

Extracellular DNA as a Prognostic and Therapeutic Target in Mouse Colitis under DNase I Treatment

(DNase / DSS-induced colitis / extracellular DNA / inflammation / inflammatory bowel disease)

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Abstract. The aim of this study was to investigate the potential of extracellular DNA as a prognostic and/or therapeutic target in inflammatory bowel disease. Fifty male C57BL/6J mice were used in the experiment. Acute colitis was induced by intake of 2% dextran sulphate sodium (DSS) for seven days followed by three days of water intake. DNase I was injected intravenously on days 3 and 7. Plasmatic levels of extracellular DNA (ecDNA) were measured on days 6 and 10. Weight loss, stool consistency and liquid intake were monitored throughout the experiment. Colon length and weight, myeloperoxidase activity and tumour necrosis factor α (TNF- α) levels were measured at sacrifice. DSS-treated mice displayed severe colitis, as shown by disease activity parameters. Both groups with colitis (DNase treated and untreated) had significantly poorer weight loss, colon length and stool consistency compared with control groups on water. No differences between the DNase-treated and untreated DSS groups were recorded. Myeloperoxidase activity and levels of TNF- α in colonic tissue were notably greater in both groups with colitis compared to controls. In addition, both bio-

chemical markers were improved in the DNase-treated group with colitis compared to the untreated group. Although the disease activity was proved by several independent parameters in both groups with colitis, levels of ecDNA did not show any difference between the groups throughout or at the end of experiment. The role of ecDNA in experimental colitis has not been confirmed. However, DNase I injection resulted in some improvement, and thus should be studied in more detail.

Introduction

Extracellular DNA (ecDNA) is a non-cellular component of DNA that is present in the plasma, saliva, urine, breast milk, and semen (O'Driscoll, 2007). It is present in the form of fragments of genomic or mitochondrial DNA. Under physiological conditions, the main source of ecDNA are blood cells, and certain concentration of ecDNA is also found in the plasma of healthy individuals (Zhong et al., 2000; Lui et al., 2003). In case of organ damage, such as myocardial infarction, stroke or burns, the concentration of plasmatic ecDNA rises (Antonatos et al., 2006; Chiu et al., 2006; Destouni et al., 2009; Boyko et al., 2011; Shoham et al., 2014).

The extracellular DNA is also a component of the inflammatory response of the body to injury. Under certain conditions, the inflammatory neutrophil activation can cause a specific type of cell death called NETosis with formation of so called NETs (NET – “neutrophil extracellular trap”) (Brinkmann et al., 2004). Although their primary purpose is to prevent damage to the body, their excessive accumulation, as with other molecules and inflammatory cells, may contribute to tissue damage (Masuda et al., 2016). Not surprisingly, NETs are an attractive therapeutic target in different types of animal models of disease. NETs are composed of a tangle of decondensed chromatin, histone and proteases, and DNA that is released from the decondensed chromatin serves

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Abbreviations: DNase – deoxyribonuclease, DSS – dextran sulphate sodium, ecDNA – extracellular DNA, IBD – inflammatory bowel disease, MPO – myeloperoxidase, NETs – neutrophil extracellular traps, PBS – phosphate-buffered saline, PCR – polymerase chain reaction, TNF- α – tumour necrosis factor α , TLR – toll-like receptor.

as a stabilizing factor for the structure of the NETs. One of the principles to prevent the action of NETs is disrupting their structure using DNase.

In recent years, the administration of DNase was shown to be effective in animal models, for example in organ damage induced by the experimental model of sepsis (Luo et al., 2014; Mai et al., 2015), dissemination of metastatic cells (Park et al., 2016), in ischemia-reperfusion renal impairment (Peer et al., 2016), ischemia-reperfusion injury of myocardium (Savchenko et al., 2014), acute lung injury (Caudrillier et al., 2012) and thrombosis (Brill et al., 2012). It is interesting that activation of TLR-9 through CpG dinucleotide motifs (of viruses and/or bacteria) can not only mediate the inflammatory response, but may also act in an anti-inflammatory manner, depending on the DNA that activates it (Krieg, 2002). One of the ways to remove pro-inflammatory activity of ecDNA and NETs is DNase administration. Given that the administration of DNase is an approach aimed to cleave the ecDNA, it analyses the efficacy of blocking the NETs, but also explains the actual role or the nucleic acid itself.

