STAT3 and importins are novel mediators of early molecular and cellular responses in experimental duodenal ulceration

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ABSTRACT

Objectives Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which directly upregulates VEGF, Ref-1, p21 and anti-apoptotic genes such as Bcl-xL. In this study, we hypothesized that STAT3 signaling is activated and provides a critical protective role that is required for enterocyte survival during the early phases of cysteamine-induced duodenal ulcers.

Methods We studied the effect of inhibition of STAT3 activity on cysteamine-induced duodenal ulcers in rats and egr-1 knockout mice using STAT3/DNA binding assay, immunohistochemistry, immunoblot, quantitative reverse transcriptase PCR analyses.

Results We found that G-quartet oligodeoxynucleotides T40214, a specific inhibitor of STAT3/DNA binding aggravated cysteamine-induced duodenal ulcers in rats by 2.8-fold ($p<0.05$). In the pre-ulcerogenic stage cysteamine induced STAT3 tyrosine phosphorylation, its translocation to nuclei, an increased expression and nuclear translocation of importin α and β in the rat duodenal mucosa. Cysteamine enhanced the binding of STAT3 to its DNA consensus sequences at 6, 12 and 24 h after cysteamine by 1.5, 1.8 and 3.5- fold, respectively and activate the expression of STAT3 target genes such as VEGF, Bcl-xL, Ref-1, and STAT3-induced feedback inhibitor, suppressor of cytokine signaling 3 (SOCS3). We also demonstrated that egr-1 knockout mice which are more susceptible to cysteamine-induced duodenal ulcers had lower levels of STAT3 expression, its phosphorylation, expression of importin α or β, and STAT3/DNA binding than wild-type mice in response to cysteamine.

Conclusion Thus, STAT3 represents an important new molecular mechanism in experimental duodenal ulceration.

Keywords STAT3 · Experimental duodenal ulcers · I**mportin α and β** · **A**nti-apoptotic genes.

Introduction

Gastroduodenal ulcer disease remains a common condition, although the reported incidence and prevalence are decreasing, but that has "not resulted in fewer hospital admissions for peptic ulcer disease-related complications" [1, 2]. Duodenal ulcers after eradication of *H. pylori* may remain healed after reduction of acid secretion despite persistent infection, in part because *H. pylori* infection decreases the bioavailability of angiogenic growth factors and the healing of wounded epithelial cells [3-5]. *H. pylori*-negative ulcers are associated with high overall patient mortality and patients with history of *H. pylori*-negative idiopathic bleeding ulcers have a high risk of recurrent ulcer bleeding and mortality [6].

Gastroduodenal ulcers develop when the protective mechanisms of the gastroduodenal mucosa, such as mucus and bicarbonate secretion, are overwhelmed by the damaging effects of gastric acid, pepsin and others; and gastroduodenal mucosal damage depends on a break in the normal balance between aggressive and protective factors. The activation of the protective mechanisms of the gastrointestinal mucosa would be great achievement in the therapy of ulcer disease. Thus, it would be valuable in the clinical setting to determine the therapeutic effect of increased resistance of the mucosa on NSAID- or other chemically induced injury of the small intestine.

Signal transducers and activators of transcription (STAT) are a family of structurally related proteins that play important roles in the intracellular transduction of signals regulated by oxidative stress, various cytokines and growth factors [7-11]. STAT3 was initially identified as a transcription factor that mediated the effects of IL-6 in acute phase response in the liver [12]. STAT proteins contain an SH2 domain and a conserved tyrosine residue near the carboxyl

terminus that is phosphorylated upon activation [9,13]. The integration of cell and tissue-specificity signaling from active JAK kinases or members of the Src family kinases and STAT proteins, leads to cell proliferation, cell survival and differentiation, the end-point of the cytokine/growth factor stimulus [9,14-17].

STAT3 is a latent cytoplasmic transcription factor and must gain entrance to nucleus to impact transcription. The nuclear trafficking of STAT3 is mediated by various importin α and importin β [18], and occurs through the nuclear pore complex by an active process facilitated by nuclear localization signal (NLS) present in protein. NLS-containing cargos are usually recognized by the importin α–β heterodimers. Importin α recognizes and binds the NLS, and importin β docks the trimeric complex to the nuclear pore, which translocates STAT3 into the nucleus [19-21].

STAT3 modulates the transcription of genes involved in the regulation of critical functions, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses. STAT3 activation correlates with the expression of Bcl-xL and Mcl-1, consistent with the induction of anti-apoptotic genes, which promote the survival and cell progression [22]. Persistent STAT3 activity promotes *in vivo* angiogenesis, in part by inducing VEGF, a potent inducer of angiogenesis [23-25]. STAT3 activity is absolutely required for small-intestine crypt stem cell survival [26].

