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Protective effects of neurokinin-1 receptor during colitis in mice: role of the epidermal growth factor receptor

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1 The role of substance P and its high affinity neurokinin-1 receptor in colitis has not been fully elucidated. We assessed the participation of neurokinin-1 receptor in colitis using the 2,4,6,-trinitrobenzensulphonic acid and dextran sulphate-induced animal models of colitis and genetically-engineered, neurokinin-1 receptor-deficient mice.

2 Clinical signs, macroscopic and histologic damage associated with 2,4,6,-trinitrobenzensulphonic acid (12 days) and dextran sulphate (5 days) colitis were more severe in neurokinin-1 deficient than in wild-type mice, while immunoreactivities for epidermal growth factor and its receptor were similar in the colon of both mice strains before and after colitis.

3 Substance P, dose-dependently induced intestinal fibroblast proliferation and enhanced epidermal growth factor-induced proliferation in intestinal fibroblasts isolated from wild-type, but not from neurokinin-1 receptor deficient mice.

4 Substance P-induced intestinal fibroblast proliferation required the presence of epidermal growth factor receptor with kinase activity. Furthermore, substance P induced epidermal growth factor tyrosine phosphorylation and activation in normal intestinal fibroblasts.

5 Our results indicate that in mice lacking the neurokinin - 1 receptor, substance P plays a protective role in prolonged experimental colitis.

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Abbreviations: CGRP, calcitonin gene-related peptide; DDS, dextran sodium sulphate; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GPCR, G protein-coupled receptor; 2,4,6-trinitrobenze sulphonic acid; TNBS; MAPKs, mitogen-activated protein kinases; PFA, paraformaldehyde; SP, substance P; NK-1R, neurokinin-1 receptor

Introduction

Substance P (SP) is an 11 amino acid peptide widely distributed throughout the intestine and the nervous system (Pernow, 1983). SP has been implicated in the regulation of several important biologic processes, such as nociception, smooth muscle contraction, exocrine and endocrine glands secretion, and connective tissue proliferation (Pernow, 1983). Several studies suggest that SP is a major neuroimmunomodulator (Pernow, 1985). For example, SP stimulates T cell proliferation, B cell differentiation, monocyte and leukocyte chemotaxis, release of proinflammatory cytokines by macrophages, monocytes and neuroglial cells, and mast cell degranulation (Brain, 1997).

SP exerts many of its biologic effects by binding to a high affinity G protein-coupled receptor (GPCR) of the neurokinin family, the neurokinin-1 receptor (NK-1R) subtype (Krause *et al*., 1992). Ligand binding to NK-1R is followed by phosphoinositide hydrolysis, calcium mobilization, rise in intracellular cyclic AMP levels, and activation of mitogen-activated protein kinase (Krause *et al*., 1992; Luo *et al*., 1996). We recently reported that binding of SP to its NK-1R causes activation of mitogen-activated protein kinases (MAPKs) and stimulates DNA synthesis in U-373 MG epithelial cells by a mechanism involving activation of the epidermal growth factor receptor (EGFR) (Castagliuolo *et al*., 2000). NK-1R is expressed on a wide variety of cells such as neurons, fibroblasts, mononuclear cells, and endothelial cells (Pernow, 1985; Brain, 1997). The level of NK-1R expression may vary in acute and chronic inflammatory processes. For example, the intestinal NK-1R expression level is increased during *Clostridium difficile* toxin A-mediated enteritis and in the colon of patients with inflammatory bowel disease (Pothoulakis *et al*., 1998; Mantyh *et al*., 1988).

The exact role of SP in the different phases of intestinal inflammation is not clear. Studies using a model of acute enteritis caused by *C. difficile* toxin A and NK-1R deficient ($NK\text{-}1R^{-/-}$) mice provided evidence for a pro-inflammatory role of SP and NK-1R in acute inflammation (Pothoulakis *et*

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al., 1994; Castagliuolo *et al*., 1997). In addition, recent studies have shown that administration of a non-peptide NK-1R antagonist reduces inflammatory damage in two different models of chronic experimental colitis in rats (Di Sebastiano *et al*., 1999; Stucchi *et al*., 2000). Another study showed that mice lacking the cell surface enzyme neutral endopeptidase that degrades SP had exacerbated inflammatory responses compared to wild-type in the dinitrobenzene sulphonic acid model of colitis (Sturiale *et al*., 1999). In contrast, studies with animals chronically pre-treated with the neurotoxin capsaicin, which causes degeneration of primary sensory neurons containing SP and calcitonin gene related peptide (CGRP), showed increased severity of chronic colitis, suggesting that sensory neuropeptides may play a protective role in chronic intestinal inflammation (Evangelista & Meli, 1989; Reinshagen *et al*., 1996).

We have recently reported that mice genetically deficient in NK-1R (Bozic *et al*., 1996), showed reduced tissue damage as compared to the wild-type animals in experimental models of acute enteritis and pancreatitis (Castagliuolo *et al*., 1998; Bhatia *et al*., 1998). The availability of NK-1R$^{-/-}$ mice allowed us to directly investigate the role of this receptor in colonic inflammation. Two different models of colitis were used: 2,4,6-trinitrobenzene sulphonic acid (TNBS) and dextran sodium sulphate (DSS). These models have been extensively used to study the pathophysiology of inflammatory bowel disease (Elson *et al*., 1995). Both colitis models are characterized by diffuse ulcerations of the colonic mucosa (Elson *et al*., 1995). TNBS-induced colitis is mostly characterized by ulceration, necrosis and infiltration of the colonic mucosa by inflammatory cells (Elson *et al*., 1995). DSS-induced colitis is characterized by erosions of the colonic epithelial crypts, extensive loss of the epithelium, oedema and mild infiltration of the mucosa with macrophages, neutrophils, and T cells (Elson *et al*., 1995; Egger *et al*., 1998).

