・OPC6535はヒト単球からのTNF-αやIL-12産生抑制効果を示した。
・EARK、p38、p65、STAD3についてはOPC-6535投与群においてもリン酸化への影響は認めなかった。
・PKA費依存性の経路で効果を示すことが示唆された。
・IL=10ノックアウトマウスの自然腸炎発症を抑制した
・他のPDE4阻害剤やcAMPアナログとの作用効果及び機序の差異は見出せなかった。

【OPC6535の他の免疫担当細胞への効果】
・T細胞の効果：　TNFα、INFγ産生に対して抑制が見られる。
・NK細胞への効果：　濃度依存的にINFγ産生が抑制されることから抗炎症効果を持つ

【炎症性腸疾患、腸管手術検体でのOPCの効果の検討】

◆免疫担当細胞は抹消血中と腸管局所では機能が異なることが報告されていることから、それに対する検討
◆クローン病腸管NK細胞での検討を進めているのでOPCでも解析を実施
【背景】
・我々はクローン病において腸管のNK細胞が健常者やUCと比べて増加していることを確認している。
・免疫染色：クローン病の腸管ではINFγを多く産生するNK細胞が増えていることが示唆された。
・クローン病の腸管からLPMを単離し、CD3とCD56で展開すると、もともとINFγのメジャーソースとしてT細胞が知られているが、サイトカイン（IL-12）刺激でINFγを産生する。同時に腸管のNK細胞からもINFγが出てくることがわかった。
・菌（死菌）刺激でもNK細胞からもINFγがたくさん出てくることがわかった。

【OPCの効果】
・サイトカインで刺激した場合
クローン病の腸管からLPMをとってきて、未刺激の状態ではINFγは出ていないが、IL-12,15の刺激により、殆どの細胞がINFγを亢産生するが、OPCを加えると濃度依存性に抑制されることが確認された。
cAMPでも同様に下がるが、その作用はかなり弱い。
・菌刺激の場合
菌刺激の場合も同様に腸管のNK細胞からINFγが出てくるが、OPCを加えると濃度依存性に抑制されることが確認された。

・腸管のT細胞に対する検討
サイトカイン刺激に対して出てくるINFγをOPCは濃度依存的に抑制することが確認された。
菌刺激に対しても同様であった。
【まとめ】
・OPC6535は末梢血中のT細胞、NK細胞からの免疫性サイトカイン産生を抑制する。
・クローン病では、腸管のNK細胞が増加しており、commensal bacterial 刺激により著明に炎症性サイトカインを産生する。
・OPC6535およびcAMP analogueはクローン病の腸管NK細胞、腸管T細胞に対しても炎症性サイトカイン産生を濃度依存性に抑制する。

<質疑応答>
Q:OPC6535はコードネームか？
A:一般名はテトミラスト。

Q:この薬剤は腸管の上皮にはどのように働くか?cAMP を上げるということは、上皮を増殖したり、下痢の原因になったりする可能性は?
A:その検討はしていない。
Q:マクロファージに対してはどうか?
A:マクロファージからのIL-23の産生も抑制している。
Q:開発の状況は?
A:米国で潰瘍性大腸炎の有効性が乏しくかったが、その後の解析ではクローン病で期待されている。

12) 新規ケモカインCXCL16制御を目的とした炎症性腸疾患に対する治療開発　(研究分担者:千葉　勉)

○ 仲瀬裕志、宇座徳光、千葉　勉(京都大学大学院医学研究科消化器内科学)

【Pathophysiology of IBD】

・バクテリアが関係している。
・APC,DC,活性化したマクロファージ、T 細胞が暴れだすと悪さをするということで、いろんなターゲットがあるが、IL-17,HGF などの増殖因子など、
・我々がターゲットとしたものはAPCをメインにするものであり、DDSをすればよくなる。

【SR-PSOX/CXCL16】

・2000年にスカベンジャーレセプターとして発見された　と同時にCXCCR6のリガンドとして同定された膜結合型のケモカインである。
・脳と骨格筋以外の臓器に広く分布する。
・消化管ではパイエル板のみに発現
・血球成分ではマクロファージとDCに発現
・4つのdomainを有する膜結合型で発現する。
・スカベンジャーレセプター

ケモカインレセプターはバクテリアをファゴサイトするファンクションを有している。
樹状細胞、マクロファージのところにCXCL16が出ており、バクテリアが取り込まれる。また、Tcell、CXCCR6がリガンドとくっついて、interactionをおこす。こういったinteractionにより、炎症の継続や生体のホメオスターシスを保っていると考える。

【目的】

実験腸炎モデルを用いてSR-PSOX/CXCL16の役割を検討する。
※preliminaryな段階で患者血清を見たところ、UC/CDともCXCL16は上がっている。

【実験デザイン】※実際に炎症を起こして、上がっているのかを見た

Mice　:CXCL16　10−12適齢、メス、SPF環境下
Induction of colitis :3% DSS　M.W.36000-50000
検討項目:

・腸炎誘導前後の大腸組織におけるCXCL16の発現を解析(real time PCR法)
・蛍光免疫染色によるCXCL16　発現細胞とその分布の検討(今回はまだconfirmしていない)

結果:
大腸組織におけるCXCL16の発現

炎症をおこすと、CXCL16 の発現はあがることが確認された。

⇒**【CXCL16 は腸炎発症に関わっているのか？】**

ノックアウトマウスで検討（DSS を用い）

［経時的体重変化］

・ノックアウトマウスでは normal と比べて体重変化が軽微であった。

［腸管長の比較］

・マクロで見てもノックアウトの方が腸管長は非常に長い　wild type とノックアウトを比較すると有意差があり、炎症はコントロール出来ているのではと考えられた。

［組織学的評価］

・wild type とノックアウトを比べてみると大腸に関しては phenotype は差が出なかった。

・DSS をもちいると Wild type では炎症細胞浸潤がかなり強くでるが、ノックアウトではクリプトは残りある程度はブロックできるが完治したとまでは行かない。組織学的スコアは差がある。
※DSS のモデルで CXCL16 をノックアウトすると炎症は軽くなることが示唆された。

［大腸組織におけるサイトカインの発現］

・TNF-α、IL-1B、INF-γは下がるが、IL-10 は上がることを予想したが、炎症が下がればカウンターで IL-10 も下がるという結果であった。

【まとめ】

・**CXCL16 は腸炎の発症に関与していることが判明した。**

【実験的腸炎モデルにおいて CXCL16 は治療のターゲットになりうるか？】

・ヒトでの検討が必要であり、抗体を作って検討した

・予防的な投与で、DSS を入れる前から投与する。
体重変化：6 日前後で変わってくる、もう少しみると差がでる。
腸管長：コントロール IgG と CXCL16 の抗体では有意差が出る。
組織学的評価：CXCL16 抗体群は完全ではないがクリプトが保たれており、組織像は改善している。

※CXCL16 の抗体をつくってブロッキングすると腸炎が改善するデータが得られた。

別なモデルで検討

⇒**TNBS 腸炎で検討**　SJL mice を使用

CXCL16 の抗体をもちいて検討
体重変化：3～4 日で差が出てくる。
組織学的評価：コントロールと比べると CXCL16 の抗体ではクリプトが戻ってくる

※現在は IL-10KO と掛け合せて、検討中である。

【ヒトでの検討】

・CXCL16 をヘルシーコントロール、活動期 CD, 非活動期 CD、活動期 UC, 非活動期 UC, で見ると 2 つとも上がっていた。

・疾患の活動性とと CXCL1 を見ると相関関係が示唆された。

・白血球除去療法でみると、UC のリスポンダーでは下がり、ノンリスポンダーでは変わらないことが確認されたので、治療のマーカーにも使えることが示唆された。

【まとめ】

・**CXCL16 は腸炎発症に関与していると考えられた。**

・**CXCL16 は病気のマーカーとして使えることが示唆された。**

<質疑応答>

Q: CXCL16の阻害剤は？

A:抑える薬剤はない。

Q:治療に生かすのは？

A:抗体療法の可能性はある。

事務局連絡

平成20年度スケジュール

平成21年

2月　6日（金）　平成20年度　第2回総会

2月　8日（金）　分担研究者報告書類提出　締め切り

総合研究報告書18年度～20年度

※昨年11月の事後評価報告書を若干変更してもらえれば良い。

3月　6日（金）　収支決算報告書提出　締め切り

Ⅸ. 研究成果の刊行物・別刷

GASTROENTEROLOGY 2006;131:1110–1121

Human Thioredoxin-1 Ameliorates Experimental Murine Colitis in Association With Suppressed Macrophage Inhibitory Factor Production

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BASIC–ALIMENTARY TRACT

Background & Aims: Thioredoxin-1 (TRX) is a small multifunctional protein with antioxidative and redox-regulating functions. In this study, we investigated the significance of TRX in patients with inflammatory bowel disease (IBD) and the ability and mechanism to ameliorate experimental colitis. **Methods:** Serum TRX and macrophage migration inhibitory factor (MIF) levels were measured in patients with IBD. The effects of TRX were evaluated in a dextran sulfate sodium (DSS)-induced colitis model by comparing TRX-overexpressing transgenic (TRX-TG) and control mice. We further evaluated the effect of recombinant human TRX (rhTRX) administration on DSS-induced colitis and colonic inflammation of interleukin (IL)-10 knockout (IL-10 KO) mice. Colonic inflammation was examined clinically and histologically. Proinflammatory cytokine levels were examined in colonic tissues, and MIF levels were measured in colonic tissues and sera in mice. The effect of TRX on MIF production was also analyzed in vitro. **Results:** Serum TRX and MIF levels were significantly higher in patients with IBD than normal controls, and TRX levels correlated with disease activity. TRX significantly ameliorated DSS-induced colitis and colonic inflammation of IL-10 KO mice. Increase of tumor necrosis factor-α and interferon-γ in colonic tissues was significantly suppressed in TRX-TG mice compared with wild-type mice. MIF levels in colonic tissues and sera were significantly lower in TRX-TG mice than in wild-type mice, irrespective of DSS administration. Anti-TRX treatment exacerbated DSS-induced colitis. In vitro studies demonstrated that rhTRX suppressed MIF production in human monocyte cells. **Conclusions:** TRX might have a potential as a novel therapeutic agent for the treatment of IBD.