The activation of STAT3 in the duodenal ulceration has not been investigated. In this study, we analyzed the possible cellular events and signaling pathways in the protective mechanisms against duodenal ulceration. We delineated the precise effects of duodenal ulcerogen cysteamine on STAT3 expression and transcription activity in duodenal mucosa, and STAT3 localization in epithelial crypt cells of “progenitor zone”. We investigated the participation of importin α and β

in STAT3 nuclear import, the level of cysteamine-induced STAT3/DNA binding and the effect of STAT3 activity on target genes expression of Bcl-xL, Ref-1, VEGF, and bFGF.

Materials and Methods

Animal Experiments

This study was approved by the Subcommittee for Animal Studies and the Research and Development Committee of VA Long Beach Healthcare System. Female Sprague-Dawley rats (180-210 g) were maintained on standard laboratory rat chow (Harlan Teklad Rodent Diet, 8604, 150-250 ppm Fe, Harlan Teklad, Madison, WI). Animals had unlimited access to food and water. Randomized groups of rats (n=5) were given either water or duodenal ulcerogen cysteamine-HCl (25 mg/100 g, Sigma-Aldrich, St. Louis, MO) by intragastric (i.g.) gavage once and euthanized 0.5 or 2 h later, twice or three times at 4 h intervals and rats were euthanized 6, 12 and 24-48 h after the first dose of cysteamine. As second duodenal ulcerogen we used mepirizole (20 mg/100 g, Sigma-Aldrich, St. Louis, MO) dissolved in 1% methylcellulose and injected subcutaneously (s.c.). In pharmacologic experiments, detailed dose- and time-response studies were performed with STAT3/DNA binding inhibitor G-quartet oligodeoxynucleotides (GQ-ODN), T40214: 5'-GGGCGGGCGGGCGGGC-3' [27] (1 mg/100 g) or scrambled control for T40214, (1 mg/100g) (Midland Certified Reagent Companyx Inc., Midland, Texas). STAT3 GQ-ODN T40214 or scrambled control plus polyethylenimine (PEI) was injected s.c. 30 min before each cysteamine treatment. PEI with MW=25 kDa was purchased from Polysciences, Inc., (Warrington, PA). T40214 was dissolved in H_2O, heated at 95˚ for 10 min and gradually cooled to room temperature. The PEI was added into T40214 solution to make the complex of T40214 at 1µg/µl with 2.0 µg/µl of PEI (PEI/ODN of 2:1). Animals were

euthanized at 6 and 48 h after first cysteamine administration. Scrapings of proximal duodenal mucosa (2.0 cm) were harvested and frozen in liquid nitrogen. Similar studies were performed in egr-1 knockout mice, which originally were received from Marilyn Thoman, PhD, Sidney Kimmel Cancer Center, San Diego, CA. These mice were treated with cysteamine (65 mg/100 g, i.g.) and euthanized 0.5, 2, 6, 12 and 48 h later to obtain the tissue from proximal duodenum for further investigation. The duodenal ulcer crater dimensions were measured in millimeters, and the ulcer areas were calculated using the ellipsoid formula.

Extraction and purification of total RNA and proteins

TriZol® (Invitrogen, San Diego, CA) and RNeasy kit (Qiagen, San Diego, CA) was used for total RNA extraction and purification. Total protein extraction was performed as described previously [28] and for nuclear and cytoplasmic protein extraction we used Active Motif Nuclear Extraction Kit (Carlsbad, CA). Protein concentration was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Real-time polymerase chain reaction (PCR)

Total RNA extraction from different tissues was performed using Trizol Reagent (Invitrogen, Carlsbad, CA) followed by purification with RNeasy Mini Kit (Qiagen, Valencia, CA). For cDNA synthesis, 5 µg total RNA was used with the First-Strand cDNA Synthesis Super Mix and M-MLV Reverse Transcriptase, (Invitrogen, Carlsbad, CA). Real-time PCR was performed using TagMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and an iQ5 Real-Time PCR detection system (Bio-Rad iCycler Real-Time PCR instrument; Bio-Rad, Hercules, CA). The specific primers for IL-6, SOCS3 were purchased from Applied Biosystems Inc.

(Foster City, CA). The thermal profile was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, finally holding at 4°C. The relative quantification of expression of the gene was normalized to the internal control gene β-actin.

Western blotting

Equal amount of proteins (100 μg) was subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) according to established techniques. The membranes were incubated for 3 hr at 20°C with antibody STAT3, Tyr705-phosphorylated STAT3, importins α and β, c-Src, p-Src (Tyr416), pJAK1, pJAK2 and pTyk2 (Cell Signaling Technology, Inc., Danvers, MA) and pErk1/2, Bcl-xL, p21, Ref-1, bFGF, VEGF, lamin A/C, α tubulin and actin/GAPDH antibodies (Santa Cruz Biotech. Inc., Santa Cruz, CA).

