-6 wk following treatment. Whereas very early symptoms are attributable to altered intestinal motor activity, mucosal injury is the prominent feature underlying symptoms later on during the course

of treatment, although altered intestinal motor activity is another contributing factor throughout the treatment course and following treatment[27-29]. Beside the fact that toxicity-grading systems are not used uniformly by investigators[30], they are being adjusted on a regular basis. More importantly however, clinical symptoms correlate poorly with objective parameters of gut damage such as altered morphology[28,31], sugar permeability tests[32-34] or treatment related parameters[30], illustrating the complexity of the pathophysiology of clinical symptoms related to cytotoxic treatment induced small bowel damage[35-37].

Because of the limitations related to the assessment of morphological endpoints in patients, investigators have used several surrogate endpoints for measuring small bowel dysfunction.

Mucosal transport and barrier function

Mucosal transport and barrier function is another frequently used item for measuring small bowel dysfunction. Surrogate endpoints are the assessment of gut barrier function through measuring absorption of test markers[38,39] or tests for nutrient malabsorption[20,40], bile acid or vitamin B12 absorption[19,22,41-43]. These function tests are qualitative tests mainly suited for diagnostic purposes. The endpoints used do not address damage to target cells. Consequently, they lack a dose response relationship. Although not as troublesome as taking mucosal biopsies, these tests are impractical for monitoring purposes during and following radiation treatment. Enterocyte transport has been used as a surrogate endpoint for epithelial cell mass. Overgaard *et al*[23] demonstrated a dose response relationship for jejunal glucose absorption in mice following single dose upper abdominal irradiation. A linear correlation was observed between jejunal glucose absorption and the absorptive surface. Kirichenko *et al*[26] used a nuclear scintigraphic technique to quantify active enterocyte transport in mice. At 3.5 d after single dose whole body irradiation (WBI) absorption of the isotope correlated significantly with a surrogate endpoint for the jejunal absorptive surface, i.e. the number of cells per villus. A strong correlation was observed between absorption and radiation dose at this time point. For the dose points used in these experiments, i.e. 4, 6, 8 and 12.5 Gy, no correlation was seen between jejunal crypt regeneration, radiation dose and absorption. The results of both experiments[23,26] indicate a cellular basis for the absorptive function and a correlation with the absorptive area. Both function tests were investigated for their applicability as a clinical assay for radiation-induced epithelial cell loss in the gut and indirectly for quantification of radiation damage to the target cell for epithelial small bowel damage. To date none of these assays have been introduced in clinical practice for routine use during and following fractionated treatment, mainly for practical reasons.

Diamine oxidase

Diamine oxidase (DAO), a cytoplasmic enzyme found in almost all organs is present in a particularly high concentration in the epithelial cells of the small intestine[44-46]. Following injury to intestinal epithelial cells DAO is released into the intestinal lumen and intercellular space where it is taken up by lymphatics and blood vessels[45]. Circulating DAO is rapidly cleared by the liver[47]. The plasma DAO activity has been suggested as a candidate marker for measuring ischemic small bowel injury[48-50]. Ely *et al*[51] demonstrated a radiation dose-dependent decline of ileal tissue and plasma DAO activity in rats. Nadir values were observed at 3 d after radiation. At this time point a linear dose-response relationship was demonstrated for plasma and tissue DAO activity at a dose range of 0-6 Gy and 2-8 Gy, respectively. DeBell *et al*[52] investigated the time course of tissue and plasma DAO activity changes following irradiation. They found that the decline of plasma DAO activity preceded the decline of jejunal tissue DAO activity. In addition, the calculated RBE values for both parameters were not the same. These data do not support a direct correlation between the changes of plasma DAO activity and intestinal tissue DAO activity as was in fact also the case for the observation made by Bounous *et al*[49] These authors observed a 7.5 and 1.4 -fold increase in serum DAO activity 24 and 30 h following the onset of symptoms in a patient with a lethal acute intestinal ischemia.