Methods

Eight to 12-week-old NK-1R$^{-/-}$ or wild-type mice matched for sex and weight were bred and maintained in the Beth Israel Deaconess Medical Center animal research facility under standard environmental conditions. NK-1R$^{-/-}$ were bred either on a mixed SV129 × C57BL/6 or Balb/c background. Mice received standard pelleted chow and tap water *ad libitum*, unless otherwise specified. Animals studies were approved by the institutional animal care and use committee of the Beth Israel Deaconess Medical Center.

Induction of colitis TNBS and measurement of colitis severity

TNBS colitis was induced as previously reported (Neurath *et al*., 1995). Briefly, following overnight fasting, NK-1R$^{-/-}$ on Balb/c background or wild-type mice were lightly anaesthetized by an intraperitoneal injection of pentobarbital sodium (70 mg kg^{-1}). A polyethylene cannula (Intramedic PE-20 tubing, Becton Dickinson, Parsippany, NJ, U.S.A.) was introduced into the colon (~3.5 cm) *via* the anus. A solution (100 μl) of 50% ethanol (vehicle) or ethanol-containing TNBS (100 mg kg^{-1}; Fluka, Ronkonkoma, NY, U.S.A.) was instilled into the colon using a 1 ml syringe. Animals were held head down for 1 min after the enema to ensure the permanence of the TNBS solution into the colon. Mice were then returned to their cages and either sacrificed after 3 days or treated with a second TNBS enema 7 days later (100 mg kg^{-1}), and then sacrificed after an additional 5 days. After recording the body weight, the whole colon was removed, opened longitudinally, washed in ice-cold saline and its length (cm) and weight (mg) recorded. The colon was examined under a dissecting microscope by two independent investigators, who were unaware of the experimental conditions, for evaluation of the macroscopic damage score (scale 0–10) using previously described parameters (Keates *et al*., 2000). The scoring was as follows: 0, no damage; 1, hyperemia without ulcers; 2, hyperemia and thickening of the bowel wall without ulcers; 3, one ulcer without thickening of the bowel wall; 4, sites of ulceration or inflammation; 5, more than two sites of inflammation or one site extending over 0.5 cm; 6–10, damage extending at least 1 cm with the score increasing by 1 for each additional 0.5 cm of involvement. The entire colon was then processed for measurement of cytokine levels or fixed in 4% paraformaldehyde (PFA) for histologic examination. Longitudinal sections (10 μm thick) were cut and stained with hematoxylin and eosin and histologic evaluation was performed on colonic sections (approximately 2 cm in length) starting 3 cm above the anus by a 'blinded' histopathologist (JZ).

Determination of colonic interleukin-1β (IL-1β)

The entire colon was homogenized in ice-cold phosphate buffer saline (pH 7.4) containing protease inhibitors (1 μM phenyl methyl sulfonic fluoride (PMSF), 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). Homogenates were then centrifuged at 40,000 *g* for 10 min at 4°C, the supernatants collected and stored at −80° C. IL-1β content was measured by a commercially available ELISA (Endogen, Woburn, MA, U.S.A.).

Induction of colitis by DSS and measurements of colitis severity

Wild-type and NK-1R$^{-/-}$ mice on mixed SV129 × C57BL/6 background were divided into two groups receiving either water alone (control) or water containing 5% (wt vol^{-1}) DSS (TdB Consultancy, Uppsala, Sweden). Body weight loss, stool consistency and presence of fecal blood were recorded daily, leading to a clinical score (scale 0–4), as previously described (Egger *et al*., 1998). The scoring system for the DSS model of colitis was as previously described (Cooper *et al*., 1993). 0, no weight loss with normal stool consistency and no occult or gross bleeding; 1, 1–5% weight loss with normal stool consistency and no occult or gross bleeding; 2, 5–10% weight loss with loose stools and Hemoccult test positive; 3, 10–20% weight loss with loose stools and Hemoccult test positive; 4, >20% weight loss with diarrhea and gross bleeding. After 5 days animals were sacrificed by a pentobarbital overdose and a segment of the proximal colon from control and DSS treated mice was fixed in 4% paraformaldehyde (PFA), and paraffin embedded. Longitudinal sections (10 μm thick) were stained with hematoxylin and eosin and histologic evaluation was performed on

representative sections as previously described (Egger *et al*., 1998).

Immunohistochemistry of EGF and EGF-R

Whole colons from wild-type and NK-1R$^{-/-}$ mice were removed, washed in 1 × TBS buffer (0.05 M Tris base, 0.15 M NaCl), fixed in 4% buffered PFA and embedded in paraffin. Longitudinal sections (10 μm) were first deparaffinized and rehydrated in 1 × TBS. After incubation for 30 min in blocking buffer (2% donkey serum, 0.3% Triton X-100 in TBS) the sections were incubated for 1 h at 22° C with either a rabbit polyclonal anti-EGF antibody or a mouse monoclonal anti-EGF-receptor antibody (Santa Cruz, Biotechnology, Inc.; Santa Cruz, CA, U.S.A. 1 : 100 dilution). After washing (3 × 10 min) in 1 × TBS, sections were incubated for 1 h at 22° C with either a donkey anti-rabbit or anti-mouse IgG (ImmunoResearch Laboratories Inc., PA, U.S.A.). After washing in TBS (× 3), the slides were mounted in a drop of 1 mg ml^{-1} of *n*-propylgallate (Sigma) to reduce photobleaching in 90% glycerol-PBS. Sections were analysed and photographed by a confocal microscope (BioRad MRC1024 (BioRad Microsciences; Cambridge, MA, U.S.A.)), using a 32 × Plan-Neofluar objective, and images were digitally stored in a Biorad COMOS software. Control slides were incubated with non-immune rabbit or mouse IgG at 1 : 100 dilution and showed no immunoreactivity (data not shown).