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis. The pathophysiology involves a number of factors such as gut microbiota-immune system interactions, genetic predisposition, lifestyle and environmental factors (Sartor, 2006). The knowledge of the role of ecDNA and NETs in the pathogenesis of IBD is somewhat limited. The current findings, however, suggest some connection with the development or progression of IBD. Endogenous DNase I activity in patients with IBD is lower compared with healthy controls (Malickova et al., 2011). In mice with dextran sulphate sodium (DSS)-induced colitis, nuclear ecDNA in the plasma increases with increased duration of colitis and is directly proportional to the number of NETs (Koike et al., 2014). Moreover, the ecDNA level in patients with ulcerative colitis is positively correlated with the clinical status of the disease. Gut proteome analysis in patients with ulcerative colitis showed high expression of proteins that are associated with NETs (Bennike et al., 2015). The expression of these proteins correlated with the degree of damage to the gut, and gut immunofluorescence confirmed the presence of neutrophils and NETs. The present study aims to clarify the role of ecDNA as a therapeutic target in IBD.

Material and Methods

Animal model

Fifty male C57BL/6J mice (Anlab, Prague, Czech Republic) were purchased at the age of 10 weeks and left to acclimate for two weeks. Mice were kept in controlled environment, five per cage, with 12/12-h light/dark cycle, standard temperature (23 °C), standard humidity (55%) and ad libitum access to standard rodent chow and tap water. All experiments were approved by the Ethics Committee of the Institute of Molecular

Biomedicine, Faculty of Medicine, Bratislava, Slovakia. Mice were randomized into four groups: H₂O + saline (N = 10), DSS + saline (N = 15), H₂O + DNase (N = 10) and DSS + DNase (N = 15). Mice in the DSS groups were treated with 2% DSS (MW ~ 40 000, AppliChem, Darmstadt, Germany) dissolved in tap water ad libitum for seven days, followed by three days of "wash-out" period, and sacrificed on the following day. Control mice (H₂O groups) had ad libitum access to tap water. Twice during the experiment (on days 3 and 7), the animals were intravenously injected with DNase I (7 mg/kg, dissolved in saline, Sigma-Aldrich, Steinheim, Germany) or with saline.

Body weights, stool consistency, rectal bleeding and liquid intake were monitored daily as described previously (Palffy et al., 2011). Blood was collected via retro-orbital puncture twice, on day 6 and at sacrifice, using K3EDTA and lithium heparin-coated tubes (Sarstedt, Nümbrecht, Germany). Blood was centrifuged at 1,600 g for 10 min and supernatants were centrifuged again at 16,000 g for 10 min and stored at -80 °C. At sacrifice, mice were anesthetized using isoflurane overdose. Colon length was measured, the colon was weighed, rinsed with cold phosphate-buffered saline (PBS), cut into pieces, snap frozen in liquid nitrogen and stored at -80 °C for further analyses. Spleen and liver were removed and weighed. A piece of liver was collected and stored as described above. The research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved by the institutional Ethics Committee.

Quantification of extracellular DNA

Extracellular DNA was isolated from 100 µl of centrifuged plasma using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total DNA was quantified fluorometrically using Qubit fluorometer 2.0 and Qubit dsDNA HS assay kit (Thermo-Fisher, Waltham, MA). For SYBR green quantitative real-time polymerase chain reaction (PCR) targeting circulating nuclear ecDNA, a mastermix (SsoAdvanced™ Universal SYBR® Green Supermix, BIO-RAD, Hercules, CA) and primers for glyceraldehyde 3-phosphate dehydrogenase (forward – GAAATCCCCTGGAGCTCTGT and reverse – CTGGCACCAGATGAAATGTG) were used. MtDNA was quantified using primers targeting cytochrome B forward – CCTCCCATTCAATTATCGCCGCCCTTGC and reverse – ATTTTGTCTGCGTCGGAGTT. Real-time PCR standard curves were created using mtDNA extracted from the isolated mitochondria with Mitochondria isolation kit for tissue (Thermo-Fisher, Waltham, MA) of liver tissues (DNeasy Blood & Tissue kit, Qiagen). Real-time quantitative PCR was carried out in Mastercycler realplex4 (Eppendorf, Hamburg, Germany) using a standard real-time PCR protocol: 98 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 30 s at the primer-specific annealing temperature (60 °C for glyceraldehyde 3-phosphate dehydrogenase, 55 °C for cytochrome B).