Fatty acid-binding proteins

Fatty acid-binding proteins (FABP) are small (15 kDa) cytoplasmic proteins. Intestinal-type FABP (I-FABP) and liver-type FABP (L-FABP) are produced in small intestinal enterocytes, mainly in the villi, not in the crypt[53,54]. I-FABP has been demonstrated to be a sensitive biomarker for intestinal disease associated with tissue necrosis. Upon small bowel enterocyte necrosis I-FABP and L-FABP are readily shed into the circulation[53]. In ischemic bowel disease a rapid increase in plasma and urinary I-FABP concentration is observed[55,56]. In contrast, I-FABP and L-FABP were not elevated in patients with intestinal disease not associated with a significant degree of tissue necrosis[57]. In transplant recipients histologic graft rejection was not preceded by increased levels of serum I-FABP[58]. Taken together, I-FABP and L-FABP seem to be sensitive biomarkers for ischemic bowel disease. However, its use for intestinal damage initially targeting clonogenic crypt cells, as in radiation induced intestinal damage[9-12] and transplant rejection[59], has been disappointing so far.

Calprotectin

Calprotectin is a protein abundant in neutrophils. The fecal concentration of calprotectin has been identified as a sensitive biomarker of intestinal inflammation[60]. In patients with Crohn's disease the marker correlates with changes in intestinal permeability[61]. The test is highly sensitive. The marker was tested in a validated animal model for late intestinal radiation injury[62,63]. Fecal excretion of transferrin, the rodent analogue of calprotectin, the first 2 wk after treatment correlated with validated endpoints for acute and late intestinal radiation injury. Interestingly, the high sensitivity of the test allows treatment of a limited volume of small bowel. However, in

contrast to these experimental conditions the marker does not allow discrimination of anatomical sites of intestinal injury due to a low specificity[64].

CITRULLINE: A BIOMARKER FOR VIABLE SMALL BOWEL ENTEROCYTES

While radiation-induced tissue damage is unlikely to be expressed or quantified by a single functional or morphological parameter[36], an assay measuring damage to relevant target cells involved in the initiation of tissue damage is of great importance for both experimental and clinical research. Ideally, such an assay must be tissue-specific, display a dose-response relationship and in case of the small intestinal epithelium also a volume-response relationship. In addition, the assay must be easily accessible in clinical practice and independent of experimental conditions such as concurrent medical conditions, medication and nutritional status. Citrulline is a candidate biomarker fulfilling most of these criteria. The assay assesses radiation-induced epithelial cell loss, an important initiating factor in the pathogenesis of acute and chronic intestinal radiation injury and one of several pathophysiological mechanisms underlying clinical symptoms. Citrulline is a nitrogen end product of small bowel enterocyte metabolism. Plasma citrulline has been identified as a biomarker for functional small bowel enterocyte mass under various clinical and experimental conditions. In addition to surgery[65-68], celiac and non-celiac diseases[69], viral enteritis[70] and acute cellular rejection following small bowel transplantation[70-75], cytotoxic treatments was identified as another event associated with decreased plasma citrulline level due to epithelial cell loss[30,76-79]. As a whole, plasma citrulline seems to be a quantitative parameter independent of the underlying cause for epithelial cell loss.