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are serious disorders. Although several intrinsic factors such as deregulated immune responses, and environmental factors such as food, are suggested to be involved, an integrated concept explaining the pathogenesis of IBD has yet to be proposed. Nevertheless, it is strongly suggested that mediators of immunoregulation and inflammation are involved in the pathogenesis.[1] In particular, recent studies have focused on proinflammatory cytokines such as tumor necrosis factor (TNF)-α and macrophage migration inhibitory factor (MIF), both of which have been investigated as target molecules for novel therapies.[2-5]

Oxidative stress caused by reactive oxygen species (ROS) is believed to be an important factor involved in the onset, as well as the development of intestinal inflammation. Indeed, excessive production of ROS directly leads to severe cell damage and ultimately to apoptosis and necrosis.[6] ROS-induced oxidative stress[6,7] and the resultant apoptotic cells in the epithelium are increased in the inflamed mucosa of IBD patients.[8-10] Thus, scavenging ROS is considered to be critical for regulating intestinal inflammation.

Thioredoxin-1 (TRX), originally cloned as a soluble factor named *adult T-cell leukemia-derived factor*,[11] is one of the most important molecules controlling the redox regulation system and contains a redox-active disulfide/dithiol within the conserved active site sequence Cys[32]-Gly-Pro-Cys[35]. TRX has a pivotal role in scavenging ROS with peroxiredoxins and thus prevents apoptosis of various cells, such as lymphocytes, monocytes, and epithelial cells, by inhibiting apoptosis signal-regulating kinase 1.[12] Moreover, intracellular TRX regulates DNA binding of several transcription factors including p53, nuclear factor-κB, and activator protein-1.[13] In addition, circulating TRX inhibits neutrophil infiltration into the sites of inflammation in an air pouch model.[14,15] These results suggest that TRX has important roles, not only as an antioxidant and antiapoptotic molecule, but also as an anti-inflammatory molecule.

Serum TRX levels are elevated in several diseases such as human immunodeficiency virus infection,[15] hepatitis C virus infection,[16,17] and rheumatoid arthritis.[18] Although these findings suggest that TRX can be a good marker for oxidative stress in various diseases, the reason for the increased TRX levels in such diseases is poorly understood. However, overexpression of human TRX (hTRX) in transgenic (TRX-TG) mice induced resistance to harmful conditions including thioacetamide- or lipopolysaccharide (LPS)-induced acute

Abbreviations used in this paper: CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; DSS, dextran sulfate sodium; EDTA, ethylenediamine-N,N,N',N'-tetraacetic acid; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor; ROS, reactive oxygen species; TBS, Tris-buffered saline; TG, transgenic; TNF, tumor necrosis factor; TRX, thioredoxin; UC, ulcerative colitis; UCDAI, Ulcerative Colitis Disease Activity Index; WT, wild-type.

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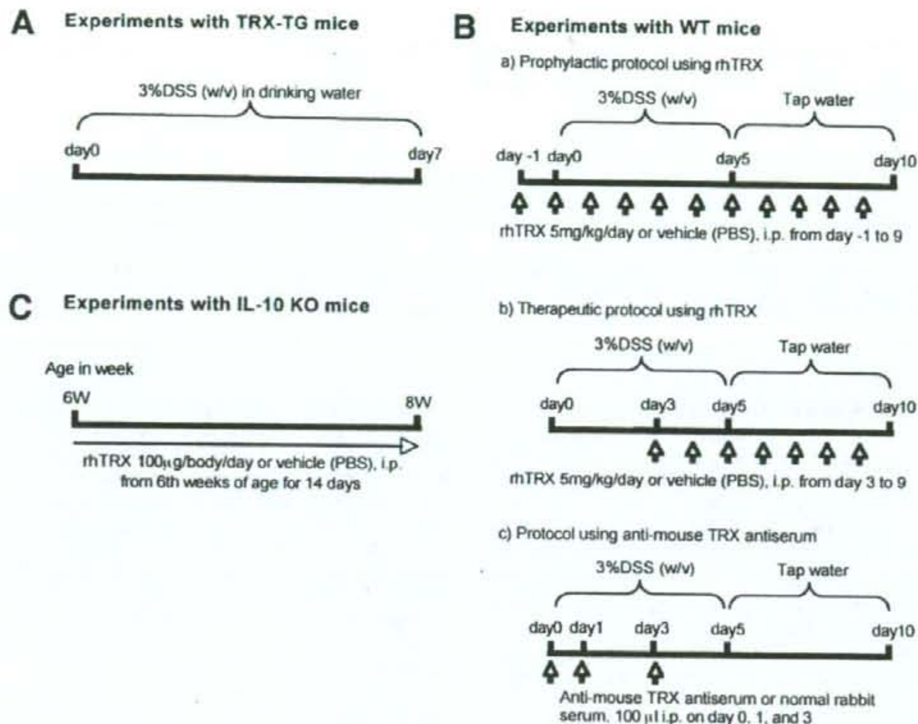

Figure 1. Experimental design for experimental colitis in mice. (*A*) Experiments with TRX-TG mice; 3% (wt/vol) DSS was administered to WT mice and TRX-TG mice from day 0 to day 7. Mice were killed on day 7. (*B*) Experiments with WT mice. (*a*) Prophylactic protocol using rhTRX. Three percent DSS (wt/vol) was administered to WT mice from day 0 to day 5 followed by a return to normal water. rhTRX (5 mg/kg) or vehicle was administered by intraperitoneal injection from day 1 to day 9 (*arrows*), and mice were killed on day 10. (*b*) Therapeutic protocol using rhTRX. Three percent DSS was administered to WT mice from day 0 to day 5 followed by a return to normal water. rhTRX (5 mg/kg) or vehicle was administered by intraperitoneal injection from day 3 to day 9 (*arrows*), and mice were killed on day 10. (*c*) Protocol using antimouse TRX serum. Three percent DSS was administered to WT mice from day 0 to day 5 followed by a return to normal water. Antimouse TRX antiserum or normal rabbit serum (100 μL/body) was administered by intraperitoneal injection on days 0, 1, and 3 (*arrows*). (*C*) Experiment with IL-10 KO mice. Daily 5 mg/kg of rhTRX or PBS alone was administered intraperitoneally to IL-10 KO mice at 6 weeks of age. Fourteen days after beginning treatment, rhTRX or PBS alone treated mice were killed for histologic analysis of colonic tissue.

hepatitis,[19] adriamycin-induced cardiotoxicity,[20] and proinflammatory cytokine- or bleomycin-induced lung injury.[21] Moreover, administration of recombinant hTRX (rhTRX) prevented ischemic lung injury,[22] cerebral infarction,[23] and myosin-induced myocarditis[24] in animal models. These findings suggest that TRX has protective effects on various diseases, possibly by its antioxidative and anti-inflammatory actions. Little is known, however, about the role of TRX in colonic inflammation.

Therefore, the present study sought to elucidate the role of TRX in the pathophysiology of IBD. For this purpose, we first investigated the serum levels of TRX in patients with IBD. Next, we examined the effects of endogenous and exogenous TRX on DSS-induced colitis in TRX-TG and wild-type (WT) mice. Furthermore, we investigated the effect of TRX on chronic colonic inflammation in interleukin (IL)-10 knockout (IL-10 KO) mice. Finally, we investigated the effects of TRX on MIF production in vitro. In addition to the antioxidative and anti-inflammatory actions previously reported, the present study suggests that TRX exerts a protective effect on experimental colitis, at least in part through the suppression of MIF production.

