

World Journal of *Gastroenterology*

World J Gastroenterol 2017 April 28; 23(16): 2819-3010



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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

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World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents[®]/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch[®]), Journal Citation Reports[®], Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports[®] released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

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I-IX Editorial Board

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER

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7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
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PUBLICATION DATE
April 28, 2017

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Basic Study

Detection and characterization of murine colitis and carcinogenesis by molecularly targeted contrast-enhanced ultrasound

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Institutional review board statement: The study was reviewed and approved by the University Hospital of Münster - Department of Medicine B Institutional Review Board.

Institutional animal care and use committee statement: All procedures using animals were reviewed and approved by the local animal subjects committee, University of Münster (permit

84-02.04.2013.A093).

Conflict-of-interest statement: None of the authors has received fees for serving as a speaker or received research funding from with regard to this study. None of the authors owns stocks and/or shares with regards to this study. None of the authors owns patents with regard to this study.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at markus.brueckner@ukmuenster.de.

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Manuscript source: Invited manuscript

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Received: December 29, 2016

Peer-review started: December 30, 2016

First decision: February 23, 2017

Revised: March 14, 2017

Accepted: March 30, 2017

Article in press: March 30, 2017

Published online: April 28, 2017

Abstract

AIM

To study mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) and vascular endothelial growth factor (VEGF)-targeted contrast enhanced ultrasound (CEUS) for the assessment of murine colitis and carcinogenesis.

METHODS

C57BL/6 mice were challenged with 3% dextran sodium-sulfate (DSS) for three, six or nine days to study the development of acute colitis. Ultrasound was performed with and without the addition of unspecific contrast agents. MAdCAM-1-targeted contrast agent was used to detect and quantify MAdCAM-1 expression. Inflammatory driven colorectal azoxymethane (AOM)/DSS-induced carcinogenesis was examined on day 42 and 84 using VEGF-targeted contrast agent. Highly specific tissue echogenicity was quantified using specialized software. Sonographic findings were correlated to tissue staining, western blot analysis and immunohistochemistry to quantify the degree of inflammation and stage of carcinogenesis.

RESULTS

Native ultrasound detected increased general bowel wall thickening that correlated with more progressed and more severe DSS-colitis (healthy mice: 0.3 mm \pm 0.03 *vs* six days DSS: 0.5 mm \pm 0.2 *vs* nine days DSS: 0.6 mm \pm 0.2, $P < 0.05$). Moreover, these sonographic findings correlated well with clinical parameters such as weight loss ($r^2 = 0.74$) and histological damage ($r^2 = 0.86$) ($P < 0.01$). In acute DSS-induced murine colitis, CEUS targeted against MAdCAM-1 detected and differentiated stages of mild, moderate and severe colitis *via* calculation of mean pixel contrast intensity in decibel (9.6 dB \pm 1.6 *vs* 12.9 dB \pm 1.4 *vs* 18 dB \pm 3.33, $P < 0.05$). Employing the AOM/DSS-induced carcinogenesis model, tumor development was monitored by CEUS targeted against VEGF and detected a significantly increased echogenicity in tumors as compared to adjacent healthy mucosa (healthy mucosa, 1.6 dB \pm 1.4 *vs* 42 d, 18.2 dB \pm 3.3 *vs* 84 d, 18.6 dB \pm 4.9, $P < 0.01$). Tissue echogenicity strongly correlated with histological analysis and immunohistochemistry findings (VEGF-positive cells in 10 high power fields of healthy mucosa: 1 \pm 1.2 *vs* 42 d after DSS start: 2.4 \pm 1.6 *vs* 84 d after DSS start: 3.5 \pm 1.3, $P < 0.01$).

CONCLUSION

Molecularly targeted CEUS is a highly specific and non-invasive imaging modality, which characterizes murine intestinal inflammation and carcinogenesis *in vivo*.

Key words: Colitis; Dextran sodium-sulfate; AOM-DSS; Carcinogenesis; Ultrasound; Contrast-enhanced ultrasound; Vascular endothelial growth factor; Mucosal addressin cellular adhesion molecule-1

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Core tip: Murine models of colitis and carcinogenesis are widely used to study novel diagnostic approaches and to preclinically evaluate promising drug candidates. However, valid assessment of severity of inflammation or cancer development routinely requires *post mortem* histological analysis. Our study provides evidence that non-invasive contrast enhanced ultrasound (CEUS) is feasible to specifically target intestinal inflammation and carcinogenesis. While MAdCAM-1-directed CEUS follows the severity of murine dextran sodium-sulfate (DSS)-colitis, the use of VEGF-targeted contrast agent allows for characterization of cancer development in the AOM/DSS-induced model of colitis-associated cancer.

Brückner M, Heidemann J, Nowacki TM, Cordes F, Stypmann J, Lenz P, Gohar F, Lügering A, Bettenworth D. Detection and characterization of murine colitis and carcinogenesis by molecularly targeted contrast-enhanced ultrasound. *World J Gastroenterol* 2017; 23(16): 2899-2911 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i16/2899.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i16.2899>

INTRODUCTION

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing and remitting inflammatory disorders of the gastrointestinal tract often resulting in challenging clinical diagnostic and therapeutic scenarios^[1,2]. Accurate assessment of inflammation is a prerequisite for achieving disease control. Furthermore, subjective clinical evaluation of IBD patients may miss subclinical inflammation and therefore endoscopy and/or cross sectional imaging techniques are crucial to improve diagnosis^[3]. Inconsistencies between disease activity indices, endoscopic phenotype^[4,5] and microscopic findings^[6,7] are commonplace. Systemic inflammatory parameters such as C-reactive protein (CRP)^[8], erythrocyte sedimentation rate^[9] or biomarkers like fecal calprotectin^[10] are of limited value for evaluating disease activity as they do not distinguish well between different degrees of inflammation^[11]. However, chronic intestinal inflammation represents a risk factor for the development of colitis-associated cancer^[12,13]. It has been reported that around 15% of all UC-related deaths result from colorectal cancer (CRC)^[14,15]. Therefore, endoscopic surveillance schedules are recommended as part of international clinical guidelines^[16,17]. Even with surveillance there remains a substantial risk that adenoma or CRC may go undetected^[18,19].

Currently, the combined approach of meticulous endoscopic evaluation and subsequent histological workup of multiple biopsies is considered to be the gold standard for accurate diagnosis and follow-up examinations of IBD patients^[16,20]. Nevertheless, repeated invasive colonoscopic examinations along

with the required bowel cleansing procedures are potentially uncomfortable and can therefore result in suboptimal investigation^[21]. Consequently, more specific and non-invasive imaging methods are necessary to improve diagnosis and monitoring in IBD patients.