Small intestinal intermediary metabolism

The small bowel epithelium plays an important role in the intermediary metabolism of amino acids, particularly glutamine, citrulline and arginine[80,81] thereby conditioning the availability of dietary amino acids to extra-intestinal organs[82]. Intestinal dysfunction resulting from intestinal diseases or injuries affect intermediary and inter-organ metabolism[83-85]. Hence, any factor affecting the intestinal mucosal cell mass will have an impact on protein and amino acid metabolism[83,86-90]. Since the pioneering work of Windmueller and Spaeth during the 1970's many research groups have demonstrated that amino acids are the major fuel for the small bowel epithelium, both under conditions of fasting and feeding[91-97]. Windmueller and Spaeth identified glutamine as the quantitatively most important arterial energy source[91,98-100] for the rat jejunum in fasted animals. Measurements in different species consistently demonstrated a concentration dependent high rate of intestinal glutamine extraction from the blood. Thus, 25%-33% of the total plasma glutamine is extracted by the small bowel in each single pass[98]. Glutamine is the most abundant amino acid in plasma and plays a key role in whole body protein and amino acid metabolism[98,101]. Organs may be classified as glutamine producers and as glutamine consumers[102]. Skeletal muscle is by far the most important producer and the small bowel the most important consumer. The gut epithelium has been identified as the predominant site of glutamine uptake and metabolism[98]. Of all epithelial cells, enterocytes are the cells mostly responsible for glutamine utilization[103,104]. The first step in enteral glutamine catabolism is the conversion to glutamate and ammonia by the mitochondrial enzyme glutaminase in a non-reversible reaction[105]. The intestinal uptake of glutamine from the blood varies with the availability of substrate in the lumen. However, the intestinal metabolism of plasma glutamine is sustained during competitive luminal substrate provision, even under conditions of luminal overloading with glutamate[91,98]. The gut epithelial cell has access to glutamine from the arterial blood supply and the gut lumen[98]. The metabolic fate of glutamine from both routes is nearly identical indicating a common metabolic pool[91]. Major glutamine carbon products are CO_2 (55%-65%), lactate (8%-16%), citrate (2%-7%), citrulline (4%-6%), proline (5%-6%), alanine (0.5%-4%) and ornithine (0.5%-2%). Major glutamine nitrogen products are ammonia (23%-36%), alanine (33%-36%), citrulline (10%-34%) and proline (7%-10%).

An endproduct of glutamine metabolism

Citrulline was identified as an endproduct of nitrogen glutamine metabolism in the rat intestine accounting for 27.6% of metabolised glutamine[96,98,99]. Citrulline is an intermediate in the urea cycle[106,107], which is comprised of 5 enzymes, 2 being mitochondrial [(CPSI) and (OCT)] and 3 being cytosolic enzymes (arginino succinate synthethase (ASS), arginino succinate lyase (ASL) and arginase). Windmueller and Spaeth[80] did not detect any urea-cycle intermediate following luminal administration of citrulline to the intestinal mucosal cells and concluded that intestinal mucosal cells contain an incomplete urea cycle. However, others suggested a complete urea cycle in rodent enterocytes[108,109]. Wu finally demonstrated urea synthesis in porcine enterocytes from ammonia, glutamine and arginine in a dose-dependent manner, thus providing the evidence that, in addition to periportal hepatocytes[106], small intestinal enterocytes[104,110] contain a complete urea cycle. Whereas an activity was observed of all urea cycle enzymes, the activity of OCT was by far the highest of all (i.e. a factor 10-20)[110]. In contrast to hepatocytes in which CPSI and ASS are considered the regulatory enzymes due to an exceedingly high arginase activity, in enterocytes the arginase activity seems to be the limiting factor for urea synthesis[110]. Given the high rate of glutamine/glutamate metabolism[91,93,96,111] and the relative abundant OCT activity[110] in small intestinal enterocytes, the majority of citrulline produced from glutamine/glutamate[80,104] will not be further metabolised in the urea cycle but instead released in the portal circulation. Thus only 5% of the glutamine-derived ammonia was converted to urea indicating the low capacity of urea synthesis from glutamine (or ammonia) in enterocytes[110]. Hence, although the small intestinal mucosa contains a metabolically significant urea cycle, the liver is without doubt the major

organ for urea synthesis in mammals[106,112]. Furthermore, citrulline can be effectively regarded as an endproduct of glutamine/glutamate metabolism of intestinal enterocytes as suggested by Windmueller and Spaeth[80,98] and confirmed in many studies since then[66,69,83,86-88,90,104,111,113-115].