Isolation of intestinal fibroblasts

Intestinal fibroblasts were isolated by a modification of the method described by Reubi *et al*. (1996). Collagen Gelfoam sponges (5 × 5 × 7 mm) (Upjohn Co., Kalamazoo, MI, U.S.A.) impregnated with 1 ng mm^{-3} acidic FGF (Boehringer and Mannheim, Indianapolis, IN, U.S.A.) were surgically implanted on the colon of wild-type and NK-1R$^{-/-}$ mice. After 2–3 weeks sponges were removed, cut in small pieces, washed in RMPI 1640 medium containing 1% penicillin-streptomycin and fungizone (2 μg ml^{-1}) (Gibco BRL, Bethesda, MD, U.S.A.), and digested with collagenase type IV (100 u ml^{-1}), Dispase (2 mg ml^{-1}) and DNase type I (0.1 mg ml^{-1}). After 60–90 min the cellular digest was collected, filtered through sterile 40-μm nylon mesh, and centrifuged (500 × *g* for 10 min). Cells were washed twice and then cultured on fibronectin (1 μg cm^{-2})-coated flasks in complete M-199 medium (Sigma) containing 2 ng ml^{-1} acidic FGF. After 4 h at 37° C the flasks were washed and adherent cells re-fed fresh M-199 medium. No gross difference in cell growth was evident during this period between cells obtained from wild-type and NK-1R$^{-/-}$ mice.

Transfection experiments

Fibroblasts were transfected using a modified $CaPO_4$-DNA precipitation procedure (Macphee *et al*., 1994). Briefly, cells were plated at a density of 25,000 cells cm^{-2} and cultured for 24 h. Two hours prior to transfection the media was replaced with fresh media and 2.5 μg per ml media of the pCHER plasmid, a dominant negative EGFR plasmid lacking kinase activity (Prywes *et al*., 1986), was introduced to the cells. After 18 hrs the media was removed, cells were washed twice with PBS, and cultured in media containing 10 μM methotrexate to select for positively transfected cells.

Immunoprecipitation and Western blotting

Fibroblasts were cultured in complete media and, when 75% confluent, incubated for 18 h in media containing 0.5% FCS. Cells were then incubated with SP (10–0.1 nM) or EGF (20–0.1 ng ml^{-1}) for the indicated times shown in Figure 5. Monolayers were washed twice with ice-cold PBS and lysed (45 min on ice) using non-denaturing RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 100 μM $NaVO_4$, 1 mM NaF, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). Particulate material was removed by centrifugation (15,000 × *g* for 5 min) and the supernatant was collected. Protein concentrations were determined by the bicinichonic acid method (Pierce; Rockford, IL, U.S.A.). Lysates (2 mg ml^{-1}) were incubated with a mouse monoclonal anti-EGF-R antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) (10 μg mg^{-1} cell lysate) for 2 h at 4°C. Protein A agarose or protein G Plus agarose (50 μl, Santa Cruz Biotechnology, Inc.) was added to the mixture and incubated for 1 h at 4°C. Beads were washed twice by centrifugation (20 s, 12000 × *g*) with ice-cold RIPA buffer, followed by one wash with ice-cold PBS and then boiled (5 min) in 30 μl of sample buffer (62.5 mM Tris, p.H. 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, and 0.1% bromophenol blue). Immunoprecipitated proteins were fractionated on a SDS-polyacrylamide gel electrophoresis (PAGE) gel and then transferred to a nitrocellulose membrane. Membranes were blocked overnight at 4°C in 5% skim milk in phosphate-buffered saline (pH 7.4), containing 0.05% Tween-20 and then phosphorylated tyrosine residues were identified using the PY99 antibody (1 : 1000 dilution, Santa Cruz Biotechnology, Inc.). Immunocomplexes were visualized using the ECL Western blotting detection reagent (Amersham Life Science, Buckinghamshire, U.K.).

Proliferation assay

DNA proliferation assays were performed as previously described (Hauck & Tranners, 1995). Briefly, fibroblasts were plated at 25,000 cm^{-2} fibronectin-coated wells and after 24 h either non-transfected, mock transfected or transfected with pCHER as described above. After 48 h media were removed and cells cultured overnight in media containing 0.5% FCS. Cells were then stimulated (12 h at 37°C) with SP (Phoenix Pharmaceutical, CA, U.S.A.) (100–0.1 nM) or EGF (100–1 ng ml^{-1}) in the presence of [^{3}H]-thymidine (0.5 μCi well^{-1}, New England Nuclear, Boston, MA, U.S.A.). Cells were washed × 3 with ice-cold PBS, lysed with 0.3 M NaOH and [^{3}H]-thymidine incorporation was determined by scintillation counting. Results were expressed as counts per minute.

Statistical analyses

Results were analysed using the SIGMA-STAT program (Jandel Scientific Software, San Rafael, CA, U.S.A.). Analyses of variance with protected *t*-test (ANOVA) were used for intergroup comparisons.