In contrast to endoscopy, high frequency ultrasound (US) of the bowel offers the possibility of performing serial follow-up investigations non-invasively^[22,23]. Furthermore, available scoring systems allow native ultrasonic examination of the small and large bowel to be standardized and more reliable, facilitating the detection of severe complications such as fistulae and stenoses^[24]. Sensitivity and specificity rates for the detection of stenoses and fistulae are comparable to that of computed tomography^[25,26] and magnetic resonance imaging (MRI)^[27,28]. CEUS refers to the intravenous delivery of gas-filled micro-bubbles (MB), which have an approximate diameter of 2.9 μm , stabilized by a lipid shell. These MBs accumulate predominantly in well-perfused tissue resulting in specific echogenic changes, which can help discriminate the differential diagnoses and is therefore advantageous compared to native US^[29]. CEUS was first clinically used in patients with liver lesions and then successfully adopted for human IBD in order to assess the degree of inflammation^[30,31]. The advancement of CEUS led to the use of targeted contrast agents, most frequently biotinylated antibodies, which are bound to streptavidin molecules located on the outer shell of MB. The contrast agent can be used to specifically target endothelial molecular components such as VEGF and mucosal specific MAdCAM-1, giving specific contrast enhancement of tissues expressing the target molecules.

The aim of this study is to evaluate the feasibility of detection and characterization of experimental colitis by the use of MAdCAM-1-targeted contrast agent ultrasound. Additionally, experimental colitis-associated cancer is studied by use of anti-VEGF-targeted contrast agent.

MATERIALS AND METHODS

Mice

All procedures using animals were reviewed and approved by the local animal subjects committee, University of Münster (permit 84-02.04.2013.A093). Female C57BL/6 mice (8-10 wk of age, 20-22 g) (Charles River Laboratories, Sulzfeld, Germany), were acclimatized for 2 wk prior to the start of experiments. Five mice were housed per cage and maintained in air-conditioned quarters with a room temperature of $23 \pm 2^\circ\text{C}$, controlled relative humidity of $50\% \pm 10\%$ and an alternating 12 h light/dark-cycle. Mice were fed with standard chow and autoclaved tap water *ad libitum*. Chows was removed from cages 12 h prior to ultrasound examination. For ultrasound examinations,

abdomen was depilated with standard depilatory cream. All animals were euthanized by isoflurane overdose before tissue collection.

Chemically induced colitis

Colitis was induced as described previously^[32]. Briefly, drinking water was replaced by 3% (w/v) DSS (MP Biomedicals, Illkirch, France) for a period of three, six or nine days. This strategy leads to a reliable and reproducible acute colitis^[33]. On the last day of administration, mice received ultrasound examination and were subsequently euthanized by an overdose of anesthesia^[34]. The colon was removed, opened and split longitudinally with the intestines being used for various analyses.

Induction of colorectal cancer

Pretreatment with a single intraperitoneal injection of mutagenic AOM (10 mg/kg) was followed by three cycles of 2.5% (w/v) DSS in drinking water (days 0 to 7, days 21 to 28 and days 42 to 49)^[35]. Mice received autoclaved water in treatment-free intervals. After 42 and 84 d, targeted contrast agent ultrasound was performed in anesthetized mice. After the final examination, mice were euthanized by an overdose of anesthesia. Colons were removed, opened and split longitudinally with the intestines being used for various analyses.

Histological colitis score

After explantation, colons were embedded in O.C.T. compound (Tissue-Tek, Sukura Fine Tek Europe, Zoeterwoude, NL) and kept frozen at -80°C . Sections of 5 μm thickness were stained with hematoxylin and eosin (HE) and then scored according to the Dieleman colitis score^[36]. Colons were graded by three blinded investigators (M.B., D.B., F.C.) by the amount of inflammation and the depth of inflammation, graded 0 to 3, as well as being graded 0 to 4 for the amount of crypt damage or regeneration detected. These changes were also quantified as percentage involvement: (1) 1%-25%; (2) 26%-50%; (3) 51%-75%; and (4) 76%-100%. Points allocated were multiplied by the factor of epithelium involvement in regards to extent and presence of inflammation (range 0 to 12) and extent of regeneration and crypt damage (range 0 to 16).

Immunoblotting

Colonic tissue samples were transferred into lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X; all Sigma-Aldrich Chemie GmbH, Munich, Germany) and sonicated on ice for 10 seconds. Total protein concentrations were determined by Bradford assay (BioRad, Hercules, California, United States). 30 mg of total cellular protein per lane were size separated on 4%-20% tris glycine gel, blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Illinois, United States) and blocked

with blocking buffer (phosphate buffered saline (PBS) containing 10% (w/v) non-fat dry milk and 1% (w/v) bovine serum albumin (BSA). Blots were incubated with purified monoclonal rat anti-mouse MAdCAM-1 (clone: MECA-367, DB, Becton, Dickinson and Company, New Jersey, United States, dilution 1:250) overnight at 4 °C. MAdCAM-1 is a tissue- and disease-specific endothelial cell adhesion molecule critically involved in lymphocyte homing^[37,38] and its expression is massively increased in inflamed intestinal tissues of IBD patients^[39,40]. Immunodetection was carried out using biotinylated goat anti-rat Ig (dilution 1:1000) (DB, Becton, Dickinson and Company, New Jersey, United States) for 1 h at room temperature, followed by streptavidin horseradish peroxidase and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, United States).

Immunohistochemistry

Cryostat colon sections of 7 µm thickness were air-dried, fixed for 10 min in pure acetone at 20 °C and blocked with blocking buffer (PBS containing 0.1% BSA and 10% goat serum, Sigma-Aldrich Chemie GmbH, Munich, Germany). A three-step staining was used, incubating the slides with solutions of purified polyclonal rabbit anti-murine VEGF₁₆₅ antibody (dilution 1:100, PeproTech, New Jersey, United States), secondly biotinylated goat anti-rabbit Ig (dilution 1:100, DB, Becton, Dickinson and Company, New Jersey, United States) and then Streptavidin Alexa Flour 546 conjugate (1:100; Molecular Probes, Luiden, NL). Finally, nuclei were counterstained with 4,6-Diamidine-2-phenylindoldihydrochloride (DAPI; Sigma-Aldrich Chemie GmbH, Munich, Germany). Positively stained cells were counted in at least 10 representative HPF per section and data were expressed semi-quantitatively.