Pathways for citrulline synthesis

The synthesis of citrulline from glutamine involves 5 mitochondrial enzymes; phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate synthase (P5CS), ornithine aminotransferase (OAT), OCT and CPSI with P5CS being the key regulatory enzyme[104,116-118]. P5CS is unique to small intestinal enterocytes[116,119-121]. PDG converts glutamine to glutamate and ammonia. Glutamate is then converted to pyrroline-5 carboxylate by P5CS. Pyrroline-5-carboxylate is then converted to ornithine by OAT. Glutamine derived ammonia plus HCO_3^- are converted to carbamoyl phosphate by CPSI. Carbamoyl phosphate and ornithine are finally converted to citrulline by OCT. Pyrroline-5-carboxylate is a common precursor of both ornithine and proline. For a long time, glutamine and glutamate have been considered the only precursor for pyrroline-5-carboxylate. Wu *et al*[122] have demonstrated proline oxidase (PROox) activity in porcine enterocytes with the synthesis of citrulline and arginine from proline being another important pathway for citrulline synthesis. This pathway involves 4 mitochondrial enzymes, being PROox[123], OAT, OCT and PCSI. Proline is converted to pyrroline-5-carboxylate by PROox[124]. The subsequent metabolic steps are the same as for citrulline synthesis from glutamine, involving OAT, CPSI and OCT. As a consequence, glutamine-derived nitrogen intermediates such as glutamate and ammonia are necessary for the synthesis of citrulline from proline[122]. Based on the relative enzyme activities[104,125] PROox and CPSI are suggested key regulatory enzymes in citrulline synthesis from proline[122]. Small intestinal PROox activity is relatively high, i.e. 10- and 6-fold greater than the activity in the liver and the kidney of piglets, respectively[123]. Furthermore, the total cell mass of small intestine is relatively large compared to the liver and kidneys, respectively, i.e. 162% (liver) and 970% (kidneys) in 6 wk old pigs[126]. Hence, the small intestine may be a major site of proline degradation and subsequent synthesis of citrulline from proline[122]. In contrast to glutamine, the luminal proline derived from the diet is the most important source of proline for citrulline synthesis[98,122].

Metabolic fate of citrulline released into the portal vein

Under physiologic conditions there is no appreciable uptake of citrulline by the liver[80]. Labelled citrulline was supplied to the liver by a continuous portal infusion at a concentration 1.5 times the usual portal blood concentration. Less than 10% of the labelled citrulline had disappeared from the perfusate after about 40 passes clearly indicating that very little citrulline in the portal blood released by the intestine is metabolised by the liver[80]. Thus citrulline produced and released by the small intestine simply passes through the liver and reaches the systemic circulation. Subsequently the kidney is the major consumer of circulating citrulline extracting about 35% of arterial citrulline in each pass[80,86]. The relevance of this pathway is demonstrated quantitatively by the increase of the plasma citrulline level observed in patients with renal failure[127].

Source of circulating citrulline

It is now generally accepted that the small intestinal absorptive epithelial cell is the major source of circulating citrulline[104,122,128]. Windmueller and Spaeth investigated the existence of alternative sources of circulating citrulline[80]. Within 5 min after exclusion of either the intestine alone or all portal drained viscera from the circulation, plasma citrulline concentration fell by only 27% and 20%, respectively. Hence, more than 70% of the plasma citrulline concentration is sustained. Based on the high rate of citrulline uptake by the kidney accounting for 83% of the citrulline released by the small bowel, it can be estimated that clearance of citrulline from the plasma should be complete after 4.3 min in the rat in the absence of any input[80]. These findings indicate the existence of extra-splanchnic production sites and/or storage sites of citrulline. The cerebrospinal fluid[129] and skeletal muscle[130] are known sites with citrulline concentrations exceeding that of plasma but could not be identified as citrulline releasing sites[80]. Measurement of arteriovenous concentration differences across the hindquarter after complete removal of the portal drained viscera revealed a small net release of citrulline accounting for only 24% of citrulline uptake by the kidney under physiological conditions. Hence, whereas skeletal muscle may be considered a storage site for citrulline, it is not a substantial source for circulating citrulline under normal physiological circumstances[80]. The liver does not release citrulline unless provided with un-physiologically high doses of ammonium in conjunction with high concentrations of ornithine or proline in the perfusate, indicating that all the citrulline formed from ornithine or proline is converted to arginine[80]. This is probably due to the efficient metabolic channelling of citrulline to ASS and the high activity of type I arginase in hepatocytes leading to a subsequent rapid hydrolysis of arginine into urea and ornithine[131]. Hence, despite the results observed with the organ exclusion experiments performed by Windmueller and Spaeth, no other site but the intestine has been identified so far that releases significant amounts of citrulline under physiological conditions. The role of the small intestine as the major source for circulating citrulline is demonstrated by experiments in which the plasma citrulline concentration is reduced by means of small bowel targeted interventions such as specific inhibitors of OAT[116] or OCT[132], yielding a similar decrease of plasma citrulline concentration as observed after small bowel resection[83,86,113,114]. Furthermore, strong lines of evidence have been obtained since then through clinical observations which are in agreement with this concept[66,69,71,73,87,90,133,134]. Experimental and clinical data suggest a non-homogenous distribution of citrulline production. The distribution of P5CS activity in rats was 26%, 31%, 33% and 10% in the duodenum, upper jejunum, lower jejunum and ileum,