Materials and Methods

Serum Samples

Serum samples were obtained from 10 patients (7 men, 3 women) with active CD, 11 (8 men, 3 women) with inactive CD, 11 (9 men, 2 women) with active UC, 12 (7 men, 5 women) with inactive UC, 11 (7 men, 4 women) with active ischemic colitis, and 10 (6 men, 4 women) with colon adenoma (as normal controls). All samples were centrifuged within 1 hour of collection, and sera were frozen at −80°C until the assays were performed. To determine disease activity, the Crohn's Disease Activity Index (CDAI)[25] was used for patients with CD and the Ulcerative Colitis Disease Activity Index (UCDAI)[26] for patients with UC. Informed consent was obtained from each patient,

BASIC-ALIMENTARY TRACT

Table 1. Scoring System for Bloody Stool

Score	Description
0	Normal to semisolid stool, no blood
1	Normal to semisolid stool, blood-tinged
2	Semisolid to fluid stool with definite evidence of blood
3	Bloody fluid

and the experimental designs of these studies were approved by the Kyoto University Hospital Ethics Committee.

Animals

Female C57BL/6 (WT) mice (9–11 weeks of age; weighing 20–22 g) were obtained from CLEA Japan (Shizuoka, Japan). The generation and maintenance of TRX-TG mice was described previously.[27] hTRX cDNA was inserted between the β-actin promoter and the β-actin terminator. There were no differences in the expression of Mn-superoxide dismutase, CuZn-superoxide dismutase, and glutathione peroxidase between WT and TRX-TG mice, analyzed by immunohistochemistry and Western blotting (data not shown). The presence of the TRX transgene was confirmed by reverse transcription-polymerase chain reaction analysis.

C57BL/6-IL-10 KO mice (aged 4–6 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions and had free access to commercial food and water. Among them, IL-10 KO mice were transferred from specific pathogen-free to conventional housing conditions at 6 weeks of age because they spontaneously develop colitis in our conventional housing conditions by 8 weeks of age. All animal experiments were performed in accordance with our institutional guidelines, and the Review Board of Kyoto University granted ethical permission for this study.

Experimental Design of DSS-Induced Colitis with TRX-TG Mice

DSS (molecular weight, 36,000–50,000) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Adult sex-matched WT mice (control group: n = 10) and TRX-TG mice (TG group: n = 10) were given 3% (wt/vol) DSS in their drinking water for 7 days, and percentage weight changes were recorded throughout DSS administration (Figure 1*A*). Percentage weight change for each mouse was calculated as follows: percentage weight change =[(weight at specific day (day 0 to day 7) − weight on day 0)/weight on day 0] × 100. Mice were killed under ether anesthesia on day 7.

Experimental Design of DSS-Induced Colitis with WT Mice

To investigate the effects of TRX on colonic inflammation, we designed 3 protocols using the DSS-induced acute colitis model. In these protocols, WT mice were fed 3% (wt/vol) DSS in their drinking water from day 0 to day 5, followed by a return to normal water, and were killed on day 10.

Prophylactic protocol using rhTRX. Five mg/kg of rhTRX (kindly supplied by Ajinomoto Co. Ltd, Kawasaki, Japan) dissolved in 100 μL of phosphate-buffered saline (PBS) or 100 μL of PBS alone was administered intraperitoneally from day 1 to day 9 (Figure 1*B*, *a*, each group: n = 10).

Therapeutic protocol using rhTRX. Five mg/kg of rhTRX or PBS alone was administered intraperitoneally from day 3 to day 9 (Figure 1*B*, *b*, each group: n = 10).

Protocol using anti-TRX antiserum. One hundred microliters of rabbit antimouse TRX antiserum or normal rabbit serum (DAKO Corp. Carpinteria, CA) was administered intraperitoneally on days 0, 1, and 3 (Figure 1*B*, *c*, each group: n = 5).[24] The insulin disulfide reduction assay was performed to confirm that the anti-TRX antiserum effectively neutralized TRX reducing activity (data not shown).

In these studies, percentage weight change was recorded throughout the period. Mice were killed under ether anesthesia, and the colons were removed and processed for pathologic studies as described later.

Experimental Design on IL-10 KO Mice

Five milligrams/kilogram per day of rhTRX or PBS alone was administered intraperitoneally to IL-10 KO mice at 6 weeks of age. Fourteen days after the start of treatment, rhTRX- or PBS-treated mice were killed for histologic analysis of colonic tissue (Figure 1*C*).

Assessment of DSS-induced Colitis

The entire colon was dissected out, and the length was recorded. In TRX-TG mice, blood content in the stool was scored using previously described criteria (Table 1).[28] For histopathologic examination, 3 distal colonic regions spaced approximately 1.5 cm apart were collected into 10% neutral-buffered formalin, processed for paraffin embedding, sectioned (4 μm), and stained with H&E. Inflammation and crypt damage were assessed using a validated scoring scheme as described previously (Table 2),[29] and the means of the scores of each region were determined. The scores were determined in a blinded manner by 2 examiners.

Assessment of Chronic Colitis in IL-10 KO Mice

Mice were monitored for clinical signs of colitis, including diarrhea and weight loss. At necropsy, samples of the colon

Table 2. Histologic Scoring System for DSS-Induced Colitis

Feature scored	Score	Description
Inflammation severity	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Crypts lost: surface epithelium present
	4	Cryps and surface epithelium lost
Percentage involvement	0	0%
	1	1%–25%
	2	26%–50%
	3	51%–75%
	4	75%–100%

BASIC–ALIMENTARY TRACT

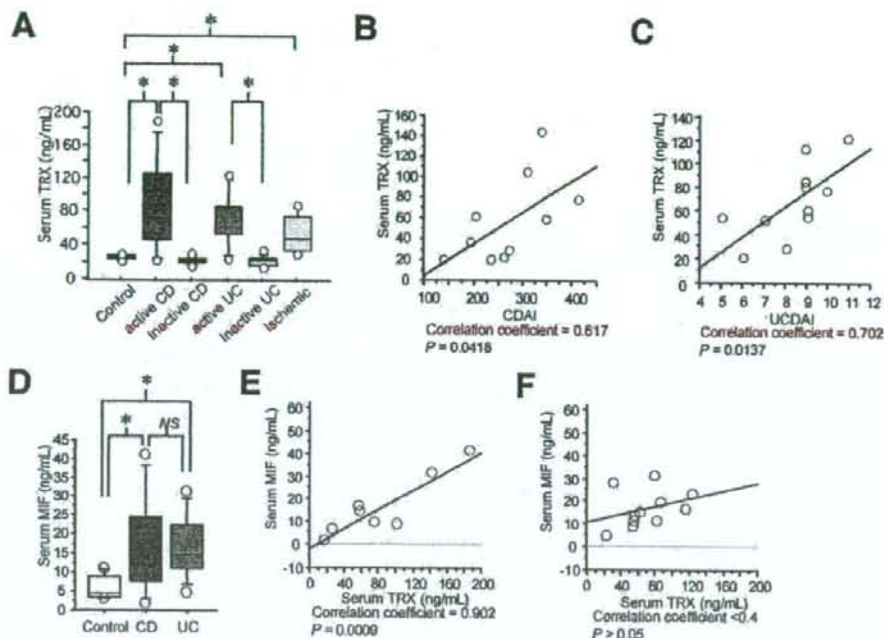

Figure 2. Serum levels of TRX and MIF in patients with IBD. Serum samples were obtained from patients with active CD (n = 10), inactive CD (n = 11), active UC (n = 11), inactive UC (n = 12), active ischemic colitis (n = 11), and colon adenoma (n = 10, as normal controls). (*A*) Serum levels of TRX. (*B*) Correlation between serum TRX levels and CDAI in patients with active CD. (*C*) Correlation between serum TRX levels and UCDAI in patients with active UC. (*D*) Serum levels of MIF. (*E*) Correlation between serum TRX and MIF levels in patients with active CD. (*F*) Correlation between serum TRX and MIF levels in patients with active UC. Results are expressed as means ± SE. *Statistically significant compared with normal controls ($P < .05$). NS, not significant.

(transverse, distal, and proximal) and the rectum were collected and histopathologically examined as described previously. For each section, inflammation (macrophage, lymphocyte, and neutrophil infiltration in the lamina propria or submucosa) was scored for severity according to the following criteria: normal, 0; minimal, 1; mild, 2; moderate, 3; marked, 4; and severe, 5. Gland loss and epithelial hyperplasia were scored by percentage of area involved: none, 0; 1, 1%–10% of the mucosa affected; 2, 11%–25% affected; 3, 26%–50% affected; 4, 51%–75% affected; and 5, 76%–100% affected. The summed scores for inflammation (lamina propria or submucosa), gland loss, and gland hyperplasia were then determined for each animal. For each section, mucosal thickness was quantitated by measuring the distance from the muscularis mucosa to the internal epithelial border in a nontangential area showing the most representative change in severity. Selected sections were scored by a second person, with excellent agreement.