In vivo contrast agent ultrasound

Mice were anesthetized with isoflurane gas (100% v/v, 1.5 Vol. %, 1.5 L/min) (Florene, Abbott, Wiesbaden, Germany) and fixed onto a preheated plate in order to maintain the body temperature. Both heart and respiratory rates as well as body temperature of the mice was monitored. Ultrasound contact gel (2 mL per animal, SAVGel-Ultraschallkontaktgel, SAV Liquid Production, Flintsbach am Inn, Germany) was instilled into the colon using a buttoned blunt metal cannula (16F) to enhance echogenicity and determination of the shape of the colon wall. Ultrasound examinations were performed using the Vevo® 2100 ultrasound system, specifically designed for small animal imaging (FujiFilm VisualSonics, Toronto, Canada). The system is fitted with a dedicated transducer detecting non-linear harmonic responses from MB and has a center frequency of 21 MHz (MS 250, FujiFilm VisualSonics, Toronto, Canada). Two-dimensional images were recorded with the transducer fixed in a retainer in the sagittal plane with imaging settings focus, image depth

and gain kept constant for all animals in an imaging session to minimize intra-examination variability. The ultrasound procedure was always performed in colon segments between the specific anatomical landmarks; (1) cranial part of the urinary bladder and left lower kidney pole; and (2) left lower and upper kidney pole. After conventional B-Mode ultrasound imaging of the colon wall, targeted CEUS was performed by application of 50 µL of contrast agent intravenously *via* the tail vein. First, Vevo MicroMarker™ Target-Ready Contrast Agent (FujiFilm VisualSonics, Toronto, Canada) was prepared with a particular isotype control antibody in order to block unspecific receptors (APC Rat IgG2a, κ Isotype Control, Biolegend, California, United States). Secondly, Vevo MicroMarker™ was prepared with biotinylated rat anti-mouse MAdCAM-1 (DB, Becton, Dickinson and Company, New Jersey, United States) or biotinylated rat anti-mouse VEGF (BioLegend, California, United States) according to the manufacturer's instructions. An infusion pump (Vevo Infusion Pump, FujiFilm VisualSonics, Toronto, Canada) ensured continuous and consistent infusion of the contrast agent at a flow rate of 20 µL per minute. With the start of contrast agent application, an image sequence of the bolus perfusion measuring time until maximal pixel contrast intensity was acquired ("time to peak flow"), providing information about the tissue perfusion. Secondly, a destruction-replenishment sequence was performed with five ultrasound pulses of high acoustic power destroying all MBs in the field of view and acquiring reperfusion with contrast agent four minutes after bolus injection. Importantly, the used destruction impulse destroyed only the MBs and did not affect the antibodies. Before initiating the destruction-replenishment sequence, measured echogenicity consisted of MBs bound to their endothelial target *via* antibody plus MBs flowing by in vessels. The applied destruction impulse destroyed all MBs in the field of view with the antibodies staying attached to their endothelial target and continually blocking it. After the destruction impulse, the measured echogenicity consisted only of MBs flowing by in vessels, as antibodies still blocked the endothelial targets preventing new compound from binding to the endothelial target. The difference between echogenicity after destruction sequence and echogenicity before the sequence led to values of echogenicity arising from highly specifically targeted MBs:

$$\text{Echogenicity (molecularly targeted MBs + flowing MBs)} - \text{echogenicity (flowing MBs)} = \text{echogenicity of molecularly targeted MBs}$$

This sequence enabled highly specific molecularly targeted CEUS, which required two sequences of application of contrast agent. First, a sequence with MBs bound to the particular isotype control antibody was recorded. Second, a sequence with MBs bound to

specific targeted antibody (MAdCAM-1 and VEGF) was recorded.

Calculation of pixel contrast intensity

All sequences were exported and analyzed with Vevo LAB Software (Version 1.7.2, FujiFilm VisualSonics, Toronto, Canada). This software application, among other functions, generates several parameters of perfusion by analyzing the kinetics of contrast-uptake. Tissue contrast-uptake is relatively quantified in mean pixel contrast intensity [LINEAR arbitrary unit (a.u.)] which is then converted into arbitrary unit decibel (dB). Contrast-uptake was measured in three regions of interest (ROI) in the descending colon of mice, where DSS-induced inflammation is reliably observed. For reliable measurements, signals from ROI of isotype control examinations were subtracted from corresponding MAdCAM-1 and VEGF examinations. Decibel-values of the isotype control subtracted from dB-values of targeted antibodies allowed calculation of dB-values specifically generated by MB targeted against MAdCAM-1 or VEGF, which directly correlate with the expression of the targeted receptor^[41]. Time until maximal pixel contrast intensity was achieved measured as "time to peak flow", providing information about tissue perfusion dynamics.

Statistical analysis

Results are expressed as mean \pm SD. Student's *t* test or analysis of variance (ANOVA) along with Tukey-HSD Test was used to compare results and the level of local significance (*P*) was 0.05. Individual experiments were performed at least three times. Statistical analyses were performed using IBM SPSS® Statistics 24.0 for Mac OS (IBM Corporation, Somers, NY, United States).

Institutional animal care and use committee statement

All procedures using animals were reviewed and approved by the local animal subjects committee of the University of Münster (permit 84-02.04.2013. A093).

RESULTS

Sonographic measurements of colonic wall thickness reflect disease severity in murine DSS-induced colitis

To establish the different degrees of colitis severity, mice (*n* = 10 per group) received DSS in drinking water for three, six or nine days. During the experiments, disease severity was monitored by determination of the relative loss of body weight (Figure 1A). Compared to healthy control mice, animals receiving DSS-treatment for three days only had a minor loss of body weight (0.99 ± 0.03), which was less pronounced compared to animals challenged for six or nine days (0.93 ± 0.03 and 0.86 ± 0.06 , respectively, *P* < 0.01; Figure 1A). *Post mortem*,

colonic histological damage was scored using the well-established scoring system according to Dieleman^[36]. In line with the finding of a significantly decreased relative body weight in groups treated with six or nine days of DSS, histological damage was also maximal in these two groups (six days, mean 5.8 ± 1.2 points and nine days mean 16.9 ± 3.8 points) as compared to animals challenged for three days or healthy controls (three days, mean 2 ± 0.9 and no loss of body weight in healthy controls, *P* < 0.01; Figure 1B). B-mode ultrasound of the colonic wall was performed analogous to human large bowel ultrasound considering the following anatomical landmarks: cranial part of the urinary bladder, left lower kidney pole and left upper kidney pole (Figure 2A and B). Colon wall thickness in healthy C57BL/6 mice was determined to be 0.3 mm (± 0.03). In contrast, colitic mice showed a significant increase in wall thickness with maximal values in animals treated for six and nine day with DSS (six days, $0.5 \text{ mm} \pm 0.2$ and $0.6 \text{ mm} \pm 0.2$, *P* < 0.05). A strong correlation between the increase in colonic wall thickness and histological damage ($r^2 = 0.86$), as well as with weight loss ($r^2 = 0.74$) was observed (*P* < 0.01; Figure 2C). Given that perfusion is enhanced in actively inflamed tissue, the ability of CEUS to visualize and quantify perfusion has proven helpful to assess inflammatory alterations^[42]. Interestingly, the time from contrast agent application to peak flow "time to peak flow" was dramatically shortened in animals DSS-treated for three, six and nine days ($6.1 \text{ s} \pm 0.7 \text{ s}$; $4.9 \text{ s} \pm 0.5 \text{ s}$ and $4.9 \text{ s} \pm 0.7 \text{ s}$, respectively) as compared to healthy control animals ($11.3 \text{ s} \pm 2.2 \text{ s}$, *P* < 0.01). These findings illustrate the additional value of the use of contrast agents in ultrasound diagnostics. Taken together, our results indicate a strong correlation between ultrasound findings, histological alterations and clinical parameters of colitis severity in mice.