Co-immunoprecipitation

0.5 – 1.0 mg of proteins from rat duodenal mucosa were used for immunoprecipitation of STAT3 with anti-STAT3 antibody and gp-130 with anti gp-130 antibody (Cell Signaling Technology, Inc., Danvers, MA). Immunoprecipitation was performed in the presence of 5 μg of the indicated primary antibody at 4 °C overnight. Immune complexes was captured by adding 50 μl of protein A-Sepharose beads (Thermo Fisher Scientific, Rockford, IL) and rotated at 4 °C for 2-4 h. After centrifugation the supernatant was discarded, protein A-Sepharose beads were washed with cold PBS for 4–5 times, and immunoprecipitates were fractionated by SDS-PAGE and importins α were detected using anti-importins α antibody. Detection of tyrosine phosphorylation of gp130 was demonstrated by anti-phosphotyrosine antibody (Cell Signaling Technology, Inc., Danvers, MA).

Immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin, sectioned and irradiated at 750W in a microwave oven with 3% hydrogen peroxide in 0.01 M sodium citrate buffer pH 6.0, dewaxed and immunostained with anti-STAT3, anti-importins, anti-Bcl-xL, anti-VEGF or anti-bFGF antibodies (Santa Cruz Biotechnology, CA) or (Cell Signaling Technology, Inc., Danvers, MA), or (Lab Vision Products Thermo Fisher Scientific, Fremont, CA) followed by biotinylated secondary antibodies and a peroxidase-labelled streptavidin-biotin staining technique.

STAT3/DNA binding activity

STAT3 binding to DNA consensus binding sequences of STAT3 was detected using Chemiluminescent TransFactor Kits (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacture's protocol. The wells are coated with the wild-type *cis*-acting DNA element for STAT3. When nuclear tissue extracts containing the STAT3 factor are incubated in the wells, its binds to its consensus sequence. Bound transcription factor was then detected by a specific anti-STAT3 antibody. The level of bindings was measured by a NOVOstar microplate reader (BMG LABTECH GmbH, Offenburg, Germany).

IL-6 detection in rat serum

We used the rat IL-6 Colorimetric ELISA Kit (Thermo Fisher Scientific Inc. Rockford, IL) according manufacture's protocol to detect IL-6 in serum of control rats and rats treated with cysteamine at different time point (from 30 min to 24 hr). Results were detected using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data are presented as means ± S.E. At least three independent experiments were carried out for each assay, and results were analyzed with ANOVA or the nonparametric Mann–Whitney *U* test. Student's *t* test was used to evaluate the significance of difference between two groups of experiments. Differences resulting in a p-value < 0.05 were considered to be significant.

Results

Inhibition of STAT3 activity by GQ-ODN T40214 aggravated experimental duodenal ulcers in rats

To demonstrate the role of STAT3 transcription activity in the pathogenesis of cysteamine-induced duodenal ulcers we used the specific inhibitor of STAT3/DNA binding which was demonstrated to inhibit pro-angiogenic gene expression and significantly decrease tumor growth [27]. The rats were pretreated with G-quartet ODN T40214 or nonspecific ODN 30 min before each of three cysteamine doses, and 48 h later the ulcer sizes were measured. The ODN T40214 aggravated duodenal ulcer formation by 2.8-fold, increasing the size of ulcers from 12.1±2.1 to 34.7±7.2 mm^2 ($p< 0.01$; Fig. 1, *A*-*C*). Histologic evaluation revealed mostly superficial ulcers in the proximal duodenum of rats pretreated with vehicle or scrambled ODN before cysteamine, whereas deep ulcers penetrating into the Brunner glands, often with perforation, were seen in rats given cysteamine after pretreatment with ODN T40214 (Fig. 1*C*).

Cysteamine-induced STAT3 protein expressions and phosphorylation in the rat duodenal mucosa

Marked elevation of STAT3 protein levels was seen in the rat duodenal mucosa 6 h after cysteamine administration. STAT3 were localized mostly in crypt area (Fig. 2*A*). Intracellular localization of STAT3 was detected in nuclear and cytoplasmic fraction of rat duodenal mucosa scrapping. Figure 2*B* demonstrated an almost complete pSTAT3 translocation to the nuclei at 2 h after cysteamine administration.