Colon Fragment Culture

Fragment culture of distal colon segments was performed following published methods.[30] Briefly, colon segments were washed in PBS containing 100 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO) and 100 μg/mL penicillin (Sigma Chemical Co.) and then kept in cold serum-free media (RPMI-1640 medium [Gibco BRL, Eggenstein, Germany] with streptomycin and penicillin). After the dry weight was measured, the organs were cut into small pieces in a Petri dish containing fresh media, and 100 mg of tissue fragments were incubated at 37°C in 1 mL fresh media for 18 hours. Culture supernatants were collected and stored at −80°C and assayed for TNF-α, interferon (IFN)-γ, and MIF secretion by enzyme-linked immunosorbent assay (ELISA).

Cell Culture

The human monocytic leukemia cell line THP-1[31] was obtained from the RIKEN Bioresource Center (No. RCB1189; Tsukuba, Japan) and maintained at 37°C and 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, 100 μg/mL penicillin, and 2 mmol/L glutamine (RPMI complete medium). Cells were cultured in 12-well plates (1×10^6 cells/well) in the presence of 16 nmol/L of phorbol-12-myristate13-acetate (Sigma Chemical Co.) for 12 hours, washed twice with PBS, and incubated with RPMI complete medium in the presence or absence of rhTRX (0.01–1 nmol/L) for an additional 24 hours. Cells were then washed twice with PBS and harvested 12 hours after stimulation with 1 μg/mL of *Escherichia coli* O55:B5 LPS (Sigma Chemical Co.) and 10 ng/mL of IFN-γ (Strathmann Biotec AG, Hamburg, Germany) in RPMI complete medium.

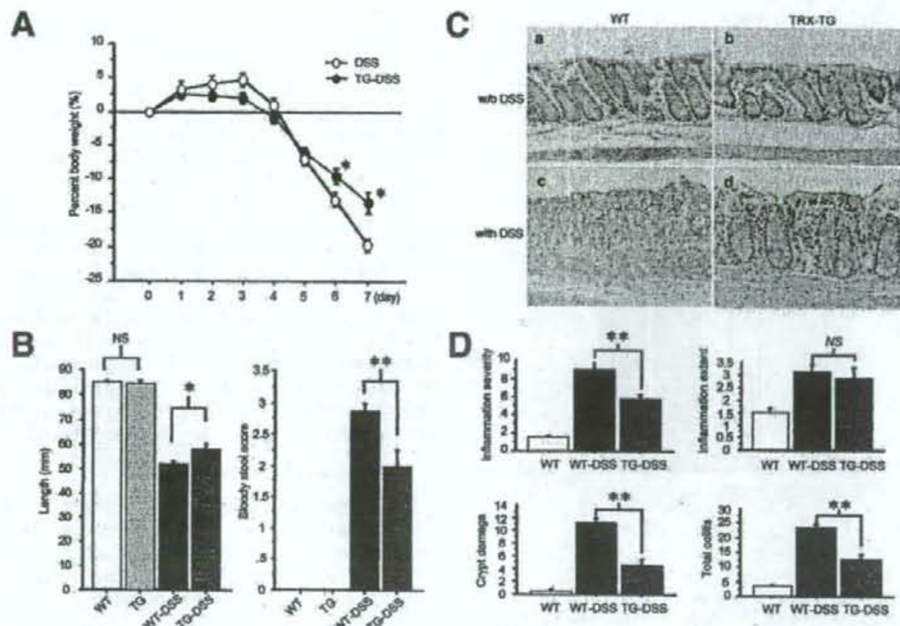

Figure 3. Overexpression of hTRX ameliorated clinical signs and histopathologic features in DSS colitis model. (*A*) Serial changes of percentage body weight during DSS administration in WT (*open circle*) and TRX-TG mice (*solid circle*). (*B*) Colon length and bloody stool score at day 7. (*C*) Representative distal colon sections stained with H&E. (*a*) WT mouse, (*b*) TRX-TG mouse, (*c*) DSS-treated (7 days) WT (WT-DSS) mouse, and (*d*) DSS-treated (7 days) TRX-TG (TG-DSS) mouse. (*D*) Histologic scores showing inflammation severity, inflammation extent, crypt damage, and total colitis. Scores reflect evaluation of 2 segments of distal colon for each animal (n = 10, in each group). Results are expressed as means ± SE. **P* < .05 and ***P* < .01 between WT-DSS and TG-DSS. NS, not significant.

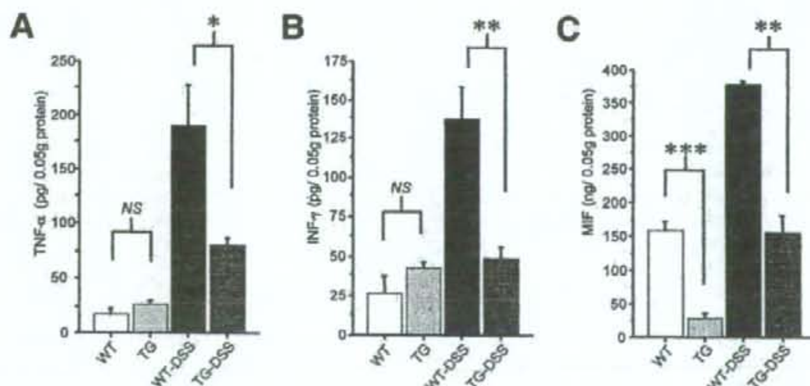

Figure 4. Overexpression of human TRX reduced the DSS-induced production of TNF-α, IFN-γ, and MIF in the distal colon. (*A*) TNF-α, (*B*) IFN-γ, and (*C*) MIF production in the supernatants of cultured colon strips of wild-type (WT) mice, TRX-TG (TG) mice, DSS-treated WT (WT-DSS) mice, and DSS-treated TRX-TG (TG-DSS) mice (n = 8, in each group). Results are expressed as means ± SE. **P* < .05 and ***P* < .01 between WT-DSS and TG-DSS mice. ****P* < .01 between WT and TG mice. NS, not significant.

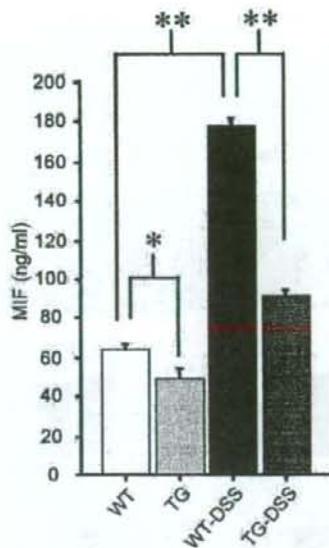

Figure 5. Overexpression of human TRX reduced the DSS-induced increase in MIF serum levels. MIF serum levels of wild-type (WT), TRX-TG (TG), DSS-treated WT (WT-DSS), and DSS-treated TRX-TG (TG-DSS) mice (n = 8, in each group) were determined by ELISA after DSS administration. Results are expressed as means ± SE. $^{*}P < .05$ between WT and TG mice. $^{**}P < .01$ between WT and WT-DSS and WT-DSS and TG-DSS.

Cytokine Assay

The serum levels of human TRX were determined by a sandwich ELISA Kit (Redox Bioscience Inc., Kyoto, Japan) as previously described.[15,32] The serum levels of human MIF were determined by ELISA using the human MIF ELISA kit (Sapporo I.D.L, Sapporo, Japan).[33] Mouse TNF-α, IFN-γ, and MIF concentrations in culture supernatants of colon fragments were determined by ELISA using the mouse TNF-α ELISA kit (R&D Systems Inc., Minneapolis, MN), mouse IFN-γ ELISA set (BD Biosciences, San Diego, CA), and mouse MIF ELISA kit (Sapporo I.D.L.). MIF concentrations in supernatants from an in vitro study were determined by the human MIF ELISA kit (Sapporo I.D.L.).

Western Blot Analysis

For preparation of colon tissue samples, tissues were lysed in RIPA buffer (1% Triton X-100, 1% Na-deoxydolate, 0.1% sodium dodecyl sulfate [SDS], 20 mmol/L Tris-HCl [pH 7.4], 5 mmol/L ethylenediamine-N,N,N',N'-tetra-acetic acid [EDTA], 150 mmol/L NaCl, 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride. Insoluble materials were removed by centrifugation at 12,000g for 10 minutes at 4°C. Supernatants boiled with sample buffer (0.05 mol/L Tris-HCl, 2% SDS, 6% β-mercaptoethanol, 10% glycerol, 1.25% bromophenol blue) were subjected to the assay. For preparation of cell samples, isolated cells were lysed in reducing Laemmli buffer (0.125 mol/L Tris-HCl/SDS, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 2.5% bromophenol blue) and boiled for 5 minutes. After determination of protein concentration with the BCA protein assay kit (Pierce Chemical, Rockford, IL), the solubilized lysates were subjected to Western blot analysis by separation of 10 or 30 μg protein per lane on Tris-glycine gels prior to transfer onto polyvinylidine fluoride membranes (PALL Corporation, Pensacola, FL) using standard protocols. Tris-buffered saline with 0.5% Tween-20 (TBS-T) and 5% skim milk was used to block nonspecific binding to the membrane. Antibodies recognizing TRX (Redox Bio Science Inc), MIF (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Abcam Ltd., Cambridge, England) were added at a dilution of 1:1000, and the membranes were incubated overnight. The membranes were washed 3 times in TBS-T, followed by incubation in TBS-T with 5% skim milk containing anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, England) at a 1:2000 dilution. Immunoreactive bands were visualized using ECL peroxidase developing solution (Amersham Pharmacia Biotech) and recorded on autoradiographic film (Amersham Pharmacia Biotech).