Contrast-enhanced ultrasound targeting MAdCAM-1 mirrors colitis activity

MAdCAM-1 expression is massively and almost exclusively increased during intestinal inflammation and is localised to the small bowel and colon. Therefore, MAdCAM-1 is a promising molecular target to detect and characterize DSS-induced colitis. For sonographic detection of MAdCAM-1 protein expression, contrast agent targeting its isotype antibodies was administered to block unspecific signals in subsequent examinations. After *in situ* destruction of all isotype-bound contrast agents by high mechanical index ultrasound pulses, CEUS targeted against MAdCAM-1 using specific contrast agents was performed (Figure 3). A mean echogenicity of $3.9 \text{ dB} \pm 1.3$ was measured in healthy control animals, which was significantly increased in mice challenged with DSS for three, six or nine days ($9.6 \text{ dB} \pm 1.6$; $12.9 \text{ dB} \pm 1.4$ and $18 \text{ dB} \pm 3.3$ respectively). The selectivity of the MAdCAM-1-targeted CEUS was

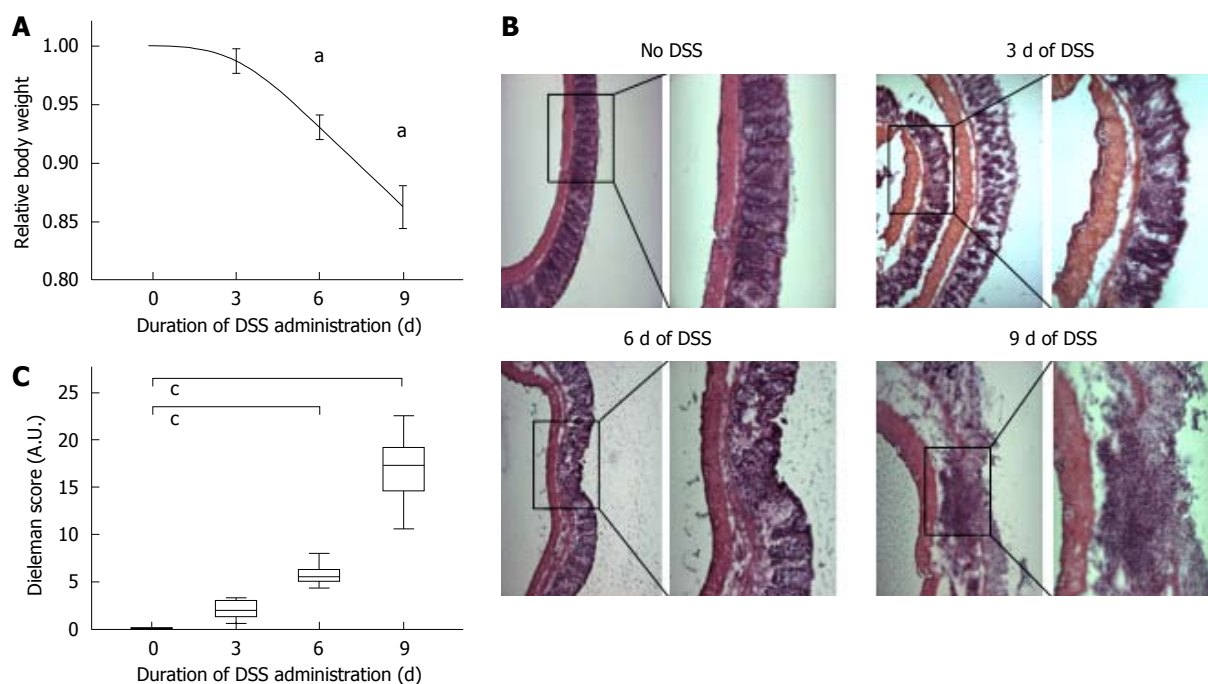


Figure 1 Assessment of dextran sodium-sulfate-induced colitis by evaluation of relative body weight loss and histological damage. C57BL/6 WT mice received 3% DSS in their drinking water for 3, 6 or 9 d, respectively. Inflammation was monitored by daily measurement of individual body weights. A: Development of relative body weight after 3, 6 and 9 d of treatment with DSS. Control group received tap water only. Data are mean \pm SD; $n = 10$ for each group, $^aP < 0.01$; B: Representative HE-stained histologic images of resected colons showing increasing inflammatory infiltrates (left panels, magnification $\times 10$; right panels, magnification $\times 20$); C: Histologic Dieleman Score (range from 0-40 points) describing inflammation, depth of inflammation and crypt damage or regeneration. Boxplot with median and whiskers, $n = 10$ for each group, $^cP < 0.01$. DSS: Dextran sodium-sulfate.