Results

Reduced severity of TNBS-induced acute colonic damage in NK-1R$^{-/-}$ mice

Previous results indicated that NK-1R play a proinflammatory role in acute small intestinal and colonic inflammation (MacCafferty *et al*., 1994; Pothoulakis *et al*., 1994; Castagliuolo *et al*., 1998). Thus, we examined the effect of NK-1R deficiency in acute (3 days) - induced colitis. Three days after colonic administration of TNBS, wild-type Balb/c and NK-1R$^{-/-}$ mice showed body weight loss, increased colonic weight/length ratio, and macroscopic colonic damage. Loss of body weight was more severe in wild-type mice than in NK 1R$^{-/-}$ animals (2.7$\pm$0.4 g vs 1.2$\pm$0.6 g, mean$\pm$s.e.m., $n=6$, $P<0.05$). Accordingly a significantly ($P<0.05$) greater colonic weight/length ratio (58.2$\pm$2.2 vs 49.7$\pm$4, respectively, $n=6$) and macroscopic damage (5.1$\pm$0.8 vs 2.8$\pm$1.1, respectively, $n=6$) was observed in wild-type mice as compared to NK 1R$^{-/-}$ animals, when animals were sacrificed three days after TNBS injection.

Increased severity of TNBS-induced colitis in NK-1R$^{-/-}$ mice

Twelve days after administration of TNBS, wild-type Balb/c mice exhibited local and systemic symptoms of colitis (Table 1). These mice showed reduced body weight gain, increased colonic weight/length ratio, macroscopic and histologic signs of colonic inflammation as compared to vehicle-treated mice (Table 1, Figure 1B). As shown in Table 1, both systemic and local signs of colitis were significantly more severe in NK-1R$^{-/-}$ mice than in wild-type animals. NK-1R$^{-/-}$ mice ex-hibited loss of body weight and a significantly ($P<0.05$) greater colonic weight/length ratio and macroscopic mucosal damage when compared to wild-type animals treated with TNBS (Table 1). Histologic evaluation revealed a more severe inflammatory infiltrate associated with more profound mucosal ulcerations in NK-1R$^{-/-}$ compared to +/+ mice (Figure 1). Additionally, colonic levels of IL-1β were higher ($P<0.01$) in NK-1R$^{-/-}$ compared to TNBS-treated wild-type mice (Table 1).

Increased severity of DSS-induced colitis in NK-1R$^{-/-}$ mice

While greatly susceptible to TNBS-induced colitis, Balb/C mice are relatively resistant to DSS-induced colitis (Egger *et al*., 1998). Thus, wild-type and NK-1R$^{-/-}$ mice of C57BL6/SV129 background, known to be more susceptible to DSS colitis (Mahler *et al*., 1998), were compared. Both wild-type and NK-1$^{-/-}$ mice receiving 5% DSS exhibited a wasting syndrome, associated with bloody diarrhea, within 5 days of treatment (Table 2). Histologic evaluation revealed superficial mucosal ulcerations associated with an inflammatory infiltrate (Figure 1C). However, body weight loss (day 5) and clinical score (days 4 and 5) were greater in NK-1R$^{-/-}$ mice compared with DSS-treated wild-type mice (Table 2). Histologic evaluation at day 5 demonstrated larger ulcers in NK-1R$^{-/-}$ compared to wild-type mice, whereas the severity of the inflammatory infiltrate and mucosal hyperplasia were comparable between the two groups (Figure 1C and F).

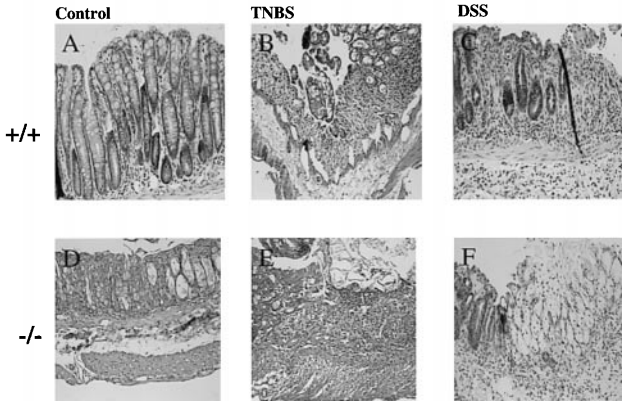

Figure 1 Increased severity of colitis in NK-1R$^{-/-}$ mice. TNBS or DSS colitis was induced in wild-type ($\pm/\pm$) (B and C) and NK-1R$^{-/-}$ mice ($-/-$) (panels E & F) as described in Methods. Animals were sacrificed after 5 days (DSS) or 12 days (TNBS) and the colon was removed, fixed in 4% PFA, and full thickness sections stained with H&E. Colon from control animals exhibited normal architecture in both wild-type (A) and NK-1R$^{-/-}$ mice (D). In contrast, animals treated with TNBS (B and E) showed damage characterized by necrosis, disruption of the mucosal architecture, granulocytic infiltrate, and mucosal ulcers. However ulcers and inflammatory damage associated with TNBS colitis were more severe in NK-1R$^{-/-}$ mice than in wild-type (compare B and E). During DDS colitis (C and F) inflammatory infiltration and superficial ulcerations were evident. However, ulcerative lesions were more pronounced in NK-1R$^{-/-}$ mice. Original magnification $\times$160.