Statistical Analysis

All results were expressed as means ± standard error (SE). Pearson correlation coefficient analysis was used to examine the relationship between serum TRX and CDAI or UCDAI. Parametric data were analyzed by the Student t test. A 2-tailed P value of less than .05 was used to indicate statistical significance.

Results

Serum Levels of TRX Were Increased in Patients With Active IBD

Serum levels of TRX were significantly higher in patients with active CD and with active UC than in controls, whereas those in patients with inactive CD and with inactive UC were not significantly different from those in controls (Figure 2*A*). Patients with ischemic colitis also had significantly higher serum TRX levels than those of controls, although their levels tended to be lower than those of active CD and UC. Furthermore, there were significant correlations between serum TRX levels and CDAI in active CD patients, and between serum TRX levels and UCDAI in active UC patients, indicating that TRX levels correlate with disease activity (Figure 2*B* and *C*). To investigate the relationship between TRX and MIF, we measured serum levels of MIF in the same patients with active CD and with active UC. Serum levels of MIF were also significantly higher in patients with active CD and with active UC than in controls (Figure 2*D*). There was a significant correlation between serum levels of TRX and MIF in patients with active CD (Figure 2*E*), whereas no such correlation was found in patients with active UC (Figure 2*F*).

Overexpression of hTRX Improved DSS-Induced Colitis

To investigate whether overexpression of TRX ameliorates colonic inflammation in DSS-induced colitis, we used TRX-TG and WT mice (Figure 1*A*). During DSS administration, the body weight of the mice gradually decreased. However, the percentage loss of body weight in TRX-TG mice was significantly lower at day 6 and day 7 than that of WT mice (Figure 3*A*). There were significant differences in colonic length and

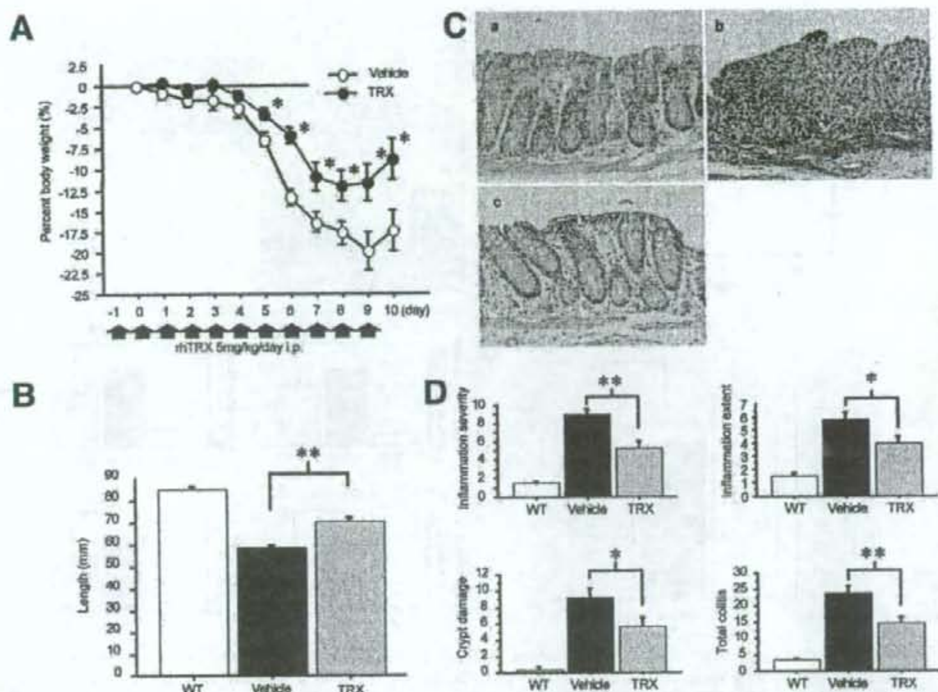

Figure 6. Prophylactic efficacy of rhTRX on DSS-induced colitis. rhTRX (5 mg/kg; TRX-treated group) or vehicle (vehicle-treated group) was administered to DSS-treated mice by intraperitoneal injection from day 1 to day 9 (*arrow*). (*A*) Serial changes of percentage body weight in the vehicle-treated group (*open circle*) and the TRX-treated group (*solid circle*). (*B*) Colon length at day 10. (*C*) Representative distal colon sections stained with H&E. (*a*) WT mouse, (*b*) vehicle-treated colitis mouse, and (*c*) rhTRX-treated colitis mouse. (*D*) Histologic scores showing inflammation severity, inflammation extent, crypt damage, and total colitis. Scores reflect evaluation of 2 segments of distal colon for each animal (n = 10, in each group). Results are expressed as means ± SE. *$P < .05$ and **$P < .01$ vs vehicle-treated group.

bloody stool score between WT and TRX-TG mice at the end of DSS administration (Figure 3*B*). DSS administration for 7 days in WT mice produced an acute colonic inflammation. Histologic analysis of distal colonic sections from DSS-treated WT mice revealed multifocal inflammatory cell infiltration and edema with crypt and epithelial cell loss and ulceration. In contrast, very little mucosal inflammation was observed in colonic sections from DSS-treated TRX-TG mice (Figure 3*C*). Histologic scores were significantly lower in TRX-TG mice than in WT mice at the end of DSS administration (Figure 3*D*).

Overexpression of hTRX Reduced Cytokine Production in DSS-Induced Colitis

To investigate the effects of TRX on proinflammatory cytokines in DSS-induced colitis, we evaluated the production of TNF-α, IFN-γ, and MIF in the colonic tissues of mice. The mean TNF-α, IFN-γ, and MIF concentrations in the supernatants of colon fragment culture were significantly lower in TRX-TG mice than in WT mice after DSS administration. Although there were no differences in TNF-α and IFN-γ concentrations between the 2 groups before DSS treatment, the mean MIF concentration in TRX-TG mice was significantly lower than in WT mice, even before DSS administration (Figure 4*A*–*C*). Western blot analysis revealed a similar tendency (data not shown). These results might suggest that TRX regulate MIF expression in colonic tissue.

Overexpression of hTRX Suppressed DSS-Induced Increase in Serum MIF Levels

Serum MIF levels in DSS-treated WT mice were significantly elevated compared with untreated WT mice. Serum levels of MIF were significantly lower in TRX-TG mice than in WT mice, both before and after DSS administration (Figure 5).

Recombinant hTRX Administration Attenuated DSS-Induced Colitis

To investigate whether exogenous TRX has therapeutic efficacy in colitis, we administered rhTRX intraperitoneally to DSS-treated WT mice using both the prophylactic and therapeutic protocols (Figure 1*B*, a,b). In the prophylactic protocol, the percentage loss of body weight from day 5 to day 10 was significantly lower in the treated group than in the vehicle-treated group (Figure 6*A*). There was a significant difference of colonic length between TRX-treated mice and vehicle-treated mice (Figure 6*B*). Consistent with observations in the experiment using TRX-TG mice, histologic assessment of distal co-

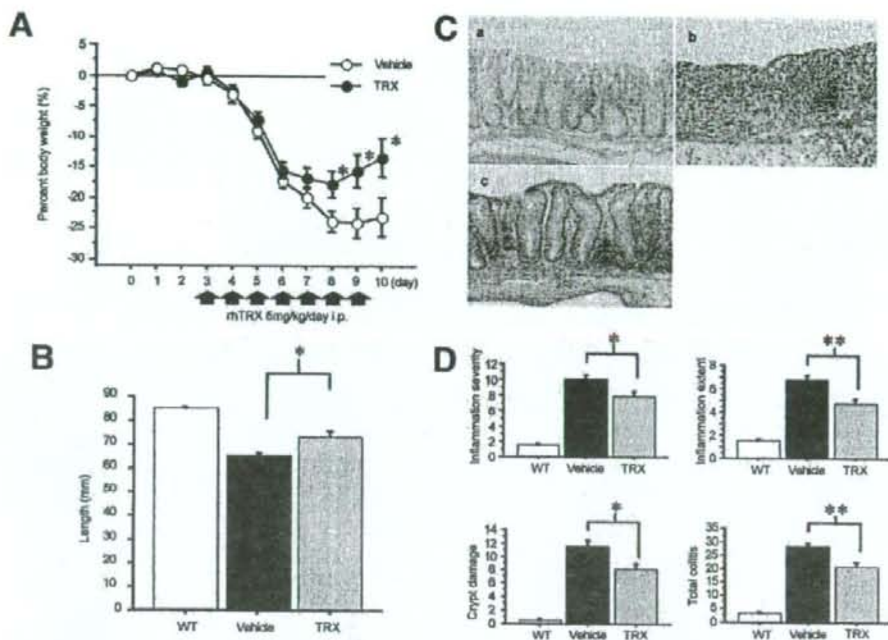

Figure 7. Therapeutic efficacy of rhTRX on DSS-induced colitis. rhTRX (5 mg/kg; TRX-treated group) or vehicle (vehicle-treated group) was administered to DSS-treated mice by intraperitoneal injection from day 3 to day 9 (*arrow*). (*A*) Serial changes of percentage body weight in the nontreated group (*open circle*) and the TRX-treated group (*solid circle*). (*B*) Colon length at day 10. (*C*) Representative distal colon sections stained with H&E (a) WT mouse, (*b*) vehicle-treated colitis mouse, and (c) TRX-treated colitis mouse. (*D*) Histologic scores showing inflammation severity, inflammation extent, crypt damage, and total colitis. Scores reflect evaluation of 2 segments of distal colon for each animal (n = 10, in each group). Results are expressed as means ± SE. *$P < .05$ and **$P < .01$ vs vehicle-treated group.