The signaling pathways induced STAT3 phosphorylation

It is known that pathway of STAT3 tyrosine phosphorylation involves the IL-6 receptor/JAK phosphorylation or Src activation [7]. In the case of IL-6 signaling, dimerization of gp130 induces intermolecular phosphorylation and activation of the associated Jaks (Jakl, Jak2 and Tyk2), which induces tyrosine phosphorylation on gp130 [17,29]. We measured the levels of IL-6 in serum of control and cysteamine-treated animals. IL-6 was not detectable in the rat blood up to 24 h after cysteamine administration. At 24 h after cysteamine treatment when duodenal ulcer almost formed, IL-6 level became elevated (Fig. 3*A*). We also detected the expression of IL-6 mRNA in duodenal mucosa using real-time PCR assay. Results showed that IL-6 mRNA of the proximal duodenum was elevated from 2 to 24 h in rats after cysteamine administration (Figure 3*B*). Figure 3*C* demonstrates that cysteamine administration resulted in phosphorylation of Src and ERK1/2 in the proximal duodenal mucosa. Cysteamine did not activate gp130, Jak1, Jak2,

and Tyk2 in the rat proximal duodenum in early stage of duodenal ulcer development (Data not shown).

Cellular localization and intracellular distribution of importin α and β in the rat duodenal mucosa after cysteamine administration

Phosphorylated dimer of STAT3 can reach nuclei by interaction with importin α and β [18]. Cysteamine enhanced expression of importin α and β time–dependently and very early. Immunohistohemistry demonstrated the localization of importin α mostly in crypt area (Fig. 4*A*). Importin α translocation to the nuclei was more prominent than that of importin β (Fig. 4*B*). Intracellular localization of importin β was detected almost equally in nuclear and cytoplasmic fractions of the rat duodenal mucosal scrapping (Fig.4*B*).

Cysteamine increased STAT3 nuclear import and transcriptional activity in the rat duodenal mucosa

To determine whether cysteamine enhance STAT3 nuclear import which is mediated by importin α/importin β heterodimer, the immunoprecipitation of STAT3 protein following the detection of importin α were evaluated. Results indicate that STAT3 nuclear import is dependent on the function of importin α (Fig.5*A*,*B*). The strong importin α expression was shown in protein complex which was immunoprecipitated by STAT3 antibody in the duodenal mucosa at 6 and 12 h after treatment of animals with cysteamine. The protein-protein interaction between importin α and STAT3 is the confirmation of activity of nuclear-pore complex in transport of STAT3 from

cytoplasm to nuclei (Fig. 5*A*,*B*). Furthermore, cysteamine induced marked activation of STAT3/DNA binding (Fig. 5*C*).

Cysteamine-induced STAT3 transcriptional activation associated with increased expression of anti-apoptotic and angiogenic factors in experimental duodenal ulceration

As STAT3 target genes we detected VEGF, bFGF, Ref-1, Bcl-xL and p21 on protein level by Western Blot. Figure 5 *D, E* demonstrates that all these proteins were time-dependently elevated in the duodenal mucosa after cysteamine administration. Activation of STAT3 led to upregulation of the negative feedback factor, suppressor of cytokine signaling (SOCS3) mRNA expression. Time-dependent enhancement of SOCS3 expression we demonstrated not only by cysteamine in rat duodenum but also by second duodenal ulcerogen mepirizole (Fig.5*F,G*).

Effect of STAT3 inhibitor (T40214) on STAT3/DNA binding and protein expression in the rat duodenal mucosa

The aggravation of cysteamine-induced duodenal ulcer was correlated with significant decrease in STAT3/DNA binding in the proximal duodenum of rats pretreated with specific inhibitor of STAT3/DNA binding T40214 (Fig. 6*A*). This figure shows that T40214 decreased STAT3/DNA binding by 3-fold. The inhibition led to markedly diminished VEGF, bFGF, p21, Bcl-xL and Ref-1protein synthesis which was detected at 6 h after cysteamine administration (Fig. 6*B*).

Inhibition of STAT3 phosphorylation and STAT3/DNA binding in the duodenal mucosa of egr-1 knockout mice after cysteamine administration

Cysteamine-induced duodenal ulcer in egr-1 knockout mice was 5-fold aggravated vs. wild-type mice (Fig.7*A*). Cysteamine enhanced STAT3/DNA binding in wild and egr-1 knockout mice but the level of STAT3 binding in egr-1 knockout mice was 2.3-fold smaller than in wild-type mice (Fig.7*B*). Untreated egr-1 knockout mice also had decreased STAT3, pSTAT3, importin α and β expressions, and the elevation in these proteins induced by cysteamine was lower than in wild-type mice (Fig. 7*C*).