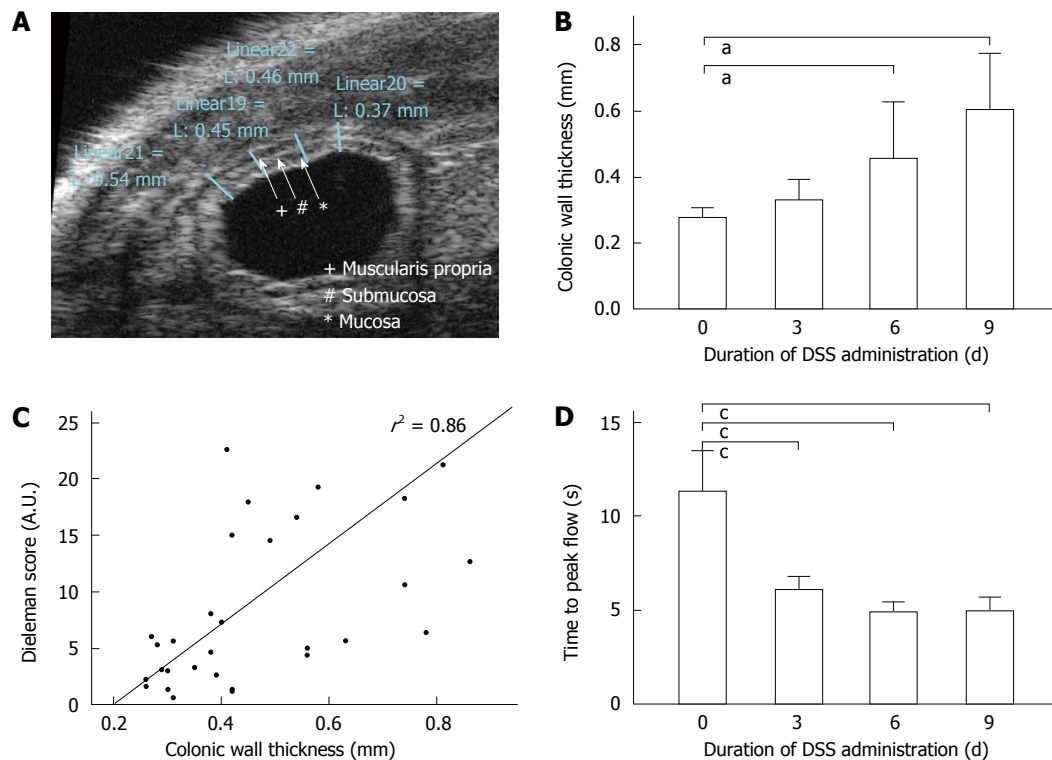


Figure 2 Contrast-enhanced ultrasound accurately detects dextran sodium-sulfate-induced colitis. A: Native ultrasound of the murine colon visualizing the bowel wall stratification. Thickness of the wall was measured in triplicate. Colon filled with ultrasound contact gel for improved testing conditions; B: Colonic wall thicknesses correlate with exposure to DSS in a time-dependent manner. DSS treatment led to a loss of sonographic bowel wall stratification (not shown). Data are means \pm SD, $n = 10$ for each group, $^aP < 0.05$; C: Linear regression between parameters thickness of colon wall and histological Dieleman score shows a significant correlation as indicated by determination coefficient $r^2 = 0.86$; D: Time to peak flow depicting start of contrast agent application until maximal pixel contrast intensity in seconds. Time to peak flow correlates with exposure to DSS in a time-dependent manner. Data are mean \pm SD, $n = 10$ for each group, $^cP < 0.01$.

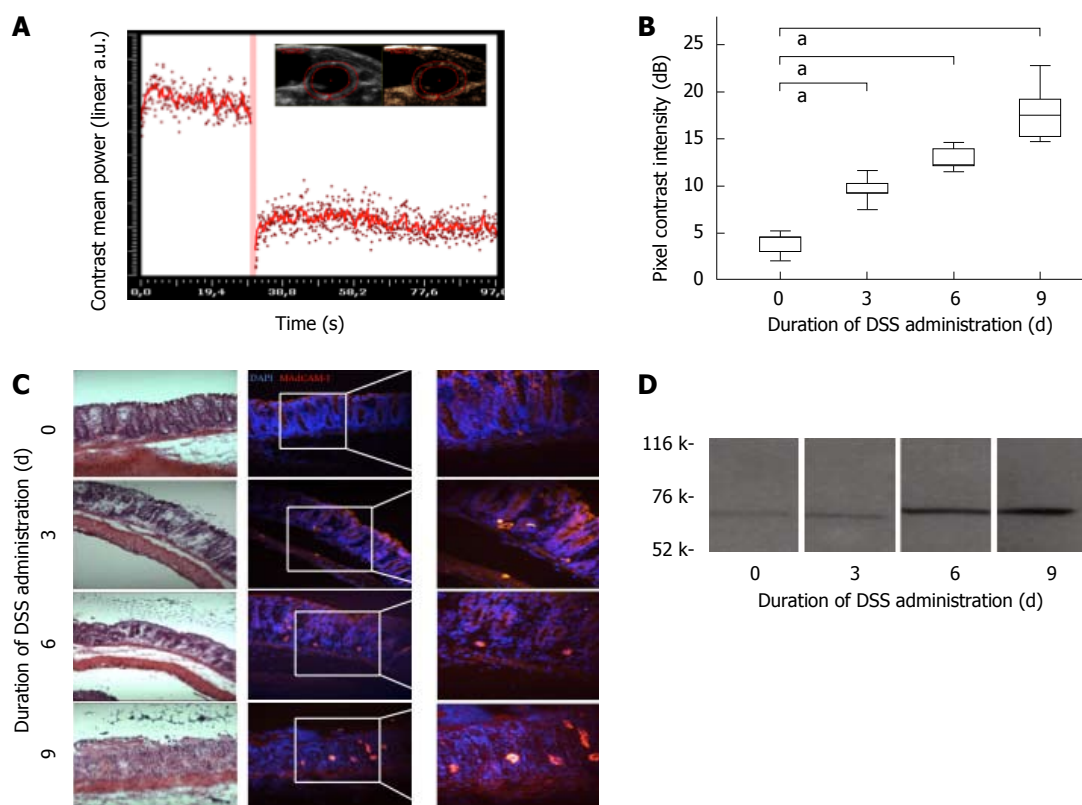


Figure 3 mucosal addressin cellular adhesion molecule-1-targeted contrast enhanced ultrasound reflects experimental intestinal inflammation. **A:** Schematic illustration of a destruction-replenishment sequence with ultrasound pulses of high acoustic power (red bar) and reperfusion of contrast agent for acquisition of highly specific molecularly targeted CEUS. Image insert showing B mode image (left) and corresponding CEUS image (right); **B:** CEUS with targeted contrast agent against MAdCAM-1. Contrast mean power of defined ROI in descending colon in arbitrary unit dB. Decibel values correlate with exposure to DSS in a time-dependent manner. Boxplot with median and whiskers, $n = 10$ for each group, $^*P < 0.05$; **C:** Immunofluorescence with staining against MAdCAM-1 (bright red), cell nuclei counterstained with DAPI (blue). Positively stained cells were counted in at least 10 representative high power fields per section and cell numbers were expressed semi-quantitatively. Left panel: corresponding HE stained sections; **D:** Immunoblot with purified anti-mouse MAdCAM-1 antibody showing a characteristic protein band at 58-66 kDa. Animals exposed to DSS for 6 and 9 d showing stronger expression of MAdCAM-1 than control animals. DSS: Dextran sodium-sulfate; CEUS: Contrast-enhanced ultrasound; MAdCAM-1: Mucosal addressin cellular adhesion molecule-1.

emphasized by a significant difference in echogenicity between all groups tested as compared to healthy control group ($P < 0.001$). To corroborate these *in vivo* findings, immunoblotting of colonic tissue samples was performed, revealing significantly stronger MAdCAM-1 expression in DSS-treated mice for 6 and 9 d as compared to those treated only for 3 d and healthy controls. In immunohistochemistry, positively stained cells for MAdCAM-1 were counted in 10 representative HPF per section and were expressed semi-quantitatively. In healthy control animals 1.7 ± 1.4 positively stained cells could be detected, animals challenged with DSS for three, six and nine days showed a significantly increased expression of MAdCAM-positive cells in IHC ($2.6 \text{ cells} \pm 1.7$, $3.5 \text{ cells} \pm 1.4$, $4.1 \text{ cells} \pm 2$, respectively, $P < 0.001$). These data underscore the increased colonic tissue expression of MAdCAM-1 in colitic mice.