lonic sections indicated that rhTRX administration reduced tissue damage such as inflammatory cell infiltration, crypt loss, and ulceration (Figure 6*C*). Each of the histologic scores in TRX-treated mice was significantly lower than in nontreated mice (Figure 6*D*).

Similar to the results in the prophylactic protocol, the percentage loss of body weight and shortening of the colon were significantly lower in TRX-treated mice than vehicle-treated mice in the therapeutic protocol (Figure 7*A* and *B*). Histologically, TRX administration reduced tissue damage (Figure 7*C*) and histologic scores compared with vehicle administration (Figure 7*D*).

Administration of Anti-TRX Antiserum Exacerbated DSS-Induced Colitis With Increase in Serum MIF Levels

To investigate the role of endogenous TRX in the attenuation of DSS-induced colitis, we used anti-mouse TRX antiserum to neutralize the endogenous TRX. With regard to percentage weight change, colon length, and histologic feature, neutralization of TRX by anti-TRX antiserum resulted in marked exacerbation of DSS-induced colitis, whereas the control serum had no such effect (Figure 8*A–D*). Notably, the neutralization of TRX significantly increased serum levels of MIF in DSS colitis mice (Figure 8*E*).

Effects of rhTRX on Colonic Inflammation of IL-10 KO Mice

All IL-10 KO mice treated with rhTRX or PBS survived the study. Histologic examination of colonic tissue from PBS-treated IL-10 KO mice demonstrated epithelial hyperplasia, crypt abscesses, and severe acute and chronic cellular infiltration in the lamina propria (Figure 9*B*). In contrast, rhTRX (5.0 mg/kg) administration improved marked colonic inflammation in IL-10 KO mice (Figure 9*C*). As shown in Figure 9*D*, colonic histologic scores in IL-10 KO mice treated with rhTRX were significantly lower than in those treated with PBS alone.

rhTRX Suppressed MIF Production In Vitro

We investigated the in vitro effect of rhTRX on MIF production in a human macrophage-like cell line, THP-1. Western blot analysis and ELISA revealed that rhTRX dose-dependently reduced not only MIF production but also its release induced by LPS and IFN-γ (Figure 10*A* and *B*).

Discussion

The present study demonstrated that serum TRX levels were significantly higher in patients with IBD than in normal controls and were significantly correlated with disease activity. More importantly, overexpression of hTRX or administration

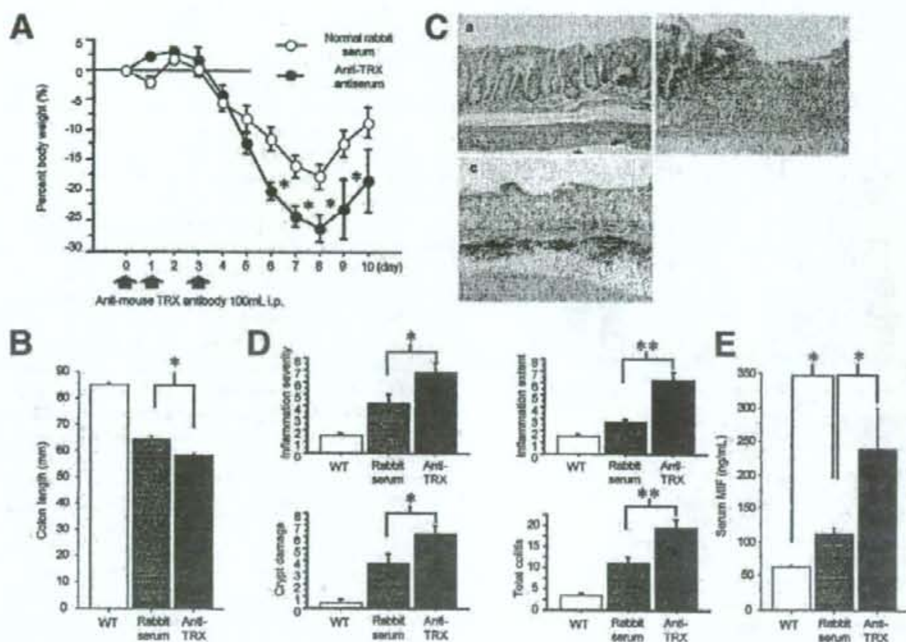

Figure 8. Exacerbating effect of anti-TRX antiserum on DSS-induced colitis. One hundred microliters/body of anti-TRX antiserum (anti-TRX group) or normal rabbit serum (rabbit serum group) was administered to DSS-treated mice by intraperitoneal injection on days 0, 1, and 3 (*arrow*). (*A*) Serial changes of percentage body weight in the rabbit serum group (*open circle*) and the anti-TRX group (*solid circle*). (*B*) Colon length at day 10. (*C*) Representative distal colon sections stained with H&E (*a*) wild-type (WT) mouse, (*b*) normal rabbit serum-treated colitis mouse, and (*c*) anti-TRX antiserum-treated colitis mouse. (*D*) Histologic scores showing inflammation severity, inflammation extent, crypt damage, and total colitis. Scores reflect evaluation of 2 segments of distal colon for each animal (n = 5 in each group). (*E*) Serum MIF levels of WT mice, anti-TRX group, and rabbit serum group (n = 5 in each group) were determined by ELISA at day 10. Results are expressed as means ± SE. $^{*}P < .05$ and $^{**}P < .01$.

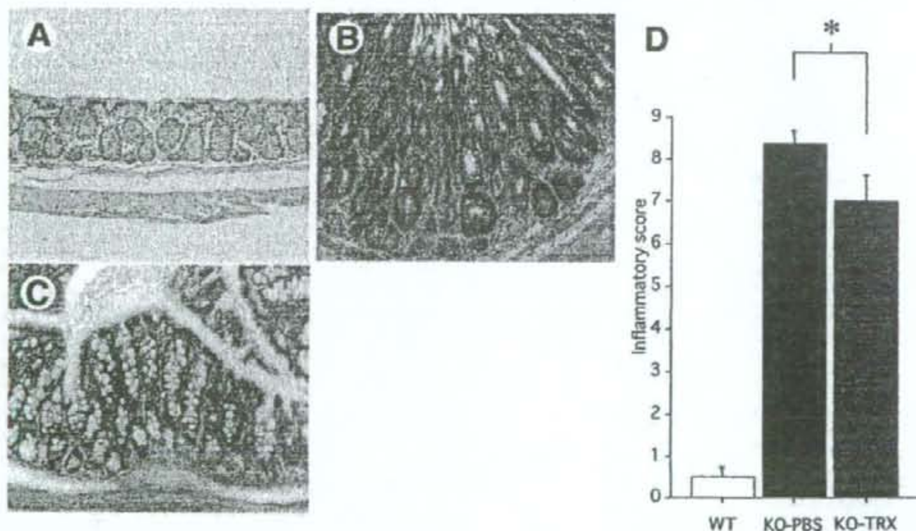

Figure 9. Administration of rhTRX ameliorated histopathologic features in IL-10 KO mice. Representative proximal colon sections stained with H&E. (*A*) WT mouse, (*B*) PBS-treated IL-10 KO mice (KO-PBS), and (*C*) rhTRX-treated IL-10 KO mice (KO-TRX). (*D*) Inflammatory score. Results are expressed as means ± SE. $^{*}P < .05$ between KO-PBS and KO-TRX.

