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Dextran Sodium Sulfate Inhibition of Real-Time PCR Amplification: A Poly-A Purification Solution

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Abstract

Dextran sulfate sodium (DSS) induces experimental colitis and promotes colitis-associated cancer in rodents. In practice we observe potent inhibition of real-time quantitative PCR (qPCR) using cDNA from DSS-exposed mouse tissues, which complicates gene expression analysis. Here we characterize DSS inhibition of qPCR in a wide array of murine tissues following ingestion of DSS. We examine different approaches to RNA purification prior to cDNA synthesis in order to optimize cDNA amplification and gene expression analysis. We find that poly-A purification of DSS-exposed RNA allows cDNA synthesis and permits reliable and cost-effective mRNA quantification for gene expression analysis in DSS-exposed tissue.

Keywords

Dextran sodium sulfate; colitis; qPCR; colitis-associated cancer

Introduction

Oral administration of a range of concentrations (typically 2–5%) of (MW 40,000–50,000) dextran sulfate sodium (DSS) produces acute colitis in mice (Okayasu et al., 1990) and has been exploited as a model of colonic epithelial injury with many features found in human inflammatory bowel disease (Cooper et al., 1993). Use of DSS colon injury models have facilitated investigation into the role of toll-like receptors (Fukata et al., 2007; Fukata et al., 2010), the unfolded protein response (Kaser et al., 2008; Brandl et al., 2009), and other pathways (summarized in (Kawada et al., 2007)) in acute colon injury, and colitis-associated neoplasia. A Pubmed search (March 7, 2011) using the search terms dextran sulfate sodium and colitis displays over 400 references illustrating the widespread use of this approach. However, despite its widespread use, little is known about the mechanism(s) by which oral administration of DSS induces colitis. Systematic characterization of the impact of DSS on mice is limited to some degree by the wide variety of experimental protocols, variance in commercial DSS preparations, and differences in murine strain susceptibilities to DSS (Suzuki et al., 2006). Here we report two novel observations in regard to DSS administration in mice. First, we demonstrate potent inhibition of qPCR RNA analysis by DSS contamination that can be avoided with poly-A purification of DSS-exposed RNA. Secondly, we show that orally administered DSS appears to traffic widely in the mouse (as implied by interference with qPCR), including to non-enteric organs. Based on these observations we describe a cost-effective solution that allows real-time quantitative RNA expression analysis in DSS-exposed tissue.

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Methods

Treatment of mice with Dextran Sodium Sulfate

8–10 week old C57BL/6 mice were offered either sterile tap water alone, or supplemented with 2.5% DSS (USB, Cleveland, OH Cat 14489, M.W. 40000–50000) for 7 days. Mice were anesthetized, sacrificed and organs harvested for RNA purification and analysis. All studies were approved by the Washington University Animal Studies Committee.

Isolation of total RNA with Trizol

50–100 mg of harvested organs were homogenized in 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA Cat 15596-018). Total RNA was prepared according to the manufacturer's protocol and quantified by UV spectrophotometry or Agilent (Santa Clara, CA) 2100 Bioanalyzer.

Ethanol RNA wash

90 μ l of 100% ethanol was added to 30 μ l of DSS-exposed RNA (approx. 0.3–1 μ g/ μ l). 1 μ l of glycogen (20 mg/ml) and 3 μ l of 3 M sodium acetate were added and the mixture incubated at –80 C for 15 minutes. The mixture was centrifuged at 12,000 $\times$ g for 10 minutes and resuspended in 30 μ l of nuclease-free water.

Silica Column RNA Cleanup

25 μ g of total RNA bound to a Qiagen (Valencia, CA) RNeasy (Cat. 74140) silica column according to the manufacturer protocol, eluted in nuclease-free water. Oligotex Resin Column mRNA Purification: 5 μ g of total RNA was used to isolate poly-A mRNA using the Qiagen Oligotex mRNA Mini Kit (Cat. 70022) according to the manufacturer protocol. Column elution was performed with 40 μ l of elution buffer.

DNase treatment of total RNA

1–10 μ g of total RNA was treated with DNase using the Ambion DNA-free kit (Cat. AM1906) according to the manufacturer protocol. DNA-free RNA was quantified by UV spectrophotometry and used in subsequent reverse transcriptase reactions.

Reverse Transcription of RNA

Poly-A purified or between 0.3–1 μ g of DNase treated RNA were used to generate cDNA using the Applied Biosystems (Carlsbad, CA) High Capacity Reverse Transcription Kit (Cat. 4368814) according to the standard protocol. cDNA was subject to real-time quantitative PCR either undiluted or after up to 4-fold dilution in nuclease-free water. The qPCR primer sequences were as follows: GAPDH: 5'-TGTGTCCGTCGTGGATCTGA-3' 5'-CCTGCTTCACCACTTCTTGA-3' 18S: 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'.

Real-time Quantitative PCR

cDNA was amplified using Applied Biosystems Sybr Green (Cat 4385612 or 4309155) using an ABI Prism 7000 sequence detection system with SDS 7000 software or ABI StepOnePlus thermal cycler with StepOne (v2.1) software.