Table 1 Increased severity of TNBS colitis in NK-1R deficient mice

	Body weight change (%)	*Macroscopic damage score*	*Colon weight/length* (mg cm^{-1})	*IL-1β* (pg mg^{-1} protein)	*Histologic score*
(+/+) vehicle	(+) 10$\pm$0.2	0	25.3$\pm$0.8	19.0$\pm$4.9	0.3$\pm$0.1
(+/+) TNBS	(+) 3.8$\pm$0.5^{++}	1.2$\pm$0.1^{++}	36.2$\pm$4^{++}	138.2$\pm$18.5^{++}	0.9$\pm$0.3^{++}
(−/−) vehicle	(+) 9.8$\pm$0.3	0	24.9$\pm$1.1	13.4$\pm$4.0	0.2$\pm$0.1
(−/−) TNBS	(−) 3.6$\pm$0.3^{++}**	2.2$\pm$0.2^{++}*	59.9$\pm$7^{++}*	485.5$\pm$22.3^{++}	1.9$\pm$0.5^{++}*

NK-1R$^{-/-}$ ($-/-$) or wild-type (+/+) mice received a colonic injection of TNBS (100 mg kg^{-1}) or vehicle (50% ethanol). After 7 days animals received a second TNBS injection and sacrificed after 5 days. Changes in body weight were recorded and expressed as per cent changes from the initial weight. The colon of each animal was removed, opened longitudinally and washed in ice-cold PBS. Colon length (cm) and weight (mg) were recorded to calculate the weight/length ratio and a macroscopic damage score was assigned to each animal (0–10). Each colon was then either homogenized to measure levels of IL-1β by ELISA or processed for histologic evaluation. Data represents the mean$\pm$s.e.m of 6–10 animals per group. $^{++}$denotes $P<0.01$ vs respective control; **denotes $P<0.01$ and *$P<0.05$ vs wild-type TNBS.

EGF and EGF-R up-regulation during colitis in wild-type and NK-1R$^{-/-}$ mice

Growth factors of the EGF family such as EGF and TGF bind the EGF receptor (EGF-R) and are involved in ulcer healing in the gastrointestinal tract (Procaccino *et al*., 1994). Since EGF and EGF-R immunoreactivities increase in tissues surrounding colonic ulcers during colitis (Wright *et al*., 1990; Sottili *et al*., 1995), we evaluated the levels of EGF and EGF-R in NK-1R$^{-/-}$ mice before colonic inflammation and at day 5 in DSS and day 12 in TNBS colitis. We observed a similar modest signal for EGF and EGF-R immunoreactivity in non-inflamed colon of wild-type and NK-1R$^{-/-}$ mice (Figures 2 and 3). As shown in Figures 2 and 3, EGF- and EGF-R immunoreactive cells were increased in both wild-type and NK-1R$^{-/-}$ mice during TNBS- and DSS-induced colitis as compared to vehicle-treated mice. Moreover, there was a similar increase in EGF and EGF-R immunoreactivities in both wild-type and NK-1R$^{-/-}$ mice in both types of colitis (Figures 2 and 3). It should be mentioned, however, that no quantitative approaches were applied to measure this response.

Reduced EGF-mediated fibroblast proliferation in NK-1R$^{-/-}$ mice

The observation of larger mucosal ulcerations in NK-1R$^{-/-}$ vs wild-type mice after colitis (Figure 1), while EGF and EGF-R levels were increased to a similar degree in both genotypes during colitis (Figures 2 and 3), suggested that other factors related to mucosal healing may be impaired in NK-1$^{-/-}$ mice. Since SP exerts a mitogenic effect on fibroblasts (Nilsson *et al*., 1995), a cell type that plays a critical role in wound healing (Jobson *et al*., 1998), we investigated the impact of the absence of NK-1R in fibroblast proliferation. Exposure of wild-type fibroblasts to either EGF (100 ng ml^{-1}) or SP (10 nM) caused a dose-related increase in ^{3}H-thymidine up-take (Figure 4A). In addition, co-incubation of fibroblasts with doses of SP (1 nM) and EGF (1 ng ml^{-1}), which individually were ineffective to induce a proliferative response, significantly increased ^{3}H-thymidine up-take (Figure 4A). In fibroblasts obtained from control NK-1R$^{-/-}$ mice, EGF at 100 and 10 ng ml^{-1} caused a proliferative response which was similar to that obtained in wild-type mice (Figure 4B). In contrast, SP failed to significantly stimulate

Table 2 Increased severity of DSS-induced colitis in NK-1R deficient mice

	Body weight change (%)	*Clinical score*	*Histological score*
(+/+) vehicle	(+) 5.0±0.2	0	0.3±0.1
(+/+) DSS	(−) 1.2±0.7^{++}	2.17±0.1^{++}	0.9±0.3^{++}
(−/−) vehicle	(+) 4.8±0.3	0	0.2±0.1
(−/−) DSS	(−) 6.3±0.9^{++}**	3.22±0.1^{++}**	1.9±0.5^{++}*

NK-1R$^{-/-}$ (−/−) or wild-type (+/+) mice received drinking water with or without 5% DSS. Animals were sacrificed after 5 days and changes in body weight were recorded and expressed as per cent changes from the initial weight. Body weight loss, stool consistency, and presence of faecal blood were recorded daily, leading to clinical score (scale 0–4). The colon of each animal was removed and full thickness segment processed for histology. Data represents the mean±s.e.m of 5–10 animals per group. $^{++}$denotes $P<0.01$ vs respective control; **denotes $P<0.01$ and *$P<0.05$ vs wild-type DSS.

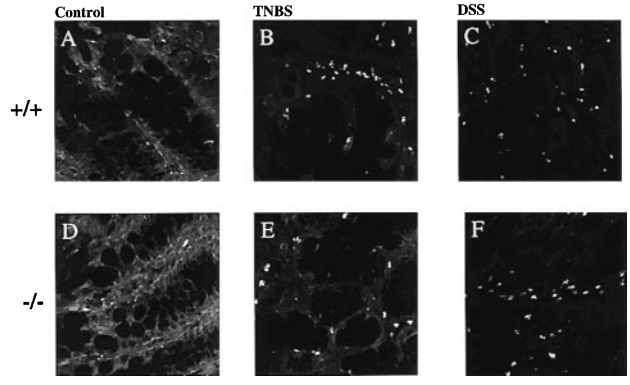

Figure 2 Expression of EGF during colitis in NK-1R$^{-/-}$ and wild-type mice. TNBS (12 days) or DSS (5 days) colitis was induced in wild-type (±/±) (B and C, respectively) and NK-1R$^{-/-}$ (−/−) (E and F, respectively) mice and colonic sections were processed for detection of EGF-like immunoreactivity as described in Methods. Confocal microscopy showed little EGF-like immunoreactivity in the normal colon of wild-type (A) and NK-1R$^{-/-}$ mice (D). EGF-like immunoreactivity increased in cells localized in the colonic lamina propria during TNBS (B and E) and DSS (C and F) colitis in wild-type and in NK-1R$^{-/-}$ mice. Results are representative of three experiments for each experimental condition. Original magnification × 300.