Detection and quantification of carcinoma-associated angiogenesis via CEUS targeted against VEGF

Longstanding chronic colitis is associated with an increased risk for colitis-associated cancer development and thus demands an appropriate diagnostic tool for

cancer surveillance. As VEGF functions as a key growth factor in tumor angiogenesis, VEGF-targeted CEUS using VEGF-specific molecular probes was performed to detect experimental colorectal cancer following AOM/DSS administration in WT mice^[43]. Carcinogenesis was examined at three different time points. While the first group was examined prior to AOM/DSS administration, two other groups were investigated 42 and 84 d after AOM/DSS treatment was commenced. After ensuring specificity by performing CEUS targeted against an isotype control, VEGF-targeted ultrasound of murine colons was performed. After correction of values for VEGF echogenicity according to healthy adjacent colonic tissue, at day 42 after AOM/DSS start, targeted CEUS of detected tumors was measured of $18.2 \text{ dB} \pm 3.3 \text{ dB}$. 84 d after AOM/DSS start, mean value of $18.6 \text{ dB} \pm 4.9 \text{ dB}$ was detected and was significantly increased as compared to healthy control mice ($1.6 \text{ dB} \pm 1.4 \text{ dB}$, $P < 0.01$) (Figure 4). No significant difference in echogenicity was observed in tumors of animals after 42 and 84 d after AOM/DSS treatment was begun. *Ex vivo*, tumours were confirmed by HE-staining and immunofluorescence staining assessed VEGF expression in malignant tissue samples. More

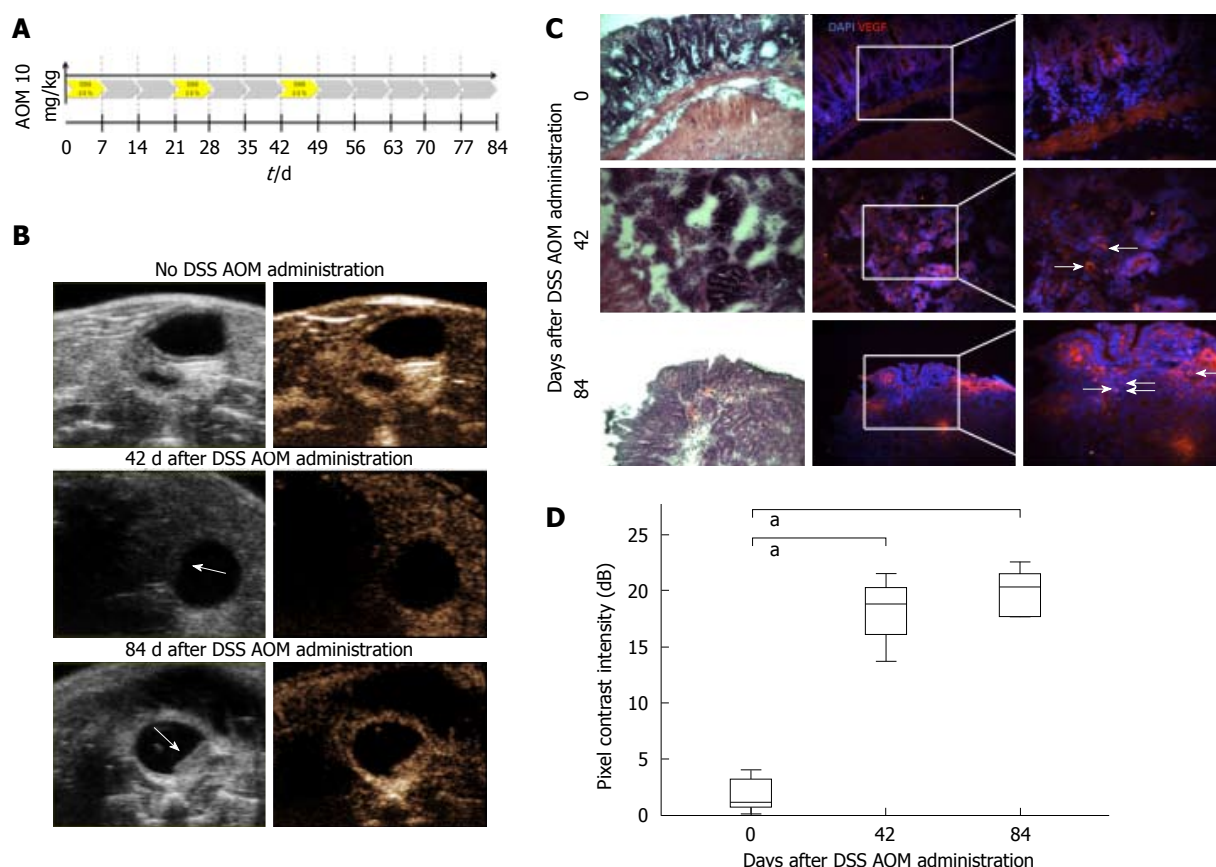


Figure 4 Evaluation of vascular endothelial growth factor expression in colorectal carcinogenesis by contrast-enhanced ultrasound. A: Schematic illustration of induction of dextran sodium-sulfate (DSS)/azoxymethane (AOM) colitis. Single intraperitoneal AOM application followed by three cycles of 2.5% (w/v) DSS in drinking water (days 0 to 7, days 21 to 28 and days 42 to 49); B: Schematic illustration of contrast agent ultrasound in highly specific molecularly VEGF-targeted CEUS. Development of tumors after 42 and 84 d after treatment with AOM. White arrows depicting colonic tumor mass. Left panel, B mode images; right panel, corresponding CEUS images; C: Immunohistochemistry staining against VEGF (bright red), cell nuclei were counterstained with DAPI (blue). Positively stained cells were counted in at least 10 representative high power fields per section and cell numbers were expressed semi-quantitatively; D: CEUS with targeted contrast agent against VEGF. Contrast mean power of defined ROI in descending colon in arbitrary unit dB. Decibel values correlate with exposure to DSS in a time-dependent manner. Boxplot with median and whiskers, $n = 10$ for each group, $^*P < 0.01$. VEGF: Vascular endothelial growth factor; DSS: Dextran sodium-sulfate; CEUS: Contrast-enhanced ultrasound; AOM: Azoxymethane.

specifically, the number of VEGF-positive cells in 10 HPF were counted and were significantly increased in tumor samples 42 and 84 d after AOM/DSS start as compared to colonic tissue samples from healthy mucosa (42 d: 2.4 ± 1.6 ; 84 d: 3.5 ± 1.3 vs healthy mice: 1 ± 1.2 ; $P < 0.01$).