Discussion

The primary finding of the present study is the critical role of STAT3-activated pathways in the protective mechanisms of duodenal mucosal cells in early duodenal ulceration. We demonstrate that STAT3 is responsible for the activation of gene expressions such as VEGF, bFGF, Ref-1, p21, and for anti-apoptotic Bcl-xL to foster resistance during chemically induced duodenal ulcer development. STAT3 activation as a result of cysteamine administration to the animals includes upregulation of Src and Erk1/2 signaling, which induces STAT3 tyrosine phosphorylation, followed by activation of the nuclear localization signals identified by a family of importin-α carriers. The importin-α proteins associate with importin-β which mediates transit via nuclear pore complexes. We identified in this study that cysteamine i

ncreased the binding of STAT3 to its DNA consensus sequences with concomitant enhancement of the expression of STAT3 target. The level of enterocyte survival during the early

phases of cysteamine-induced duodenal ulcers was abrogated by administration of STAT3 specific inhibitor T40214 which blocked the STAT3/DNA binding, inhibited the STAT3-induced gene expressions (VEGF, bFGF, Ref-1, Bcl-xL and p21) and aggravated cysteamine-induced duodenal ulcers. We also demonstrated that egr-1 knockout mice which are more susceptible to cysteamine-induced duodenal ulcers had lower levels of STAT3 expression, STAT3 phosphorylation, expression of importin α or β and STAT3/DNA binding than wild-type of mice in response to cysteamine.

Our present data suggest that STAT3-dependent signaling in the proximal duodenal mucosa of rats treated with cysteamine was not initiated by activation of IL-6-type cytokine signaling as it was demonstrated in many different cases [7,17,29,30]. The tyrosine phosphorylation of STAT3 in the proximal duodenum was detectable at 2-6 h after cysteamine administration, increase of IL-6 mRNA in duodenal mucosa was found also 2 h after cysteamine treatment but IL-6 protein in blood was present only after 12-24 h. The results suggests that activation of gp130/Jak1/2/Tyk2 was not essential for cysteamine-induced STAT3 phosphorylation in the duodenal mucosa as it was described in the different studies under other conditions [29,31]. Our work provides significant new insights into the mechanisms of cysteamine-induced STAT3 phosphorylation in the proximal duodenum which involves ERK1/2 and Src activation. Persistent *in vivo* activation of ERK1/2 and STAT3 phosphorylation was observed in mu-specific agonist-injected mice during DSS-induced intestinal injury response, mu-specific opioids as promoters of colonocyte migration/restitution and proliferation in the intestine involving the activation of STAT3-dependent pathways [32]. Activation of endogenous cellular STAT3 by the various kinases including c-Src and ERK1/2 leads to specific gene regulation and cell

transformation [33], but dephosphorylation of STAT3 led to the suppression of proteins involved in proliferation, survival, and angiogenesis [34,35].

Our observations strongly suggest that duodenal ulcerogens activate STAT3 nuclear trafficking which is a key regulatory mechanism of STAT3 transcriptional activity. Cysteamine efficiently stimulates STAT3 tyrosine phosphorylation, leading to prominent STAT3 nuclear localization similar to nuclear localization of importin-α. Distribution of importin-β is also prevalent in nuclear fraction of duodenal mucosal cells but still with relatively strong expression in cytoplasm and also upregulated by cysteamine. In addition, our data showed the ability of importin-α to bind STAT3 and this binding was enhanced within first 12 h of cysteamine ulcerogenic activity. It was previously shown using a live cell imaging technique that importin-α3 direct binding to STAT3 plays a critical role in STAT3 nuclear import [36]. Furthermore, STAT3 nuclear import was inhibited after reduction of the cellular levels of importin-β1 RNA by RNA interference [19]. These data together demonstrate that STAT3 is imported into the nucleus by importin-α/importin-β1-mediated active transport, while cysteamine augments the STAT3 import into the nucleus.

The activation of STAT3 transcription activity seems to be an important new molecular mechanism in experimental duodenal ulceration. We showed that the duodenal ulcerogen augments STAT3/DNA binding in the proximal duodenal mucosa in early pre-ulcerogenic stage of ulcer development and increased the expression of STAT3 target genes (VEGF, bFGF, Ref-1, Bcl-xL and p21). We also demonstrated the activation of SOCS3 not only by cysteamine but also by mepirizole (second model of chemically induced duodenal ulcer), and these results suggest that STAT3 transcriptional activation may play important role in experimental duodenal ulceration independently of the chemical used to induce duodenal ulcer. The deletion of

STAT3/DNA binding using inhibitor GQ-ODN T40214, markedly enhanced experimental ulcerogenesis and reduced STAT3 target gene expression. So, despite the activation of protective pathways, why duoendal ulcers still develop? The likely explanation is that although STAT3 was activated and VEGF is upregulated as a response to injury, but because of an angiogenic imbalance or 'inappropriate angiogenic response' [37,38] in duodenal ulceration, in addition to VEGF, the anti-angiogenic angiostatin and endostatin are also upregulated. Hence the beneficial effect of angiogenic factors cannot be manifested and ulcer still develops.