respectively[120]. The release of citrulline measured as venous minus arterial concentration in patients admitted for elective gastrointestinal surgery was 30.4 ± 4.0 μmol/L and 8.4 ± 1.7 μmol/L for the jejunum and ileum, respectively[111].

Determinants of intestinal citrulline synthesis and plasma citrulline level

The activity of intestinal citrulline-synthesizing enzymes changes as a function of the feeding regimen, i.e. during the suckling and (post) weaning period. Weaning-induced changes in plasma cortisol levels are suggested to play a role in the difference observed between suckling and weaning animals, rather than developmental changes related to age[135-138]. Except for the interaction of metabolites with specific enzymes[137-141], substrate availability is another determinant of the citrulline production by enterocytes[104,122,140,142]. Several inborn errors[143-148] may give rise to specific changes in citrulline concentration. Enhanced NO synthase activity in patients with SLE has been associated with hypercitrullinemia[149]. Taken the central role of glutamine metabolism in the small intestinal citrulline synthesis[104,122], any metabolic condition substantially influencing intestinal glutamine metabolism is likely to have a major impact on citrulline synthesis as well. In this respect cumulative data indicate an important role for glucose metabolism[140,141,150]. A major determinant, however, under steady state conditions for the rate of citrulline released into the portal and subsequently the systemic circulation is the actual number of functional enterocytes[65-69,83,86,87,90,113,114,134,151]. This was further demonstrated by clinical data on small bowel transplantation[70-75,152]. In addition to a variation in citrulline synthesis, alterations in citrulline utilization will have an influence on the plasma citrulline concentration[80,119,127].