DISCUSSION

In this study, we demonstrate that native small-animal ultrasound is feasible as a diagnostic procedure to detect and follow the course of DSS-induced colitis in mice. There was a strong correlation of ultrasound findings with conventional measures of experimental colitis such as loss of body weight and histological damage. Furthermore, applying anti-MAdCAM-1-labeled MBs allowed us to characterize and quantify MAdCAM-1 expression during the course of experimental intestinal inflammation. Finally, in the murine model of AOM/DSS-induced carcinogenesis, the use of labeled MBs directed against VEGF enabled the specific detection and quantification of a key promoter of carcinogenesis and a

clinically relevant therapeutic target.

Over recent decades, endoscopic examinations have been established to assess the gastrointestinal tract in patients with IBD^[44,45]. However, besides being not comfortable for patients, this modality is invasive and also has risks of serious complications such as perforation, especially in IBD patients with active colonic inflammation^[46,47]. Alternative diagnostic approaches such as cross-sectional imaging have been developed but may be cost-intensive in terms of magnetic resonance imaging or associated with radiation exposure with regard to computed tomography^[48,49]. In contrast, ultrasonography is a non-invasive and inexpensive imaging modality that is well tolerated by patients. In addition, a large meta-analysis demonstrated comparable diagnostic accuracy for ultrasound and cross-sectional imaging modalities regarding the diagnosis of IBD^[50]. Therefore, several current guidelines recommend bowel sonography for evaluating the terminal ileum and the large bowel^[51]. Accordingly, ultrasound has already been integrated in the diagnostic work up for IBD patients in several European countries

such as Germany and Italy^[52]. In contrast to the clinical investigation of IBD patients, experimental assessment of the intestinal inflammation using small-animal ultrasound is limited. Nevertheless, over recent years, technical characteristics of transducers have significantly evolved providing appropriate resolution, frequency and penetration for ultrasound examination of mice^[53,54]. The methodological settings used in our study employing a 13-24 MHz nonlinear contrast transducer enables accurate morphologic evaluation of anatomical structures in animals < 250 g body weight. Of note, specific recommendations regarding the positioning of mice and transducer as well as suggestions about the appropriate use of cleansing methods prior to ultrasound are available and necessary to ensure valid and reproducible findings^[55]. However, small animal bowel ultrasound does require a high level of technical expertise. As a prerequisite, long ultrasound examinations demand continuous monitoring of vital parameters of the animals; stable venous cannulation is needed for safe application of contrast agent. For reliable testing conditions and comparable accurate results, moderate bowel cleansing can be reached *via* a short overnight starving and rectal instillation of ultrasound contact gel. To avoid interference of bowel peristalsis on the US examination, repeated scans at fixed anatomical landmarks like the upper or lower kidney pole are advisable. Following this approach, the chance of missing an inflammatory or tumorous colonic lesion is minimized. Since there remains a risk, especially in early tumorous lesions, of missing a diagnosis in murine two-dimensional ultrasound of the bowel, the technique of three-dimensional ultrasound can be considered for long-segment examination of the colon. Automated movement of transducers allows for a three-dimensional acquisition of native and CEUS pictures^[56].

The observed increase of colonic wall thickness over the course of colitis is in line with ultrasonography findings from human studies in IBD patients. Most recently, a prospective multicenter study followed 234 CD patients with an acute flare of disease over a period of 12 mo. After medical treatment escalation, a successful induction of clinical remission was mirrored by a significant reduction in the Harvey-Bradshaw index, an index of clinical disease activity. Along with the clinical improvement of disease activity, a significant decrease of colonic wall thickening was measured, which correlated well with a decrease in serum CRP-concentrations three months after treatment escalation^[57]. Another study by Novak *et al.*^[58] showed that ultrasound evaluation of bowel wall thickness and other variables was able to detect residual disease activity in patients with clinically quiescent CD. As a gold standard to measure colitis severity, we determined changes in body weight as well as histological damage, assessed by the Dieleman Score. Both parameters showed a marked correlation with increase of bowel wall thickness determined by

ultrasonography.

Facilitated by advances in gene array analyses, molecular characterization of tumors has become clinically relevant in recent years with the advent of targeted therapies^[59]. In particular, the addition of anti-angiogenic therapies has greatly enriched treatment regimens and improved survival rates, successful due to the ability of malignant tumors to facilitate their own blood supply^[60,61]. In advanced stages of colon cancer, selected patients benefit from the addition of anti-VEGF antibodies (bevacizumab), which target a key promoter of tumor angiogenesis^[62]. Our studies demonstrate a successful quantification of VEGF expression by CEUS in mice, which might be useful to accelerate preclinical drug testing or as an objective endpoint when testing individual therapies. Since efficient targeting of tumor receptors with antibodies is frequently challenging because of a low diffusion rate to tumors or rapid uptake by the liver, it is essential to allow for sufficient distribution of the antibody and enrichment in the desired target region^[63]. In our hands, CEUS was highly capable in readily visualizing VEGF expression in a murine model of colon cancer.

Chronic inflammation of the bowel exposes patients to an increased risk of developing cancer^[14]. International guidelines therefore recommend endoscopic surveillance regimens for affected patients with IBD such as UC^[64]. Recently, sophisticated endoscopic imaging technologies such as dye-based chromoendoscopy and endomicroscopy-aided molecular imaging have been described as effective tools to detect mucosal inflammation and neoplasias in IBD patients and might provide a rational basis for patient tailored therapy. It was demonstrated that *in vivo* endoscopic molecular imaging of mucosal inflammatory patterns by identification of membrane-bound tumor-necrosis factor on mucosal cells as a potential driver of disease activity in CD could optimize current treatment approaches on a personalized basis^[44,65]. However, endoscopy-based approaches require an invasive procedure and are limited to the luminal site of the intestine. Ultrasound in combination with molecular imaging might allow for a non-invasive assessment of the entire intestinal wall, which could be of special interest in transmural inflammation detectable in Crohn's disease.