A recent study demonstrated that selective blockade and inhibition of STAT3 tyrosine phosphorylation and its nuclear translocation in EGF-stimulated mouse fibroblast suppresses the viability, survival, and malignant transformation of the human breast and pancreatic cancer cell lines and down-regulated the expression of c-Myc, Bcl-xL, survivin, the matrix metalloproteinase 9, and VEGF [39]. Another compound (ENMD-1198) with antiproliferative and antiangiogenic activity which reduced STAT3 transcriptional activity resulted in lower VEGF mRNA expression, together with a significant reduction in tumor growth, tumor vascularization, and numbers of proliferating of human hepatocellular carcinoma cells [40]. STAT3 regulates myocardial apoptosis, cellular proliferation, and the immune response after ischemia/reperfusion. STAT3 is necessary for the production of VEGF by mesenchymal stem cells, which are known to reduce myocardial injury after ischemia/reperfusion. Intracoronary infusions of mesenchymal stem cells improve postischemic left ventricular function and reduce proapoptotic and proinflammatory signaling via a STAT3-dependent mechanism [41].

Our studies also present important observation about possible interaction of immediate early growth response, egr1 transcription factor with STAT3 function in the mouse proximal duodenum. We demonstrated that egr-1 knockout mice had aggravated duodenal ulcers and

decreased level of STAT3, pSTAT3, STAT3/DNA binding, expression of importin α and β. These data suggest that egr-1 and STAT3 as both are antiapoptotic transcription factors may act together to potentiate protective mechanisms in duodenal cells. The relations of STAT3 and egr-1 were also observed in a study showing that neurotrophin-induced activation of STAT3 is required for several downstream functions of neurotrophin signaling in PC12 cells. The knockdown of STAT3 expression using the RNA interference approach attenuated nerve growth factor-induced transcription of immediate early genes in PC12 cells such as egr-1, junB, and cyclin D1 [42].

Conclusions

In summary, this study demonstrates that STAT3 is a key transcription factor in the multifactorial protective mechanisms against duodenal ulceration because its transcriptional activation upregulates the expression of angiogenic and antiapoptotic factors. Namely, the inhibition of STAT3/DNA binding significantly aggravated experimental duodenal ulcers and inhibited the expression of STAT3 target genes such as VEGF, bFGF, Ref-1, Bcl-xL and p21 in the rat duodenal mucosa. Thus, STAT3 transcription factor seems to be important molecules in the pathogenesis of experimental duodenal ulceration.

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Disclosures

All authors do not have any potential conflicts (financial, professional, or personal) that are relevant to the manuscript.

References

1. Manuel D, Cutler A, Goldstein J, et al. Decreasing prevalence combined with increasing eradication of *Helicobacter pylori* infection in the United States has not resulted in fewer hospital admissions for peptic ulcer disease-related complications. *Aliment Pharmacol Ther*. 2007;25:1423-1427.
2. Calam J. Clinical science of *Helicobacter pylori* infection: ulcers and NSAIDs. *British Medical Bulletin*. 1998;54:55-62.
3. Hobsley M, Tovey FI, Holton J. Controversies in the *Helicobacter pylori*/duodenal ulcer story. *Trans R Soc Trop Med Hyg*. 2008;102:1171-1175.
4. Sandor Z, Vincze A, Jadus M., et al. Effect of Vac+ and Vac- *Helicobactor pylori* supernatants on the bioactivity of basic fibroblast growth factor and platelet-derived growth factor in vitro. *Gastroenterology*. 1996;110:A249.
5. Szabo S, Shing Y, Fox J, et al. Inactivation of basic fibroblast growth factor (bFGF) by gastric *helicobacters* and not by *E. coli*. *Gastroenterology.* 1994;106:A190.
6. Wong GL, Wong VW, Chan Y, et al. High incidence of mortality and recurrent bleeding in patients with *Helicobacter pylori*-negative idiopathic bleeding ulcers. *Gastroenterology*. 2009;137:525-5231.
7. Akira S. IL-6-regulated transcription factors. *Int J Biochem Cell Biol.* 1997;29:1401–1418.

8. Boccaccio C, Ando M, Tamagnone L, et al. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature.* 1998;391:285–288.

9. Darnell JE Jr. STATs and gene regulation. *Science*.1997;277:1630–1635.

10. Gong G, Waris G, Tanveer R, et al. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci U S A.* 2001;98:9599-9604.

11. Waris G, Turkson J, Hassanein T, et al. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. *J Virol.* 2005;79:1569-1580.

12. Akira S, Nishio Y, Inoue M, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gpl30-mediated signaling pathway. *Cell.* 1994;77:63-71.

13. Kaptein A, Paillard V, Saunders M. Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. *J Biol Chem.* 1996;271:5961–5964.

14. Akira S. Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene*. 2000;19:2607–2611.

15. Ihle JN. The Janus protein tyrosine kinase family and its role in cytokine signaling. *Adv Immunol*. 1995;60:1–35.

16. Nelson KL, Rogers JA, Bowman TL, et al. Activation of STAT3 by the c-Fes protein-tyrosine kinase. *J Biol Chem*. 1998;273:7072–7077.