Plasma citrulline a surrogate endpoint for enterocyte mass

Effectively, citrulline can be regarded as an endproduct of glutamine and/or proline metabolism of intestinal enterocytes[80,98,104,110,122]. Several enzymes are involved in the synthesis of citrulline from glutamine and/or proline[104,110,122]. Whereas OAT[116,121] can be categorized as a ubiquitous enzyme, OCT, CPSI, PROox are highly polarized and P5CS is extremely polarized[119]. Thus high activities of OCT[153], CPSI[153] and PROox[121,123] are found in the small intestine and liver. P5CS activity is almost exclusively found in small intestinal enterocytes[116,120,121]. This unique enzymatic profile, the unique role of the small intestine in whole body glutamine metabolism[96,154] and the relatively high small bowel enterocyte cell mass[126] make the small bowel epithelium the most important source of circulating citrulline[128,131]. Taken together these data indicate a high specificity for circulating citrulline, i.e. small intestinal enterocytes. Thus under steady state conditions, citrulline can be considered a marker for the functional epithelial cell mass of the small bowel, a concept amply demonstrated in experimental and clinical studies[65-75,80,83,86-88,90,104,111,113-116,132-134,152]. Of notice, the lower plasma citrulline level observed in short bowel patients was sustained up to one year after treatment[66] emphasizing its strict dependence on the epithelial cell mass. As such, plasma citrulline concentration has been proposed as a biological marker for viable small bowel epithelium[65-67,69,71,73-75,152]. Crenn *et al*[69] have recently correlated plasma citrulline concentration with histologically graded villous atrophy in 42 patients with celiac and 10 patients with non-celiac villous atrophy disease. These authors identified a threshold value of 10 μmol/L (25% of the mean normal baseline value) to be predictive for severe and extensive villous atrophy and 20 μmol/L to be predictive for severe villous atrophy, whatever the extent. The plasma level of citrulline was thus indicative for the degree of villous atrophy. This finding is indicative for a possible volume effect. Crenn *et al*[69] demonstrated the use of plasma citrulline for monitoring treatment response in patients with celiac disease, indicating the simplicity of the marker in clinical practice. The accuracy of the assay has been assessed for various clinical settings. Crenn *et al*[66] measured plasma citrulline level in 57 patients with nonmalignant short bowel syndrome defined by a postduodenal remnant small bowel length of less than 200 cm. Minimal follow up was 2 years after definite digestive circuit modification. The threshold of plasma citrulline that best discriminated short bowel patients from controls was 30 μmol/L yielding a sensitivity, specificity, PPV and NPV of 77%, 75%, 76% and 77%, respectively. The best threshold of plasma citrulline for discrimination of transient from permanent intestinal failure was 20 μmol/L yielding a sensitivity, specificity, PPV and NPV of 92%, 90%, 95% and 85%, respectively. In a series of 52 patients with celiac and nonceliac villous atrophy Crenn *et al*[69] correlated plasma citrulline and mucosal atrophy assessed by endoscopic mucosal biopsies. The threshold of plasma citrulline for discrimination between nondestructive and destructive mucosal lesions (modified Marsh classification) was 20 μmol/L yielding a sensitivity, specificity, PPV and NPV of 95%, 90%, 88% and 96%, respectively. Gondolesi *et al*[75] measured plasma citrulline in 49 intestinal transplant recipients within 12 h before or after endoscopic biopsies taken according to a protocol (i.e. twice weekly for 6 wk, once weekly until 6 mo and monthly until 1 year postintestinal transplant). The sensitivity and specificity of the citrulline assay for diagnosing transplant rejection in adults was 80% and 58%, respectively.

PLASMA CITRULLINE: A SURROGATE ENDPOINT FOR RADIATION INDUCED EPITHELIAL CELL LOSS

Taken together, plasma citrulline is a candidate marker for measuring radiation-induced epithelial small bowel damage. The data indicate that this biomarker is tissue-specific, i.e. small intestinal epithelium. The biomarker corresponds with an important morpholocal endpoint, i.e. mucosal atrophy, and is easily accessible in clinical practice. Although experimental[120] and clinical data[111] suggest a non-homogenous distribution of citrulline production, a volume effect is suggested by the data provided by Crenn *et al*[66,69].

A decrease of intestinal absorptive function following irradiation has been correlated to the loss of