Biochemical analyses

The concentration of tumour necrosis factor α (TNF- α) in the colon was measured using a commercially available ELISA kit (R&D systems Inc., Minneapolis, MN). The activity of myeloperoxidase (MPO) was measured in colonic tissue by a modified protocol published by Kim and as described previously (Kim et al., 2012; Babickova et al., 2015). One hundred mg of colonic tissue was homogenized in 500 μ l of ice-cold PBS and centrifuged at 4 °C; 13,400 g for 10 min. Fifty μ l of the supernatant was used for analyses as per manufacturer's instructions. Results for TNF- α and MPO are presented as pg/mg of proteins and U/mg of proteins, respectively. Concentration of proteins was measured using a bicinchoninic acid kit (Sigma-Aldrich).

Statistical analysis

Data were analysed using one- or two-way ANOVA with Bonferroni post-hoc test where appropriate. Data are presented as mean \pm standard deviation. P values less than 0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA).

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12h/12h light/dark, 55% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by isoflurane overdose for tissue collection.

Results

Macroscopic observations

Intake of 2% DSS resulted in significant weight loss in both DSS groups (DNase and saline) starting from day 5 compared to the respective control groups on water ($P < 0.001$ each day) (Fig. 1a). However, no difference had been observed between the two DSS groups (DNase vs. saline) throughout the experiment. Similar results were observed with stool consistency, where both DSS groups showed poorer score starting from day 2 and day 3 ($P < 0.001$ each day), respectively, compared to the respective control groups (saline and DNase, respectively) (Fig. 1b). However, no significant difference was reported between the two DSS groups (DNase vs. saline). The colon length was measured at the end of the experiment. Both DSS groups (DNase and saline) showed shortened colon compared to the control groups (both $P < 0.001$) (Fig. 1c). However, no difference was observed between the two DSS groups (DNase vs. saline).

Levels of ecDNA

Extracellular DNA was measured on days 6 and 10. Fluorometric measurement of total DNA showed no significant differences between the groups (data not shown).

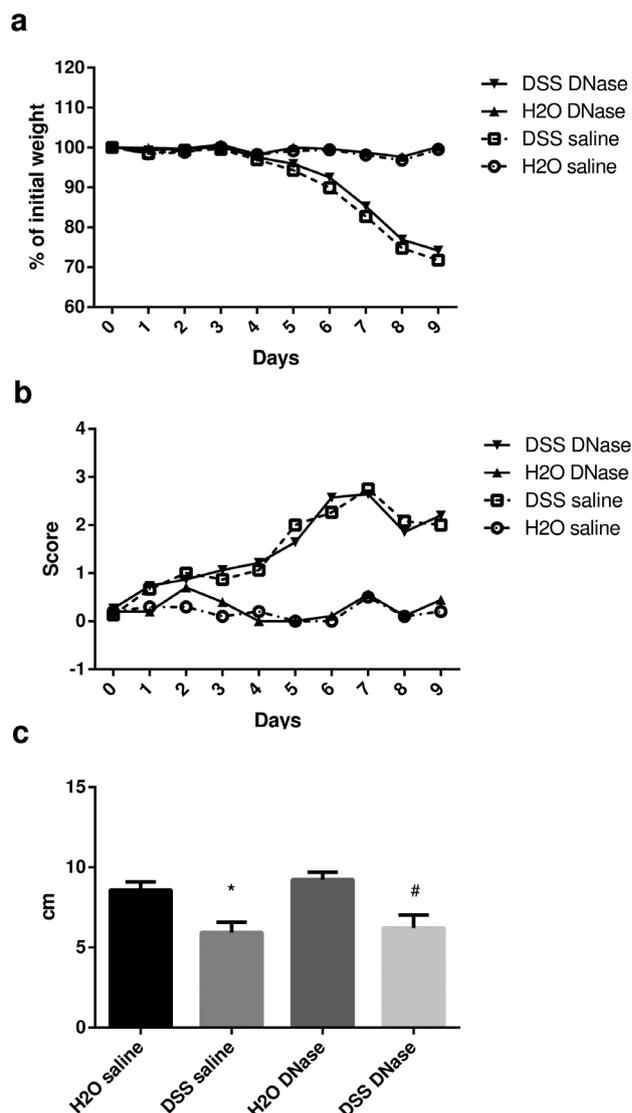


Fig. 1. Disease activity markers. **a)** Percent weight loss of mice during the experiment. Both groups with colitis (DSS DNase and DSS saline) showed significant weight loss compared to the respective controls (H₂O DNase and H₂O saline, respectively) starting from day 5 ($P < 0.001$). **b)** Stool consistency score. Both DSS groups showed poorer stool consistency compared to their respective controls starting from day 3 ($P < 0.001$). **c)** Colon length at the end of experiment. Shortened colon is a marker of ongoing inflammation. Both groups with colitis (DSS saline and DSS DNase) had significantly lower colon length (*** $P < 0.001$ vs H₂O saline; ### $P < 0.001$ vs H₂O DNase). Data are expressed as mean \pm SD.