BASIC–ALIMENTARY TRACT

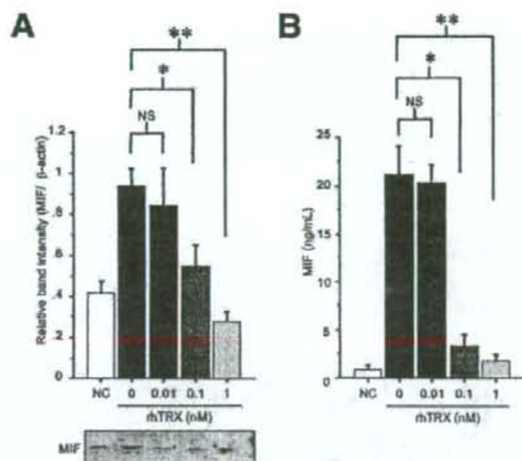

Figure 10. Effect of exogenous rhTRX on MIF expression in THP-1 cells. THP-1 cells were cultured in 12-well plates in RPMI complete medium with 16 nmol/L phorbol-12-myrisate 13-acetate for 12 hours and then incubated in RPMI complete medium in the presence of rhTRX (absence, 0.01, 0.1, and 1 nmol/L). Cells were harvested 12 hours after stimulation with 1 μg/mL LPS and 10 ng/mL IFN-γ in RPMI complete medium. Control cells were cultured without TRX, LPS, and IFN-γ in the same conditions (NC). (*A*) Western blot measurement of MIF in cell samples was performed as described in the Materials and Methods section. Results are expressed as relative band intensity quantified by densitometric analysis of MIF expression normalized with β-actin expression. (*B*) MIF released into supernatants of cultured THP-1 cells. *Bars* represent mean values ± SE of 3 independent experiments. $^*P < .05$ and $^{**}P < .01$ vs control. NS, not significant.

of rhTRX decreased the severity of DSS-induced colitis in mice as shown by clinical, histologic, and immunologic parameters. Moreover, administration of rhTRX decreased the severity of colonic inflammation in IL-10 KO mice. These findings strongly suggest that TRX is involved in the pathophysiology of IBD and that TRX has a therapeutic effect on experimental colitis.

TRX is induced in various inflammatory conditions and has a role in preserving cellular homeostasis through maintaining the redox state. Increased ROS levels and oxidative damage occur in inflamed mucosa of patients with IBD[7] and ischemic colitis,[34] and such an imbalance in the redox state appears to induce TRX production[13] Therefore, it is possible to speculate that the elevation of serum TRX levels is a host defense response against oxidative stress in patients with colitis such as IBD and ischemic colitis.

One important issue for this study was to clarify the mechanisms by which TRX attenuates colitis. For this purpose, we used the DSS-induced colitis model in mice, which is a well-established model of colonic inflammation.[35] Colonic inflammation induced by DSS administration is mainly attributed to direct chemical injury of colonic epithelial cells by DSS, with a resultant increase in oxidative stress and activation of macrophages.[36,37] Previous studies reported that overexpression or administration of antioxidants reduces oxidative stress in experimental colitis models.[38-40] In this study, we clearly demonstrated that TRX ameliorated DSS-induced colonic inflammation. TRX down-regulated TNF-α, IFN-γ, and MIF production from cultured colon strips of DSS-induced colitis mice. In addition, rhTRX decreased the severity of colonic inflammation in IL-10 KO mice, a typical model of T-cell-mediated colitis, as documented by the histologic parameters. This beneficial effect was similar to results in the acute DSS model.

It should be emphasized that, in the present study, colonic and serum MIF levels were lower in TRX-TG mice than in WT mice before DSS administration, as is in contrast with TNF-α and IFN-γ that showed similar levels between TRX-TG and WT mice before the treatment. Therefore, it is possible that down-regulation of MIF levels is more important than that of TNF-α or IFN-γ for the ameliorating effect of TRX on colonic inflammation. Thus, to clarify further the direct relationship between TRX and MIF in vivo, we examined whether neutralization of TRX affects MIF production. As expected, administration of anti-TRX antiserum resulted not only in exacerbation of DSS-induced colitis but also an increase in serum MIF levels. Furthermore, in vitro studies demonstrated that rhTRX down-regulated MIF production and its release from THP-1 cells, suggesting that TRX and MIF counteract each other.

MIF was originally identified as a lymphocyte mediator that inhibits macrophage migration.[41,42] Thus, MIF has been assumed to have anti-inflammatory activities. MIF, however, induces various inflammatory cytokines,[43] nitric oxide, and superoxide anions.[44] Moreover, MIF enhances macrophage and lymphocyte proliferation.[45] During inflammatory processes, MIF is produced mainly by activated macrophages and lymphocytes[46] and has an important role in the pathophysiology of inflammatory conditions, such as septic shock, rheumatoid arthritis, and lung diseases.[46] Accordingly, MIF is now believed to have a potent inflammatory action rather than an anti-inflammatory action. Because of its potent inflammatory effect, MIF is also thought to be involved in the pathogenesis of IBD. In fact, as shown in this study, serum MIF levels are increased in patients with UC and CD.[3,4] Moreover, trinitrobenzene sulfonic acid does not induce colitis in MIF-deficient mice, and transfer of CD45RBhi T cells from MIF-deficient mice to severe combined immunodeficieny mice also does not induce colitis.[4] In addition, administration of anti-MIF antibody ameliorated DSS-induced colitis.[3] Taken together with our present study, MIF appears to have an important role in the development of IBD.

The TRX superfamily contains a conserved catalytic site consisting of a Cys-X_1-X_2-Cys sequence that is essential for its oxidoreductase activity.[47] Interestingly, MIF has a similar conserved catalytic site consisting of Cys^{57}-Ala-Leu-Cys^{60} (CALC motif); the CALC motif is indispensable for MIF activity.[48,49] Thus, MIF is now included in the TRX superfamily. However, the MIF activity is very different from that of TRX in several areas. For example, in contrast to TRX, MIF induces ROS and does not have an antioxidative activity. Furthermore, MIF inhibits the anti-inflammatory and immunosuppressive effects of steroids,[43] whereas TRX directly interacts with the glucocorticoid receptors in the nucleus, allowing transcriptional activation of the glucocorticoid receptor under oxidative conditions.[50] MIF also down-regulates activity of peroxiredoxin-1 that exhibits antioxidative effects by reducing electrons provided by TRX.[51-53] In these respects, overexpression of intracellular rhTRX down-regulates MIF production in Jurkat T cells.[32] In support of our previous data, we also demonstrated that

exogenous TRX inhibits production and release of MIF from a human monocyte cell line. Moreover, we clearly showed that blocking of TRX activity resulted in the increase of serum MIF levels coupled with exacerbation of DSS-induced acute colitis in mice. Taken together, our study suggests that TRX and MIF counteract each other in various aspects during the inflammatory process.

In conclusion, we demonstrated that TRX is involved in the pathophysiology of IBD. Moreover, we showed that TRX ameliorates colonic inflammation not only by its antioxidative and anti-inflammatory effects but also by down-regulating MIF production. Considering the potent protective role of rhTRX in experimental colitis, rhTRX might be a new therapeutic molecule for treatment of IBD.

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RESEARCH ARTICLE

Attenuation of mouse acute colitis by naked hepatocyte growth factor gene transfer into the liver

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Abstract

Background Hepatocyte growth factor (HGF) has multiple biological effects on a wide variety of cells. It modulates intestinal epithelial proliferation and migration, and critically regulates intestinal wound healing.

Aims To investigate the therapeutic effect of HGF gene transfer, we introduced the HGF gene into the liver of mice with acute colitis.

Methods The rat HGF expression plasmid vector, pCAGGS-HGF, was injected via the tail vein into C57BL/6 mice, followed by dosing with dextran sulfate sodium in distilled water. Firstly, the HGF gene was injected once on day 0. Secondly, the HGF gene was injected on day 0 and again on day 2.

Results Injection of the HGF gene ameliorated colitis with inhibition of both loss of body weight and shortening of colon length. It protected the colon from epithelial erosions and cellular infiltration. Expression of mRNAs for IFN-γ, IL18, and TNF-α was reduced in the colon. In contrast, expression of mRNA for IL-10 was increased. The numbers of BrdU-positive intestinal epithelial cells were increased, and the numbers of TUNEL-positive apoptotic cells were decreased. Furthermore, a second injection prolonged the elevation of serum HGF levels, and ameliorated the symptoms better than a single injection. The empty pCAGGS plasmid did not ameliorate acute colitis.

Conclusions HGF gene transfer attenuated acute colitis by facilitating intestinal wound repair as well as inhibiting inflammation, suggesting a new strategy for treatment of IBD. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords HGF; c-Met; inflammatory bowel disease; DSS colitis; naked gene transfer

Introduction

Inflammatory bowel disease, which comprises ulcerative colitis and Crohn's disease, is characterized by chronically relapsing inflammation of the bowel of unknown origin [1]. Therapy for inflammatory bowel disease has been aimed predominantly at the regulation of the inflammatory cells and their production of various inflammatory mediators, such as arachidonic acid metabolites, chemokines, and pro-inflammatory cytokines [2,3]. Conventional therapy for inflammatory bowel disease involves 5-aminosalicylates, corticosteroids, and immunosuppressive drugs such as azathioprine. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been investigated as target molecules for the development of newer therapeutic approaches for inflammatory bowel disease [4]. Anti-TNF-α antibodies have been established

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for the treatment of Crohn's disease [5], and anti-IL-6 receptor antibodies have been tried clinically with great success [6]. These agents are beneficial and established as a standard therapy for inflammatory bowel disease; however, they have limited response rate and serious side effects. Therefore, newer therapeutic approaches are required.