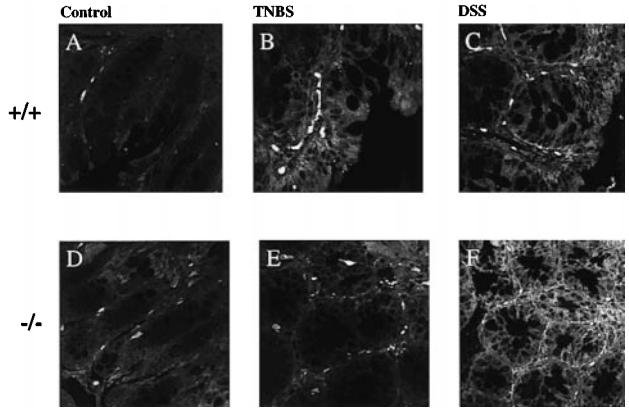

Figure 3 Expression of EGF-R during colitis in NK-1R$^{-/-}$ and wild-type mice. TNBS (12 days) or DSS (5 days) colitis was induced in wild-type (±/±) (B and C, respectively) and NK-1R$^{-/-}$ (−/−) (E and F, respectively) mice and colonic sections were processed for immunohistochemical detection of EGF-R as described in Methods. Sections were examined by confocal microscopy. Note the presence of modest signal for EGFR in the normal colon of wild-type (A) and NK-1R$^{-/-}$ mice (D). EGFR-immunoreactivity increases in the colonic mucosa both in wild-type and NK-1R$^{-/-}$ mice during TNBS (B and E, respectively) and DSS colitis (C and F, respectively). Results are representative of three experiments for each experimental condition. Original magnification × 300.

significant ^{3}H-thymidine up-take and had no synergistic effect on EGF (1 ng ml^{-1})-induced DNA-synthesis as measured by ^{3}H-thymidine up-take (Figure 4B).

Mechanism of substance P-induced proliferation of intestinal fibroblasts

Stimulation of G protein coupled receptors (GPCRs) can activate signal transduction pathways, including tyrosine kinases (Diverse-Pirluissi *et al*., 1997). Our recent results also demonstrated communication between the NK-1R and EGF-R is critically involved in SP-induced proliferation in U-373 MG epithelial cells (Castagliuolo *et al*., 2000). To determine the nature of the SP-EGF-R interaction in mouse colonic mucosa, we examined whether SP-induced DNA synthesis required a functional EGF-R using intestinal wild-type fibroblasts transfected with pCHER, a construct carrying a kinase defective dominant negative mutant of EGF-R shown to block EGFR phosphorylation and translocation (Prywes *et al*., 1986). While both SP and EGF stimulated ^{3}H-thymidine up-take in mock transfected fibroblasts (Figure 5), these responses were significantly inhibited in pCHER-transfected cells (Figure 5). Since these results suggest that SP-induced DNA synthesis in colonic fibroblasts is dependent on activation of EGF-R we next examined whether SP binding to NK-1R activates the EGF-R in colonic fibroblasts. Our results show that SP induces a transient increase in tyrosine phosphorylation of EGF-R in intestinal fibroblasts, which is detectable 15 min after SP exposure (Figure 6).