Quantitative real-time PCR was used for quantification of nuclear and mitochondrial circulating DNA. There was no significant difference between the groups in any of these parameters (data not shown).

Changes in biochemical markers

TNF- α concentration was measured in distal colon homogenates. A significantly higher TNF- α level was

found in the groups treated with DSS when compared with controls ($P < 0.001$ for DSS saline vs. H₂O saline; $P < 0.01$ for DSS DNase vs. H₂O DNase) (Fig. 2a). Although the DNase-treated mice with colitis showed clearly lower TNF- α levels compared to the saline control, the difference did not reach statistical significance. MPO activity in the colon was found to be higher in both DSS groups compared with respective controls ($P < 0.001$ for DSS saline vs. H₂O saline; $P < 0.05$ for DSS DNase vs. H₂O DNase). In addition, the DSS group treated with DNase had significantly lower MPO activity than the DSS group with saline ($P < 0.05$) (Fig. 2b).

Discussion

To explore the role of ecDNA in IBD, a mouse model of acute colitis was used with administration of 2% DSS. All measured parameters of the disease activity

have shown severe colitis in both groups receiving DSS. Moreover, a low variability of the measured values indicates a well-standardized protocol and appropriate experimental conditions.

Colitis was treated by intravenous injection of DNase twice during the experiment. Most of the disease activity parameters showed no significant improvement upon DNase treatment. However, the levels of MPO were lower in colonic tissue of DNase-treated DSS mice compared with the untreated DSS group. Similarly, TNF- α levels showed some improvement, although in this case it was not significant. These findings suggest that there might be some effect of DNase on colonic tissue, which is, however, not sufficient for improvement of the disease.

To our knowledge, this is the first study that investigated the effects of intravenous administration of DNase on DSS-induced colitis. During the experimental design, we chose two time-points of administration based on the course of the experimental colitis – day 3 and day 6. In our hands, day 3 represents the early onset of clinical symptoms of colitis, while day 6 is characterized by a full-blown disease. We also aimed to analyse whether the plasmatic concentrations of extracellular DNA were different between DSS and H₂O groups on day 6 (full-blown disease) and at sacrifice. However, no significant differences were observed. This is partly in line with the observations made by Koike et al. (2014), who did not report increase in the concentrations of ecDNA in the experimental groups before day 7 of colitis. On the other hand, the authors showed elevated concentrations of ecDNA on day 14, after 7 days of “wash-out”, which we were unable to show in our study after only three days of the “wash-out” period.

These discrepancies might be caused by various reasons:

- i) we used a thorough centrifugation protocol to harvest the extracellular DNA, which might yield lower overall concentration of ecDNA in our samples, and which failed to prove differences between the DSS and H₂O groups. Our protocol followed the recommendations of the laboratory of Professor Dennis Lo (Tsui et al., 2012; Jiang et al., 2015), pioneer in the field of ecDNA research; the centrifugation g is not specified in the study by Koike et al. (2014).
- ii) Different methods were used to quantify ecDNA, real-time PCR and fluometric assay using Qubit fluorometer 1.0 in the present study compared to QuantiTMM PicoGreen dsDNA Assay Kit and multiphoton microscopy in the study by Koike et al. (2014), which could provide a better resolution to study ecDNA, especially the multiphoton microscopy.
- iii) Different manufacturer of DSS and supplier of the animals should always be considered as a factor when contradictory results are reported.

This study has several limitations. To reduce the stress of repeated blood collection in mice, only two time-points to study the concentrations of ecDNA were analysed. The kinetics of ecDNA was best described in pregnant