Leukocyte recruitment to the intestinal wall is a crucial step in the initiation and perpetuation of IBD-associated inflammation and is regulated by chemokines and adhesion molecule interactions. Several integrins participate in this mechanism, *e.g.*, the interaction between the $\alpha 4\beta 7$ -integrin on T-cells and MAdCAM-1 addressin on endothelial cells promote the accumulation of pathogenic T-cells in the inflamed mucosa^[66]. Therapeutical blockade of T-cell homing *via* the integrin $\alpha 4\beta 7$ and the cellular adhesion molecule MAdCAM-1 has been intensively studied during the last years as T cells represent a key player in the perpetuation of intestinal inflammation^[67]. Most recent

work by Wendt *et al.*^[68] demonstrated that even classic glucocorticoids act partially *via* MAdCAM-1 by reducing C-C chemokine receptor type 9-mediated chemotaxis of T-lymphocytes. Agents targeting leukocyte trafficking have been developed and some anti-integrin antibodies are already available for IBD therapy while others will follow in the near future^[69]. Vedolizumab is a humanized monoclonal antibody that recognizes the gut-specific $\alpha 4\beta 7$ subunit^[70,71]. It has been approved by EMA and FDA in moderate-to-severe UC or CD. Etrolizumab is an IgG1 humanized monoclonal antibody that binds the $\beta 7$ subunit of the $\alpha 4\beta 7$ and the $\alpha E\beta 7$ integrin heterodimers in the intestine^[72]. An IgG₂ monoclonal antibody that selectively targets gut-specific MAdCAM-1 and blocks its ability to act as a ligand for integrin $\alpha 4\beta 7$ (PF-00547659) is currently undergoing phase II evaluation for the treatment of UC^[73,74]. Recent research reveals differences between CD and UC with regard to the gut homing of T effector cells under treatment with vedolizumab. It could be shown that there is an unchanged homing of T effector cells in the colon of CD patients as the $\alpha 4\beta 1$ expression is increased in T effector cells during vedolizumab treatment, which leads to adhesion *via* the $\alpha 4\beta 1$ integrin and vascular cell adhesion molecule-1^[75]. This observation underlines that homing of T-cells proceeds *via* different and nonredundant pathways in IBD affecting treatment strategies in anti-integrin therapy and underlining the importance of monitoring long-term effects for the recruitment of T-cells very closely. Our results show that CEUS allows for direct visualization of MAdCAM-1 upregulation in inflamed areas of the bowel, which enables the detection of intestinal inflammation, visualizes the recruitment of inflammatory cells and provides an objective endpoint to assess the degree and extent of intestinal inflammation during evaluation of novel therapeutic approaches.

In summary, newly targeted diagnostic methods working on a molecular level are sustainable and lead to higher precision and individualization in distinct diagnostic questions. Applied in non-invasive ultrasound, longitudinal follow-up examinations appear promising for the evaluation of disease course and preclinical drug testing. The local application of highly concentrated drugs in the gut *via* targeted MB is a new therapeutic approach that needs further research and offers other possibilities aside from blocking endothelial receptors. In the hands of an experienced physician, the use of targeted CEUS in humans could lead to a more specific non-invasive diagnostic workup in IBD patients.

ACKNOWLEDGMENTS

We thank S. Dufentester and E. Weber for expert technical support. Thanks to Stefan Brückner for medical informatics support. Thanks to FujiFilm VisualSonics for close technical cooperation. We also

acknowledge the Interdisciplinary Centre for Clinical Research (IZKF, core unit PIX), Münster, Germany for their help to analyze the sonographic measurements, namely Dr. Sven Hermann and Richard Holtmeier.

COMMENTS

Background

Accurate clinical assessment of inflammation in inflammatory bowel diseases (IBD) requires elaborate diagnostic workup consisting different imaging techniques as well as histological examinations. Drawbacks are potentially occurring inconsistencies in the diagnostic process as well as repeated invasive follow-up examinations in terms of repeated endoscopies. Consequently, more specific and non-invasive imaging methods are necessary to improve diagnosis and monitoring in IBD patients. High frequency ultrasound (US) of the bowel offers the possibility of performing serial follow-up investigations non-invasively with good rates for sensitivity and specificity. Contrast-enhanced US (CEUS) refers to the intravenous delivery of gas-filled microbubbles (MB), which accumulate predominantly in well-perfused tissue resulting in specific echogenic changes, which can help discriminate the differential diagnoses, *e.g.*, to assess the degree of inflammation in IBD. The advancement of CEUS the use of targeted contrast agents with antibodies bound to the outer shell of MBs. This contrast agent can be used to specifically target endothelial molecular components giving specific contrast enhancement of tissues expressing the target molecules. Antibodies find their specific target and increase echogenicity *via* the MB they're bound to.

Research frontiers

For reasons of patient's comfort and to further improve current diagnostic procedures, non- or less invasive serological and technical diagnostic procedures are in focus of current IBD research. The use of different ultrasound techniques is in line with these efforts. Further, there is an intensive research for new anti-inflammatory therapies reaching beyond anti-TNF-therapy. Molecular targets like the integrin MAdCAM come in focus of research.

Innovations and breakthroughs

In this manuscript, authors were able to evaluate small animal ultrasound and targeted CEUS in a standard model of colitis, the DSS-model. With the use of MBs targeted against one of the main integrins specifically upregulated in IBD in the bowels, highly specific molecular changes of the disease became visible and helped to distinguish different stages of colitis.

Applications

Targeted CEUS aiming at endothelial molecules is a potential advancement even in the diagnostics of human IBD. The use of CEUS was originally adopted from the field of hepatology and can help to improve sensitivity and specificity of US examination. Molecularly targeted CEUS for the first time allows for visualisation of molecular changes related to IBD. This technique could improve human diagnostics as well in near future.

Terminology

CEUS: contrast enhanced ultrasound. Sonographic technique referring to the intravenous delivery of gas-filled MB, which accumulate predominantly in well-perfused tissue resulting in specific echogenic changes. Tumors or inflammation show changes in echogenicity. Targeted CEUS: targeted contrast agents with antibodies bound to the outer shell of MBs. This contrast agent can be used to specifically target endothelial molecular components giving specific contrast enhancement of tissues expressing the target molecules. MAdCAM: anti-integrin predominantly expressed in the bowels. As endothelial molecule it helps T-cells entering the site of inflammation.

Peer-review

Follow up on the long-term effects of binding up receptors important for the recruitment of T cells to manage the progression of inflammation. It is not clear if an additional destruction sequence is performed with ultrasound pulses

to destroy the targeted antibody or what the long term consequence of this interaction may be.

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P- Reviewer: Gardlik R, Henderson AL, Hakim GD **S- Editor:** Yu J
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ISSN 1007-9327