Bead-based Oligo (dT) Isolation of mRNA

Oligo (dT) purification of mRNA was performed using the Invitrogen Dynabeads mRNA Purification kit (Cat. 610-06, 610-11, or 610-12). 5–10 μ g of DSS-exposed total RNA (estimated to contain 50–250 ng of mRNA) was isolated and mixed with 0.25 mg of

Dynabeads Oligo (dT)₂₅ in a total volume of 200 μ l (including binding buffer). The beads/binding buffer were washed according to the manufacturer protocol and eluted with 20 μ l of Tris-HCl. 10 μ l of the eluate was used for cDNA synthesis reaction.

Results

DSS exposure results in partial (but not complete) degradation of purified colonic total RNA

To test whether DSS resulted in degradation of the RNA during the RNA purification process, we analyzed the resultant total RNA using an Agilent 2100 Bioanalyzer. Although DSS exposure resulted in some degradation of the total RNA, as evidenced by the increase in small RNA fragments, there remained intact 18S and 28S RNA making it unlikely that complete inability to amplify short cDNA fragments from DSS exposed tissue resulted from DSS-induced RNA degradation (Figure 1a).

DSS exposure results in complete inhibition of qPCR at between 1 nM and 10 nM

To test whether exogenously supplied DSS would directly inhibit qPCR, we utilized cDNA synthesized from non-DSS exposed colonic total RNA as a standard for GAPDH mRNA expression analysis. Addition of exogenous DSS in varying concentrations (calculated using an average DSS MW of 45,000) demonstrated no inhibition of PCR amplification at concentrations of 1 nM or less. Conversely, complete inhibition of amplification was observed at DSS concentrations of 10 nM or greater (Figure 1b). Similar results were obtained using cDNA generated from colon and liver. To exclude the possibility that DSS was simply quenching the SYBR fluorophore, DSS-exposed colonic cDNA was amplified in a similar fashion and electrophoresed on a 2% agarose gel. Ethidium bromide staining demonstrated a lack of PCR amplification product (data not shown) suggesting that DSS inhibits PCR amplification and not simply quantitative PCR fluorophore detection. To further examine whether tissue DSS was present in sufficient concentration in tissue-derived cDNA to inhibit PCR amplification, cDNA was generated from the anorectum of both DSS-exposed and control mice. While GAPDH amplification was observed from control animal-derived cDNA (Ct~20), no amplification was observed from DSS-exposed animal-derived cDNA. When the two cDNA preparations were mixed in a 1:1 ratio and subject to qPCR with GAPDH primers, no GAPDH amplification was observed (Figure 1c). Similar results were observed with 18S ribosomal RNA amplification. These data suggest that orally administered DSS results in colonic tissue DSS concentrations sufficient to contaminate RNA and cDNA preparations and inhibit qPCR amplification.

Orally administered DSS interferes with PCR amplification of cDNA derived from multiple tissues

We found that administration of DSS to mice interfered with qPCR analysis of RNA from liver as well as colon. We hypothesized that orally administered DSS may enter the portal and systemic circulation and reach non-enteric organs. To test this hypothesis we treated mice for 7 days with 2.5% DSS in drinking water. Control and DSS-treated mice were sacrificed and multiple tissues harvested for cDNA preparation (Figure 1d). Each DSS-exposed cDNA sample was paired with a control cDNA sample from the same organ and the difference in GAPDH Ct between DSS-treated and control tissue measured. The average delta Ct comparing DSS and control was calculated for each organ (n=3–5 DSS-control pairs/tissue) to provide an estimate of DSS presence in indicated tissues. If no amplification was detected, presumably due to DSS contamination, Ct was arbitrarily set at 40 cycles (the duration of the amplification program). Results are summarized in Figure 1d. As previously observed, there was a significant impairment of PCR amplification of colon-derived cDNA. On average, DSS-exposed colon cDNA required ~17 additional PCR cycles for crossing

threshold amplification compared to control colon-derived cDNA. Impaired cDNA amplification of cDNA was observed from several organs (including liver, spleen and lung) in DSS-exposed mice, although DSS exposure did not impair amplification of cDNA derived from brain, or heart. The mechanisms accounting for this differential tissue accumulation remain open.