functionally active enterocytes constituting the absorptive mucosal surface[23-26]. The correlation between radiation-induced epithelial cell loss and plasma citrulline level was demonstrated in mice by Lutgens *et al*[76] Following treatment with a single whole body irradiation (WBI) (dose range 0-14.9 Gy) blood and jejunal tissue were sampled for analysis. At 84 h and 4 d after WBI a dose response relationship was observed for plasma citrulline level. At this time point plasma citrulline correlated with mucosal surface, a surrogate endpoint for functional enterocyte mass. Plasma citrulline level decreased as a function of dose and time after WBI. Whereas the time effect was significant for all dose levels used, a significant dose-response relationship was observed only at d 4 after WBI. Remarkably, a rapid decline of plasma citrulline was observed at the first 2 d after WBI independent of the WBI doses used whereas recovery was more rapid for the lowest dose (i.e. 8 Gy) and incomplete during the observation period for the highest dose levels used (i.e. 11 and 12 Gy). This time and dose pattern is in agreement with the radiation effect on the hierarchically structured intestinal epithelium[155]. Interestingly, using the epithelial surface lining as a parameter did not yield significant changes except for the 4 d time point for the highest dose levels (i.e. 11 and 12 Gy) whereas for citrullinemia significant changes were observed for all dose levels used at the 4 d time point. Furthermore, plasma citrulline levels remained significantly decreased at the 11 d time point. For the dose range used in our experiments, mean values for mucosal surface lining ranged between 56% and 130% of control values, whereas for citrullinemia mean values ranged between 6% and 121% of control values. Thus citrullinemia seems to be more sensitive for detecting and monitoring small bowel radiation-induced epithelial cell loss than the representative morphologic endpoint used in these experiments. After WBI doses of 1-3 Gy no effect on citrullinemia could be demonstrated whereas this parameter was inversely proportional to WBI doses of 3-12 Gy. The threshold dose for the citrulline assay (about 3 Gy) is significantly lower as compared to the microcolony assay (about 8 Gy). Furthermore, in contrast to the microcolony assay the citrulline assay permits repeated measurements within the same animal. Therefore the citrulline assay and the microcolony assay are supplementary, both with regard to the dose range as with regard to their applicability.

The use of plasma citrulline as an assay for acute small bowel epithelial radiation injury was demonstrated by Lutgens *et al*[76] Amifostine was administered to mice as a radioprotective agent with a consistently found dose modification factor (DMF) of 1.6 using the microcolony assay as endpoint[156]. The DMF observed for citrulline (1.5) was in complete agreement with literature data. Vanclee *et al*[79] have used the citrulline assay to demonstrate a protective effect of keratinocyte growth factor on cytotoxic treatment induced intestinal injury.

The feasibility of plasma citrulline as a surrogate marker for radiation-induced small bowel injury was demonstrated by Lutgens *et al*[30] in a prospective clinical study in patients treated with fractionated radiotherapy for abdominal and/or pelvic cancer sites. A dose and volume effect was observed using dose volume histogram parameters and plasma citrulline levels as endpoints. Median nadir citrulline levels were observed during the 3rd wk of fractionated radiotherapy. This time course of plasma citrulline was further established in two clinical studies using archive material of patients treated with intensive myeloablative therapy[77,78]. Following conditioning treatment with high dose chemotherapy and fractionated WBI nadir plasma citrulline levels were observed around 7 d after hematopoietic stem cell transplant. Sensitivity and specificity of the citrulline assay were better compared to standard endpoints used for assessment of gut damage[77].

CONCLUSION

Radiation-induced small bowel damage is unlikely to be expressed or quantified by a single functional or morphological parameter. Several biomarkers are currently available differing with respect to kinetics, related target cells and pathophysiological processes involved and the convenience for clinical use. It is thus challenging to choose a (set of) biomarker(s) that is best suited to a specific experimental or clinical setting. Citrulline is a promising candidate biomarker. A dose-response relationship[76] and a correlation with epithelial cell mass[76,79] have been recently demonstrated in experimental studies. The time course of plasma citrulline following radiation[30,76,78] is in agreement with well known radiation effects on the hierarchically structured intestinal epithelium[155] and clinical observations of acute intestinal injury. The feasibility of the marker was demonstrated in a series of patients treated with fractionated radiotherapy for pelvic and/or abdominal cancers[30]. Unlike most other used endpoints, the citrulline assay can be applied to both experimental and clinical settings facilitating translational research. Also citrulline can be repeatedly measured enabling monitoring of treatment effects. Finally, the assay is simple to apply and relatively cheap. Like surgery[65-68], celiac and non-celiac disease[69] and acute cellular rejection following small bowel transplantation[70-73,75,152], ionizing irradiation has been demonstrated to be an additional event associated with reduced small bowel epithelial cell mass that can be monitored by plasma citrulline[30,76-79].

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