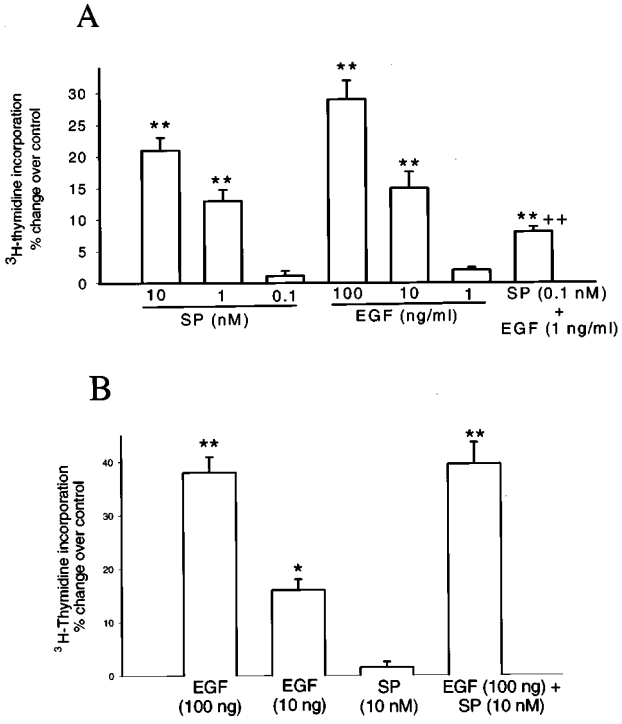

Figure 4 SP enhances EGF-R-mediated DNA synthesis in intestinal fibroblasts. Intestinal fibroblasts from wild-type (A) and NK-1R$^{-/-}$ (B) mice were plated in 12-well plates (2.5×10^4 cells cm^{-2}) pre-coated with fibronectin as described in Methods. After 48 h cells were serum-starved for 24 h and then incubated with SP (100–0.1 nM), EGF (100–1 ng ml^{-1}) or SP and EGF (1 nM and 1 ng ml^{-1}, respectively) in the presence of [^{3}H]-thymidine (0.5 μCi well^{-1}). After 12 h, cells were collected and [^{3}H]-thymidine incorporation was determined. (A) In fibroblasts isolated from NK-1R$^{\pm/\pm}$ mice, SP and EGF dose dependently increased DNA synthesis. Co-incubation of intestinal fibroblasts with SP and EGF, at doses ineffective to induce DNA synthesis, significantly increased DNA synthesis. (B) In fibroblasts obtained from NK-1R$^{-/-}$ mice EGF caused a proliferative response, which was similar to that obtained from NK-1R$^{\pm/\pm}$ mice. SP, however, failed to stimulate a significant [^{3}H]-thymidine uptake and had no synergistic effect on EGF (1 ng ml^{-1}) - induced [^{3}H]-thymidine uptake. Results are expressed as % change of DNA synthesis over control and are representative of three different experiments each with triplicate determinations. *, and ** denote $P<0.05$ and $P<0.01$ vs SP-treated cells, respectively; $\pm\pm$, denotes $P<0.01$ vs EGF alone (1 ng ml^{-1})-treated cells.

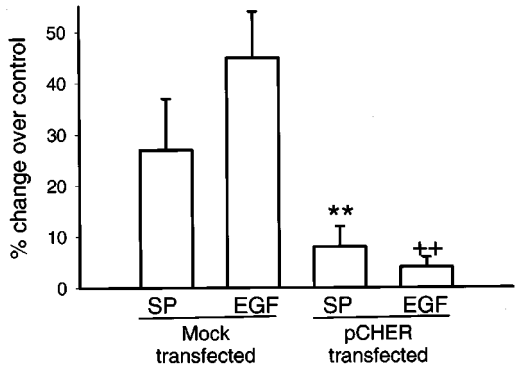

Figure 5 SP-induced DNA synthesis in intestinal fibroblasts requires transactivation of the EGF-R. Mock or pCHER transfected fibroblasts were serum-starved and incubated with SP (10 nM) or EGF (10 ng ml^{-1}) in the presence of [^{3}H]-thymidine (0.5 μCi well^{-1}) for 6 h. Cells were collected and placed in scintillation vials to determine [^{3}H]-thymidine incorporation. Results were expressed as per cent change of DNA synthesis over control. SP and EGF increased DNA synthesis in mock-transfected cells while pCHER-transfected cells, SP- and EGF-induced DNA synthesis was significantly inhibited. Data are expressed as mean$\pm$s.e.m. and are representative of three different experiments each with triplicate determinations. ** indicates $P<0.01$ vs SP-treated mock transfected cells and ++ indicates $P<0.01$ vs EGF-treated mock transfected cells.

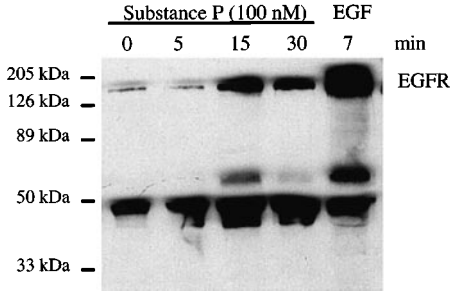

Figure 6 SP induces tyrosine phosphorylation of EGF-R and the formation of an activated EGF-R complex. Intestinal fibroblasts incubated with SP (10 nM) or EGF (20 ng ml^{-1}) for the indicated time periods were lysed in RIPA buffer. Soluble proteins were immunoprecipitated with an anti EGF-R antibody and then fractionated on a 7.5% SDS–PAGE gel. Proteins were detected using an anti-phosphotyrosine antibody. SP, time dependently, induces increased tyrosine phosphorylation of EGF-R and multiple co-precipitating proteins. Data are representative of four different experiments that gave similar results.

Discussion

Following tissue injury, SP is rapidly released and acts through the NK-1R expressed on a variety of cells such as neurons, endothelial cells, macrophages, eosinophils and mast cells. This interaction leads to increased vascular permeability, up-regulation of the expression of adhesion molecules, and release of inflammatory mediators such as IL-1, tumour necrosis factor α, IL-6, and histamine which will further amplify SP-mediated inflammation (Pernow, 1985; Brain, 1997). Studies with NK-1R antagonists indicate that SP and its NK-1R are involved in the pathophysiology of acute inflammation (Pothoulakis *et al*., 1994; MacCafferty *et al*., 1994; Neugebauer *et al*., 1995). Studies with mice lacking the NK-1R$^{-/-}$ also showed reduced inflammation and tissue neutrophil infiltration in experimental models of acute inflammation (Bozic *et al*., 1996; Castagliuolo *et al*., 1998; Bhatia *et al*., 1998; Castagliuolo *et al*., 1999). In agreement with these studies, our results here show that in the acute (3 days) phase of TNBS-induced colitis NK-1R promotes colonic inflammation. Thus, inhibition of SP-mediated actions during an acute inflammatory response is likely to reduce tissue injury (Pothoulakis *et al*., 1994; Bozic *et al*., 1996; Castagliuolo *et al*., 1998; Bhatia *et al*., 1998; MacCafferty *et al*., 1994; Neugebauer *et al*., 1995). The mechanism of NK-1R-induced proinflammatory mechanism appears to be mediated by a direct activation of the nuclear factor κB leading to release of proinflammatory cytokines from target cells (Lieb *et al*., 1997; Castagliuolo *et al*., 1997).