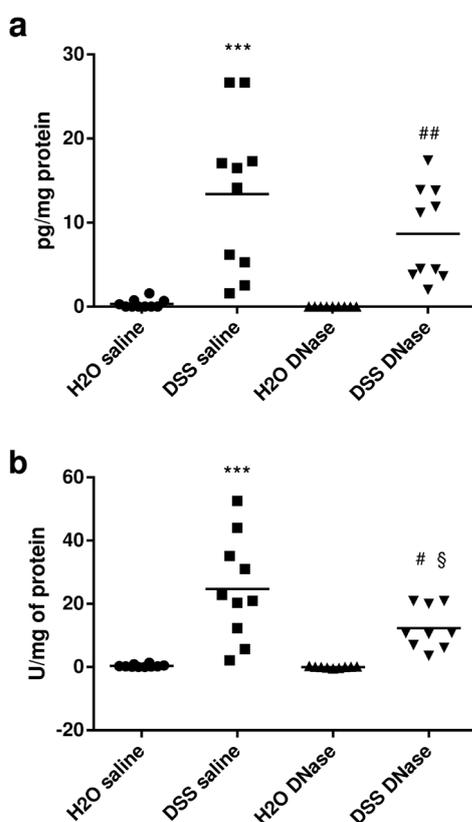


Fig. 2. Biochemical markers of colitis. **a**) TNF- α concentration in distal colon. Both groups on DSS had significantly higher TNF- α levels at the end of experiment, compared with the respective controls ($***P < 0.001$ vs H₂O saline; $##P < 0.01$ vs H₂O DNase). **b**) Myeloperoxidase concentration in distal colon. Both groups with colitis had higher levels of myeloperoxidase compared with controls ($***P < 0.001$ vs H₂O saline; $#P < 0.05$ vs H₂O DNase). In addition, mice with colitis treated with DNase (DSS DNase group) showed lower levels compared to untreated mice with colitis ($§P < 0.05$ vs DSS saline). Data are expressed as individual values and mean.

women, by analysing foetal ecDNA, which showed the highest clearance 2 h after delivery and no presence after 48 h (Lo et al., 1999). Thus, although the situation is rather different during the diseased condition, such restricted time-points of blood collection likely do not reflect the overall dynamics of ecDNA degradation in the bloodstream. Similarly, the kinetics of the injected DNase might be another factor that influenced the effectivity. The dose of the injections was based on previous studies that used 2.3 mg/kg and 10 mg/kg of the enzyme, respectively, for i.v. injections (Trejo-Becerril et al., 2016; Laukova et al., 2017). However, the design of these studies was considerably different, including the single dose of DNase I. Therefore, the dose and time-points chosen for the current study were based on experimental assumptions, since no previous studies have employed such experimental design.

On the other hand, the effectiveness of intramuscular and intraperitoneal administration of DNase I was previously documented (Patutina et al., 2010; Wen et al., 2013; Trejo-Becerril et al., 2016). Moreover, DNase I as an exogenous protein undergoes proteolysis, so that rare i.v. injections are probably less effective. Moreover, given the duration of the experiment (nine days), we aimed to investigate whether the administration of DNase I had any long-term effects on the course of the disease and on the level of ecDNA other than an hour after the injection, which has already been described (Patutina et al., 2010; Wen et al., 2013; Trejo-Becerril et al., 2016). However, it seems that in the light of DNase I kinetics, intraperitoneal or intramuscular administration might be more meaningful and should be considered in future studies.

Another interesting aspect that should be considered is the desirability of overall removal of ecDNA. For example, a recent *in vitro* study on human THP1 monocytic cell line showed stimulation of mRNA expression of TNF- α after treatment by DNase (Zinkova et al., 2017). On the other hand, several experimental *in vivo* studies showed that the treatment with DNase I reduced inflammation and improved survival of animals (Mai et al., 2015; Laukova et al., 2017; Vokalova et al., 2017). Also, worth noticing is the antimicrobial activity of DNA, an important part of the activated NETs (Halverson et al., 2015). In the complicated niche of inflammatory bowel disease where microbiome plays a crucial role, any anti-microbial activity should be further explored in this regard.

The concentrations of cytokines, DNase and NETs in the plasma were not assessed because a significant amount of the collected plasma was used for analysis of ecDNA. To reduce the number of animals used while retaining sufficient statistical power, only one concentration of DNase was tested.

Taken together, intravenous treatment with DNase proved unsuccessful in treating DSS-induced colitis. This suggests that neutralization of NETs and ecDNA in colitis as the primary targets is insufficient to treat the disease. On the other hand, some of our results (TNF- α ,

MPO) indicate there might be some effect of DNase on the colonic tissue, although not apparent in the clinical parameters. Moreover, the proven increase of ecDNA and NETs, as well as decreased expression of DNase in human patients with IBD (Malickova et al., 2011; Bennike et al., 2015), warrants further research of these possible targets, e.g., as a co-treatment to standard therapy. In conclusion, different experimental settings using altered concentrations of DNase are needed to support the results in the future experiments.

Discloser of conflict of interest

The authors declare that they have no conflict of interest.

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