17. Schindler C, Darnell JE Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem*. 1995;64:621-651.

18. Ushijima R, Sakaguchi N, Kano A, et al. Extracellular signal-dependent nuclear import of STAT3 is mediated by various importin as. *Biochem Biophys Res Commun.* 2005;330:880-886.

19. Cimica V, Chen HC, Iyer JK, et al. Dynamics of the STAT3 transcription factor: nuclear import dependent on Ran and importin-β1. *PLoS One*. 2011;6:e20188.

20. Macara IG. Transport into and out of the nucleus. *Microbiol Mol Biol Rev*. 2001;65:570-594.

21. Ohno M, Fornerod M, Mattaj IW. Nucleocytoplasmic transport: the last 200 nanometers. *Cell*. 1998;92:327–336.

22. Zhuang L, Lee CS, Scolyer RA, et al. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. *Mod Pathol.* 2007;20:416-426.

23. Niu G, Wright KL, Huang M, et al. Constitutive Stat3 activity upregulates VEGF expression and tumor angiogenesis. *Oncogene*. 2002;21:2000-2008.

24. Yahata Y, Shirakata Y, Tokumaru S, et al. Nuclear translocation of phosphorylated STAT3 is essential for vascular endothelial growth factor-induced human dermal microvascular endothelial cell migration and tube formation. *J Biol Chem*. 2003;278:40026-40031.

25. Xie TX, Huang FJ, Aldape KD, et al. Activation of Stat3 in Human Melanoma Promotes Brain Metastasis. *Cancer Res.* 2006;66:3188-3196.

26. Matthews JR, Sansom OJ, Clarke AR. Absolute requirement for STAT3 function in small-intestine crypt stem cell survival. *Cell Death Differ*. 2011;18:1934-1943.

27. Jing N, Zhu Q, Yuan P, et al. Targeting signal transducer and activator of transcription 3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer. *Mol Cancer Ther*. 2006;5:279-286.

28. Khomenko T, Szabo S, Deng X, et al. Role of iron in the pathogenesis of cysteamine-induced duodenal ulceration in rats. *Am J Physiol Gastrointest Liver Physiol*. 2009;296:G1277-G1286.

29. Heinrich PC, Behrmann I, Müller-Newen G, et al. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J*. 1998;334:297-314.

30. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*. 1994;264:95-98.

31. Funamoto M, Fujio Y, Kunisada K, et al. Signal transducer and activator of transcription 3 is required for glycoprotein 130-mediated induction of vascular endothelial growth factor in cardiac myocytes. *J Biol Chem*. 2000;275:10561-10566.

32. Goldsmith JR, Uronis JM, Jobin C. Mu opioid signaling protects against acute murine intestinal injury in a manner involving Stat3 signaling. *Am J Pathol.* 2011;179:673-683.

33. Turkson J, Bowman T, Garcia R, et al. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. Mol Cell Biol 1998;18:2545–2552.

34. Phromnoi K, Prasad S, Gupta SC, et al. Dihydroxypentamethoxyflavone down-regulates constitutive and inducible signal transducers and activators of transcription-3 through the induction of tyrosine phosphatase SHP-1. *Mol Pharmacol*. 2011;80:889-899.

35. Sugimoto K, Ogawa A, Mizoguchi E, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest*. 2008;118:534-544.

36. Liu L, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. *Proc Natl Acad Sci U S A*. 2005;102:8150-8155.

37. Deng X, Xiong X, Khomenko T, et al. Inappropriate angiogenic response as a novel mechanism of duodenal ulceration and impaired healing. *Dig Dis Sci*. 2011;56: 2792-2801.

38. Sandor Z, Deng XM, Khomenko T, et al. Altered angiogenic balance in ulcerative colitis: A key to impaired healing? *Biochem Biophys Res Commun.* 2006;50:147-150.

39. Zhang X, Yue P, Fletcher S, et al. A novel small-molecule disrupts Stat3 SH2 domain-phosphotyrosine interactions and Stat3-dependent tumor processes. *Biochem Pharmacol*. 2010;79:1398-1409.

40. Moser C, Lang SA, Mori A, et al. ENMD-1198, a novel tubulin-binding agent reduces HIF-1alpha and STAT3 activity in human hepatocellular carcinoma (HCC) cells, and inhibits growth and vascularization in vivo. *BMC Cancer*. 2008;8:206.

41. Poynter JA, Herrmann JL, Manukyan MC, et al. Intracoronary mesenchymal stem cells promote postischemic myocardial functional recovery, decrease inflammation, and reduce apoptosis via a signal transducer and activator of transcription 3 mechanism. *J Am Coll Surg.* 2011;213:253-260.