We report here that the severity of experimental colitis is increased in NK-1R$^{-/-}$ mice as compared to wild-type animals (Figure 1, Tables 1 and 2), suggesting a protective role for SP during prolonged colonic inflammation. Our findings agree with previous reports suggesting a protective role for SP-containing sensory nerves in several models of tissue injury (Evangelista and Meli, 1989; MacCafferty *et al*., 1994). In addition, SP-containing sensory nerves grow on the margin of tissue wounds (Dunnick *et al*., 1996), and administration of capsaicin delays wound healing (Kjartansson *et al*., 1987), whereas SP stimulates this process (Benrath *et al*., 1995). *In vitro* studies indicate that SP stimulates proliferation of cells required for wound repair such as fibroblasts (Nilsson *et al*., 1995), endothelial cells (Ziche *et al*., 1990), and epithelial cells (Kim *et al*., 1995). However, the effects of SP during wound healing may be more complex since SP also modulates cellular attachment, cell migration and differentiation (Nakamura *et al*., 1997), and stimulates angiogenesis by inducing the formation of capillary-like structures from endothelial cells (Wiedermann *et al*., 1996).

Our results differ from those of Di Sebastiano *et al*. (1999) and Stucchi *et al*. (2000) who suggested a proinflammatory role for NK-1 receptors in both the TNBS and the DSS models of colitis, respectively. The reason(s) for these different results are not entirely clear. We speculate that the complete absence of the NK-1 receptor in NK-1R deficient mice used in our study and the possibility of only partial inhibition of the NK-1R in the studies in rats (Di Sebastiano *et al*., 1999; Stucchi *et al*., 2000) may explain these different responses.

Maintenance of mucosal integrity and repair after injury requires complex interactions between the epithelium and lamina propria cells (i.e., epithelial cells and fibroblasts) involving a variety of peptide mediators. Peptides of the epidermal growth factor family, including EGF and TGFα, appear to be key mediators for protection and repair of the colonic mucosa during colitis. Colonic EGF mRNA is found in both normal subjects and patients with inflammatory bowel disease (Wright *et al*., 1990; Sottili *et al*., 1995; Chowdhury *et al*., 1996) and up-regulation of EGF-R occurs in the ulcerated mucosa in experimental colitis (Sottili *et al*., 1995). Animal studies showed that EGF and TGFα are important anti-inflammatory factors in colitis (Egger *et al*., 1998; Procaccino *et al*., 1994; Egger *et al*., 1997). Recent results also demonstrate interactions between growth factors and SP. SP enhances EGF-induced proliferation of skin fibroblasts (Kahler *et al*., 1996). SP also promotes insulin-like growth factor-1-induced stimulation of epithelial cell attachment to fibronectin and cell migration, and increases mRNA levels of α5 integrin (Nakamura *et al*., 1998). Here, we report that SP enhances EGF-induced proliferation of mouse intestinal fibroblasts. Since mucosal ulcerations characterize both TNBS-and DSS-induced colitis, the lack of a synergistic effect between SP and EGF to stimulate fibroblast proliferation in NK-1R$^{-/-}$ mice may be responsible for the development of larger ulcers and increased severity of colitis observed in our study (Figure 1, Tables 1 and 2).

Mitogenic pathways are tightly regulated by a variety of molecular events, such as phosphorylation-dephosphorylation of tyrosine residues on several key effector proteins. Typically, growth factors such as EGF-R ligands bind to membrane receptors with an intrinsic tyrosine kinase activity, causing auto-phosphorylation of the receptor. Once activated, EGF-R recruit adapter proteins to the cell plasma membrane, forming an activated complex that initiates a signalling cascade leading to cell proliferation (Pazin & Williams, 1992). Although SP binds to a GPCR (Krause *et al*., 1992), it ultimately activates signalling pathways similar to the cascade activated by growth factors involving protein tyrosine kinases leading to cell proliferation. For example, SP activates MAPKs and non-specific tyrosine kinase inhibitors, such as tyrphostin, inhibit SP-induced proliferation (Luo *et al*., 1996). Since GPCRs lack intrinsic kinase activity, transactivation of growth factor receptors has been suggested as a mechanism by which ligand binding to GPCRs can activate mitogenic signal transduction pathway (Daub *et al*., 1996). The EGF-R has been identified as essential in GPCR-mediated activation of MAPK-dependent pathways (Li *et al*., 1998), including in NK-1R-induced MAPKs activation and cell proliferation (Castagliuolo *et al*., 2000). Here we demonstrate that SP binding to NK-1R causes tyrosine phosphorylation of EGF-R which can be inhibited in cells transfected with a dominant negative EGF-R construct lacking kinase activity (Figure 6). These results indicate that SP-induced DNA synthesis in mouse colonic fibroblasts requires the presence of a functional EGF-R kinase domain.

In summary, our study provides substantial evidence that, in addition to their well-recognized role in acute inflammation, SP and NK-1R also play a key role in stimulating fibroblast proliferation during chronic intestinal inflammation. This activity requires a critical communication between SP and the EGF-R leading to cell proliferation. Moreover, SP-EGF-R interactions may have other pathophysiologically relevant consequences in view of the up-regulation of both NK-1R and EGF-R during gastrointestinal diseases charac-

terized by ulcer formation (Pothoulakis *et al*., 1998, Sottili *et al*., 1995). Enhanced sensitivity to the proliferative effects of SP and EGF may eventually result in aberrant growth and neoplasia formation since chronic intestinal inflammatory diseases, such as ulcerative colitis, are associated with a high incidence of cancer (Solomon & Schnitzler, 1998). Understanding the mechanisms by which SP and NK-1R activate genes and proteins involved in cell growth and proliferation may provide insights into the possible involvement of SP/NK-1R in ulcer healing and carcinogenesis.

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