Poly-A purification of mRNA enables qPCR of cDNA from DSS-exposed tissues

We next sought a cost-effective technique to separate the total RNA from DSS and allow measurement of gene expression by qPCR (Figure 2). Trizol RNA purification was not sufficient to purify DSS-exposed mRNA. We utilized a glycogen and ethanol precipitation, either once or twice, following Trizol isolation of RNA, which was inadequate to allow amplification of cDNA from DSS exposed tissue (data not shown). We then evaluated the RNEasy kit, a silica-column based RNA clean-up protocol/kit from Qiagen, used previously to examine gene expression in DSS-exposed tissue, albeit several weeks after cessation of DSS treatment (Barrett et al.). However this approach resulted in variable efficiency of cDNA amplification from DSS exposed tissues (data not shown). We then investigated poly-A mRNA purification using the Invitrogen Oligotex poly-A spin column. This resulted in reproducible cDNA amplification of GAPDH (Ct~17–18) and other targets (Cox-2, Xbp-1, IL-23) from murine colon (n=6) that were non-amplifiable prior to poly-A purification of RNA. Because the cost of column poly-A purification is high (~\$23/sample), we sought alternative approaches. The Invitrogen Dynabeads kit allowed magnetic oligo (dT) bead purification of mRNA, though the cost per sample (based on use of 0.25 mg of oligo (dT) beads/prep) is still ~\$10/prep. Using the Dynabeads mRNA DIRECT Kit (Cat. 610-12), and preparing our own binding buffer, we were able to decrease the cost to approx. \$3.40/sample with the primary cost being the oligo (dT) beads. Using this method, we have been able to reproducibly, and in a cost-permissive fashion measure gene expression in DSS exposed tissue including colon.

Discussion

DSS is widely used to induce experimental colon injury and colitis-associated cancer. To gain mechanistic insights from these studies, it would be beneficial to measure gene expression at both the RNA and protein levels. Here we demonstrate that DSS contamination of colonic tissue results in potent (nM) inhibition of qPCR cDNA amplification. We show that analysis of mRNA expression by qPCR in DSS exposed tissue requires that the DSS be separated from RNA. We examined a variety of RNA “clean up” techniques, and found that the most reliable methods of separating mRNA from DSS were via poly-A based purification techniques. Column based poly-A purification techniques, such as the Invitrogen Oligotex kit also worked well, though the cost per sample might become prohibitive when working with a large number of samples. We have found that magnetic oligo (dT) beads such as Invitrogen Dynabeads allow modification of the standard protocol (ie. use of 0.25 mg instead of 1 mg of beads) to further decrease cost. Individual investigator refinement of these protocol modifications may be necessary depending on the specific tissue involved and the tissue DSS concentration.

Though we could find no mention of challenges of PCR amplification in the GI literature, DSS interference with nucleotide polymerases has been described in the literature for several decades. In 1978, Shamida and colleagues, seeking to describe the biochemical properties of sea urchin polymerases, noted that polyanions (including dextran sulfate) could potentially inhibit polymerase- β at between 1 and 100 mcg/ml (Shimada et al., 1978). DSS use during viral DNA preparation resulted in the inability to use the resultant DSS-contaminated DNA as a template for DNA polymerase suggesting inhibition of DNA polymerase by DSS (Hitzeman et al., 1978). Inhibition of DNA polymerase- α by an extract of *Physarum*

polycephalum (a microplasma) was described by Fischer in 1989 (Fischer et al., 1989). This extract was found to contain an anionic polyester that could bind reversibly with polymerase α , and result in competitive inhibition at concentrations of 10 ng/mL. A variety of plant polysaccharides including dextran sulfate, mannin, starch, and others have been assayed for effects on PCR amplification (Demeke and Adams, 1992). DSS was found to inhibit PCR amplification, and could not be reversed with addition of low concentrations of Tween 20. Reversible inhibition of polymerases by non-nucleic acid polyanions was utilized in a patent filed by Lars-Erik Peters in 2003 that proposed the use of these polyanions to improve the specificity of PCR amplification (US Patent 6667165, 2003).

DSS inhibition of polymerase amplification from various tissues suggests that orally administered DSS distribute widely in a mouse. Though DSS concentrations may be highest in the stomach, small intestine, and colon, impaired amplification of cDNA generated from other organs (liver, spleen, and lung) suggest that intact or fragmented DSS likely reaches these organs. It is important to consider that extraintestinal DSS and resultant tissue or organ dysfunction may contribute to morbidity, anemia, and weight loss in DSS exposed mice. These data should facilitate gene expression analysis in experimental models of DSS colon injury. These data also suggest that further studies on the impact of DSS on whole animal physiology and cellular nucleotide polymerases may be warranted to better understand this commonly used experimental tool.

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Figure 1.

a) Total RNA extracted from colon of control or DSS-treated mice was analyzed using an Agilent 2100 Bioanalyzer demonstrating partial degradation of total RNA extracted from DSS-exposed tissue, b) Exogenous addition of DSS to cDNA prior to RT-qPCR amplification results in complete inhibition of PCR amplification between 1 and 10 nM DSS. c) GAPDH and 18S PCR fragments are efficiently amplified from control but not DSS-contaminated cDNA. Mixing of control and DSS contaminated cDNA results in complete inhibition of cDNA PCR amplification, d) Comparison of the GAPDH qPCR crossing thresholds (Ct) between cDNA procured from organs of DSS-treated and untreated animals (TI-terminal ileum). (n=3–5/treatment group/tissue)

Table 1
RNA purification approaches to allow amplification of DSS-contaminated cDNA

	Approach	Result (GAPDH Ct)
2.5% DSS	Control (no DSS)	14–20
	Trizol	None
	EtOH Precipitation/Wash	None/Rare
	RNEasy (Qiagen)	Variable
	Oligotex mRNA Mini Kit	~18
	Invitrogen Dynabeads	~14–15