42. Ng YP, Cheung ZH, Ip NY. STAT3 as a downstream mediator of Trk signaling and functions. *J Biol Chem*. 2006;281:15636-15644.

Figure Legends

Fig. 1. Effect of STAT3/DNA binding inhibitor GQ-ODN, T40214 on cysteamine-induced duodenal ulcers in rats. *A: g*ross appearance of duodenal ulcers 48 h after cysteamine administration. Animals were pretreated with either water (n = 10), T40214 (1 mg/100 g) (n = 10) or scrambled control for T40214 (n = 7), (1 mg/100g) (Midland Certified Reagent

Companyx Inc., Midland, Texas) plus PEI s.c., 30 min before each cysteamine treatment. *B*: duodenal ulcer crater dimensions were measured in millimeters, and the ulcer areas were calculated using the ellipsoid formula. Sizes of duodenal ulcers are expressed as means ± SE. * $p < 0.01$. *C*: light microscopy of duodenal ulcers in rats pretreated with either water (showing superficial ulcer; left), scrambled control ODN (similar ulcer size; middle), or T40214 ODT at 1 mg/rat before cysteamine (deep ulcer; right) with hematoxylin and eosin staining, 40X.

Fig. 2. Cellular localization and intracellular distribution of STAT3 and pSTAT3 proteins in the duodenal mucosa. *A*: immunofluorescent staining of STAT3 proteins in the rat duodenal mucosa in control animals and in rats at 6 h after cysteamine administration. STAT3 proteins were localized mostly in crypt area, x100. *B*: intracellular distribution of STAT3 and pSTAT3 proteins in the nuclear and cytoplasmic fraction of the duodenal mucosal cells.

Fig. 3. The role of IL-6 and different kinases in cysteamine-induced activation of STAT3 pathway in pre-ulcerogenic stage of duodenal ulcer development. *A*: ELISA results of IL-6 concentration in serum of control and cysteamine-treated rat. IL-6 concentrations are expressed as means ± SE. * $p < 0.01$. *B*: time-dependent IL-6 mRNA expression in the duodenal mucosa of rats treated with cysteamine was detected by Real-time PCR. *C.* western blotting results showing the level of Src, and ERK1/2 phosphorylation in the duodenal mucosa of control and cysteamine-treated rat. Data are results of at least 3 separate sets of experiments.

Fig. 4. Cellular localization and intracellular distribution of importin α and β in cysteamine-induced duodenal ulcer. *A*: Immunohistochemistry results of cysteamine treatment that markedly

increased importin α in the epithelial cells of mucosal crypts (“progenitor zone”). *B*: intracellular distribution of importin α and β in nuclear and cytoplasmic fractions of proximal duodenal mucosa of control and cysteamine-treated rats, x200. Data are results of at least 3 separate sets of experiments.

Fig. 5. Cysteamine-induced nuclear import of STAT3 follows by enhanced of STAT3 transcriptional activation. *A*: Results of western blotting with STAT3 immunoprecipitation and importin α detection in nuclear fraction of duodenal mucosal scrapping. *B*: Relative density of importin α expression in enhanced STAT3/importin α complex induced by cysteamine. *C*: Time-dependent increased STAT3/DNA binding in the nuclear fraction of the duodenal mucosa of cysteamine-treated rats. *D/E*: Western blotting results of increased protein synthesis of VEGF, bFGF, Ref-1, Bcl-xL, and p21 as a result of cysteamine-induced upregulation of STAT3 transcriptional activity. *F*: real-time PCR of SOCS3 mRNA expression in the duodenal mucosa of rats treated with cysteamine. *G:* real-time PCR of SOCS3 mRNA expression in the duodenal mucosa of rats treated with mepirizole. Data are results of at least 3 separate sets of experiments, * = $p < 0.05$.

Fig. 6. Effect of T40214 on STAT3 transcriptional activity in the duodenal mucosa of cysteamine-treated rats. *A*: T40214 downregulated STAT3/DNA binding activity which was detected at 6 h after cysteamine administration. *B*: Inhibition of STAT3/DNA binding which aggravated duodenal ulcers decreased VEGF, bFGF, p21, Bcl-xL, Ref-1protein synthesis. Every experiment had n = 5, * = $p < 0.05$.

Fig. 7. Cysteamine-induced duodenal ulcers and STAT3 transcription activity in egr-1 knockout mice. *A*: egr-1 knockout mice showed the marked increased duodenal ulcers vs. wild-type mice. *B*: inhibition of STAT3 transcription activity after cysteamine administration in egr-1 knockout compared to wild-type mice. C: western blotting results of decrease in STAT3 phosphorylation, and significant decrease in importin α and importin β expression especially after cysteamine treatment in egr-1 knockout compared to wild-type mice. Every experiment had n = 7, * = $p < 0.05$.