Epidermal growth factor (EGF) is a potent mitogenic peptide produced by the salivary and duodenal Brunner's glands and stimulates several components of the healing response [7,8]. In a recent report, EGF enema has been shown to be an effective treatment for active left-sided ulcerative colitis [9]. Thus, newer therapeutic approaches for inflammatory bowel disease should be aimed at regeneration and repair of the wounded intestinal epithelial cells of the bowel as well as inhibition of inflammation.

Hepatocyte growth factor (HGF) is a pleiotropic factor initially identified as a growth factor for hepatocytes [10–12]. It has mitogenic, motogenic, and morphogenic functions in various types of cells, including gastrointestinal epithelial cells [13–15], through its high-affinity receptor tyrosine kinase, Met, that is encoded by the c-*met* proto-oncogene [16]. HGF activator, HGF activator inhibitor type-1, and HGF-associated molecules involved in the activation of HGF in injured tissues are associated with colonic mucosal repair [17]. HGF expression has been reported to be up-regulated in the inflamed colonic mucosal tissue in patients with ulcerative colitis [18], and plasma HGF levels are increased in animal models of acute colitis [19]. Recently, the administration of recombinant human HGF has been shown to facilitate colonic mucosal repair in rats with dextran sulfate sodium (DSS)-induced colitis [20], and in HLA-B27 transgenic rats [21].

Liver is an important target organ for gene transfer because of its high capacity for synthesizing serum proteins and its involvement in numerous genetic and acquired diseases [22]. Among the various gene delivery systems available, naked DNA-mediated gene transfer is the simplest, and techniques for introducing DNA into hepatocytes have been the most intensely studied methods for generating therapeutic amounts of gene product [23]. High levels of foreign gene expression can be achieved in mouse hepatocytes by rapid tail vein injection of a large volume of naked DNA solution, the 'hydrodynamics-based procedure' [24]. In this study, we investigated the therapeutic effect of rat HGF gene transfer into the liver by the hydrodynamics-based method in a murine model of colitis.

Materials and methods

Animals and induction of colitis

Female C57BL/6 (B6) mice (7–8 weeks old) were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and maintained in the Animal Center of Niigata University School of Medicine under specific pathogen-free conditions. Colitis was induced in the mice by the administration of 3–5% DSS (molecular weight 36 000–50 000; Wako, Osaka, Japan) in distilled water *ad libitum* for 7 days. Three hours before the administration of DSS at day 0 and/or day 2, we injected HGF or control vector into the mice as described below. Body weight, stool consistency (scores: 0, normal stools; 1, soft stools; 2, liquid stools), hemoccult positivity and the presence of gross blood (scores: 0, negative fecal occult blood; 1, positive fecal occult blood; 2, visible rectal bleeding) were assessed daily. The disease activity index (DAI) was determined as a combination of the above parameters according to the scoring criteria as we described previously [25]. All animal experiments were performed according to the 'Guide for Animal Experiments' of Niigata University School of Medicine.

Plasmid DNA injection techniques

Plasmid pCAGGS-rat-HGF was constructed by inserting the rat HGF cDNA into a unique EcoRI site in the pCAGGS expression vector, which has the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter, and grown in *Escherichia coli* DH5α (Toyobo, Osaka, Japan). The plasmid was prepared using a Qiagen Endofree plasmid Giga kit (Qiagen GmbH, Hilden, Germany), as described previously. The empty pCAGGS plasmid was used as a control. The plasmid DNA was diluted in 2 ml (approximately 1/10 of the body weight) of Ringer's solution (Ohtsuka, Tokushima, Japan) at room temperature. At day 0 and/or day 2, we anesthetized the mice with diethyl ether, and injected 10 µg of either pCAGGS-HGF or pCAGGS plasmid into the tail vein through a 27-gauge needle with a <3 s injection time.

Measurement of HGF in plasma

To determine the concentration of HGF at various time points after injection, we used an enzyme-linked immunosorbent assay (ELISA) kit for rat HGF (Institute of Immunology, Tokyo, Japan) including an antibody that cross reacts with mouse HGF. Values are expressed in ng/ml.

Evaluation of histology

The degree of colonic injury was assessed by colon length and histological score. The entire colons (10 mice per group) were sampled and their lengths were recorded immediately. The entire colon was fixed in 4% formalin, embedded in paraffin, and transverse sections were stained with hematoxylin and eosin. According to the preliminary histological interpretation, we analyzed the distal colon tissue section located approximately 10 mm from the anal verge to calculate the number of infiltrating

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cells in the lamina propria of the colon. The crypt length of the colon of each mouse was also calculated as a mean value of five different crypts.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) to detect cytokine mRNAs

Total RNA was extracted from colon specimens with Trizol (Gibco BRL) according to the standard protocol then reverse-transcribed. Thereafter, cDNA was amplified using the ABI 7700 sequence-detector system (Applied Biosystems, Foster City, CA, USA) with a set of primers and probes corresponding to IFN-γ, TNF-α, IL-1β, IL-12, IL-18, IL-4, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.

X-gal and immunohistochemical staining

pCAGGS-lacZ expresses *E. coli* β-galactosidase. The lung, heart, liver, spleen, and kidneys were harvested for X-gal staining 1 day after the injection of 10 µg of pCAGGS-lacZ, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan), and then frozen in a mixture of dry ice and acetone. Serial sections (5-µm thick) were cut with a cryostat and placed on glass slides coated with 3-aminopropyltriethoxysilane. Then the sections were fixed in 1.5% glutaraldehyde at room temperature for 10 min, washed three times in cold phosphate-buffered saline (PBS) (5 min/wash), and incubated in an X-gal staining solution containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 0.5% Nonidet P-40 in PBS, pH 7.4, at 37 °C for 3 h, followed by counterstaining with nuclear fast red.

Immunohistochemical staining

All mice were injected with BrdU (Sigma) (500 µg/100 µl in PBS) 1 h before killing. To detect replicating cells, tissue sections were reacted with the antibody and reagents using a BrdU staining kit (ZYMED, South San Francisco, CA, USA) according to the manufacturer's instructions. Crypts that had five or more BrdU-labeled nuclei were defined as surviving crypts. The numbers of BrdU-labeled nuclei of colonic epithelial cells for each group were compared.

Terminal deoxynucleotide transferase labeling

Apoptotic cells were identified using an *in situ* apoptosis detection kit (Takara Biomedicals, Japan) according to the manufacturer's instructions. In brief, acetone-fixed 5-mm fresh-frozen colon sections were permeabilized on ice and incubated with the terminal deoxynucleotide transferase mixture for 1 h at 37 °C. FITC-labeled dNTP were treated with anti-FITC HRP for 30 min, visualized with DAB, and counterstained with hematoxylin.

Monoclonal antibodies

The following monoclonal antibodies were used for immunofluorescence and flow cytometric analyses: anti-CD4 (clone GK1.5, IgG2b), anti-CD8 (clone 53-6.7, IgG2a), anti-B220 (clone RA3-6B2, IgG2a), anti-Mac-1 (clone M-70.15, IgG2b), anti-mouse INF-γ (clone XMG1.2), and anti-mouse IL-10 (clone JES5-16E3).

For immunostaining of HGF and c-Met, a goat polyclonal IgG antibody raised against a peptide mapping at the carboxyl terminus of HGFα (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the carboxyl terminus of c-Met p140 of mouse origin (SP260; Santa Cruz Biotechnology) was used.

IF staining procedure

Frozen sections of the colon were prepared in a cryostat and stained with several fluorescent dye-conjugated anti-mouse antibodies as described above. The sections were observed by fluorescence microscopy.

Ex vivo colonic tissue culture

Ex vivo colon tissue culture was done by the established method with modification described as below [26]. The entire colon was taken from B6 mice with DSS colitis on days 2 and 5, or from those without colitis. The left side of the colon was cut into small pieces, each of which weighed 15 mg. Each colon sample was washed three times using RPMI 1640 culture medium containing 10% FBS (fetal bovine serum), penicillin G (100 IU/ml), and streptomycin (10 ng/ml), and placed in a center well of an organ culture dish (Falcon; Becton Dickinson Labware, NJ, USA). The center well of the dish was filled with 1 ml of the same medium, and incubated for 2 h and 24 h at 37 °C under 95% air and 5% CO_2. To evaluate the direct effect of HGF on *ex vivo* colon tissue, each sample was treated with recombinant human HGF (IBL, Gunma, Japan) at a concentration of 10 ng/ml at the beginning of tissue culture. The total RNA was extracted from each colon tissue and analyzed as described above for determining the mRNA expression levels for IFN-γ, IL-10, mouse HGF, and c-Met.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed using the unpaired

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