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Los resultados de los estudios que constituyen la base de la presente tesis doctoral han sido recogidos en las siguientes publicaciones:

Artículos originales

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Trefoil peptide TFF2 treatment reduces VCAM-1 expression and leukocyte recruitment in experimental intestinal inflammation.
Journal of Leukocyte Biology 2004; 75: 214-223. FI: 4.180.

Soriano-Izquierdo A, Gironella M, Massaguer A, Salas A, Gil F, Piqué JM, Panés J. Effect of cyclosporin A on cell adhesion molecules and leukocyte-endothelial cell interactions in experimental colitis.
Inflammatory Bowel Diseases 2004; 10: 789-800. FI: 3.023.

Revisión

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Artículo 1

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VCAM-1, but Not ICAM-1 or MAdCAM-1, Immunoblockade Ameliorates DSS-Induced Colitis in Mice

Antonio Soriano, Antonio Salas, Azucena Salas, Miquel Sans, Meritxell Gironella, Monserrat Elena, Donald C. Anderson, Josep M. Piqué, and Julián Panés

Department of Gastroenterology (ASo, AzS, MS, MG, JMP, JP), Institut de Malalties Digestives, and Department of Biochemistry (ME), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, and Department of Pathology (ASa), Hospital Mutua of Terrassa, Barcelona, Spain; and Discovery Research (DCA), Pharmacia and Upjohn Laboratories, Kalamazoo, Michigan

SUMMARY: Adhesion molecule immunoneutralization is envisioned as a promising therapy for inflammatory bowel disease, but the relative value of selective blockade of different adhesion molecules has not been established. The aims of this study were to measure expression and functional relevance of endothelial intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) in leukocyte recruitment in experimental colitis and to compare the therapeutic effectiveness of their selective blockade. For this purpose, cell adhesion molecule expression was measured by the dual radiolabeled antibody technique in mice with dextran sulfate sodium-induced colitis and controls. Leukocyte-endothelial cell interactions were determined in colonic venules by fluorescence intravital microscopy. Therapeutic effects of chronic treatment with anti-ICAM-1, anti-VCAM-1, or anti-MAdCAM-1 antibodies were also assessed. Whereas colonic endothelial ICAM-1 was constitutively expressed and had a mild up-regulation in colitic animals, constitutive expression of VCAM-1 and MAdCAM-1 was low, but markedly increased after induction of colitis. Leukocyte adhesion was abrogated by immunoneutralization of VCAM-1 or MAdCAM-1 but not by treatment with an anti-ICAM-1 antibody. Chronic administration of anti-VCAM-1 antibody, but not anti-ICAM-1 or anti-MAdCAM-1, resulted in significant attenuation of colitis in terms of disease activity index, colon length, ratio of colon weight to length, and myeloperoxidase activity. In conclusion, VCAM-1 plays a central role in leukocyte recruitment in colitis and blockade of this adhesion molecule has higher therapeutic effect than immunoneutralization of ICAM-1 or MAdCAM-1 in this experimental model. (*Lab Invest 2000, 80:1541–1551*).

The immune responses that mediate local inflammation in inflammatory bowel disease (IBD) are not fully characterized. Nevertheless, there is little doubt that many cardinal signs of this inflammation are due to the rapid and prolonged infiltration of leukocytes together with the release of inflammatory mediators such as proteases, cytokines, arachidonic acid metabolites, and reactive oxygen species. These inflammatory mediators can damage cells either directly or indirectly through a mechanism secondary to increased leukocyte adhesion and infiltration (Kubes, 1993). Thus, central to the pathology of IBD is the role of molecules that regulate the recruitment of leukocytes such as cell adhesion molecules (CAMs) (Springer, 1990).

Both leukocyte and endothelial CAMs participate in transmigration of leukocytes from the vascular compartment to tissue sites of inflammation or immune reactions. This process results from a complex series of events involving rolling, activation, firm adhesion, and subsequent migration of leukocytes across the vascular endothelium.

Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) are endothelial CAMs of the immunoglobulin superfamily with a critical role in mediating the firm adhesion of leukocytes to endothelial cells in a variety of acute and chronic inflammatory diseases (Henninger et al, 1997; Panés and Granger, 1998). Immunohistochemistry studies of intestinal tissue samples from patients with IBD have demonstrated an increased expression of various CAMs, but there is no uniform agreement on their pattern of activation (Koizumi et al, 1992; Oshitani et al, 1995). The contradictory results of these studies may be in part related to limitations in quantification of CAM expression by this technique. Moreover, the functional significance of changes in expression of

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Address reprint requests to: Dr. Julián Panés, Gastroenterology Department, Hospital Clínic, Villarroel 170 08036 Barcelona, Spain. Fax: 34 93 451 68 77; E-mail: panes@medicina.ub.es

these CAMs in colonic venules on leukocyte-endothelial cell interactions has only been directly assessed in a recent study in a model of trinitrobenzene sulfonic acid (TNBS)-induced colitis (Sans et al, 1999). Since immunoneutralization of CAMs is regarded as a new strategy for treatment of IBD, it is crucial to elucidate the functional relevance and potential therapeutic benefit of selective immunoneutralization of the different CAMs involved in the process of leukocyte recruitment in IBD.

In the present investigation we have used a well-standardized model of mouse experimental colitis induced by oral administration of dextran sulfate sodium (DSS), characterized by morphological changes that mimic human ulcerative colitis (Okayasu et al, 1990). Using the dual radiolabeled monoclonal antibody (MAb) technique, an accurate method to quantify expression of CAMs in vivo (Eppihimer et al, 1996; Keelan et al, 1994; Langley et al, 1999; Panés et al, 1995), we have evaluated the pattern of CAM expression in DSS-induced colitis. To assess the functional significance of changes in CAM expression we have used intravital videomicroscopy, studying leukocyte-endothelial cell interactions and its molecular determinants by means of selective acute immunoneutralization. Finally, we have investigated the effects of chronic treatment with anti-ICAM-1, anti-VCAM-1, and anti-MAdCAM-1 MAbs on tissue injury and clinical signs of IBD.

Results

Study 1: Characterization of Inflammatory Changes

Clinical, macroscopic, histological, and biochemical changes in control and colitic mice are summarized in Table 1. Administration of DSS induced a significant loss in body weight that was maximum at Days 7 and 8 after induction of colitis. Starting 24 hours after induction of colitis, all animals had a positive fecal occult blood test and diminished stool consistency when compared with control animals. A Disease Activity Index (DAI) combining these parameters, as detailed in Table 2, was increased in DSS-treated mice. Colitic mice also had a significant reduction in colon length and an increase in colon weight, as well as an increase in the ratio of colon weight to length. Histological damage was seen only in colitic animals. Myeloperoxidase (MPO) activity in colonic samples were 4-fold higher in colitic animals than in control mice.

Table 2. Criteria for Scoring Disease Activity Index[1] (reproduced from Murphy et al, 1993)

Score	Weight loss (%)	Stool consistency[2]	Rectal bleeding
0	None	Normal	Negative
1	1–5	Loose	Negative
2	5–10	Loose	Hemoccult positive
3	10–15	Diarrhea	Hemoccult positive
4	>15	Diarrhea	Gross bleeding

[1] Disease activity index = combined score of weight loss, stool consistency, and bleeding/3.

[2] Normal stools = well-formed pellets; loose stools = pasty stool that does not stick to the anus; and diarrhea = liquid stool that sticks to the anus.

Study 2: In Vivo Expression of ICAM-1, VCAM-1, and MAdCAM-1

Values for colon endothelial surface area per gram of tissue, estimated from endothelial binding of anti-intercellular adhesion molecule 2 (ICAM-2) MAb 3C4, were 350.0 ± 92.0 ng MAb/g tissue in colitic animals and 277.2 ± 27.8 ng MAb/g tissue in controls; this difference was not significant ($p = 0.47$). Binding of the anti-ICAM-2 MAb was also similar in control and colitic mice in other organs, including liver, pancreas, mesentery, stomach, small intestine, and cecum (Table 3). Therefore, values for endothelial anti-ICAM-1, anti-VCAM-1, and anti-MAdCAM-1 binding were not corrected for changes in endothelial surface area relative to organ weight that might result from edema or capillary recruitment.

Endothelial ICAM-1 expression in the colon of colitic mice, estimated as bound ng MAb/g tissue, was significantly increased compared with noncolitic mice, although this increase was relatively mild (Fig. 1). In contrast, colonic endothelial VCAM-1 and MAdCAM-1 expression showed a marked increase (3-fold over control values) 10 days after induction of colitis (Fig. 1). ICAM-1 expression in organs other than the colon and the stomach remained unmodified in colitic animals (Table 3). However, VCAM-1 expression was also significantly increased in mesentery, ileum, and cecum and MAdCAM-1 expression was up-regulated in mesentery, stomach, and cecum (Table 3). No accumulation of the anti-MAdCAM-1 antibody was observed in liver, spleen (Table 3), or other extra-abdominal organs such as heart or lung (data not shown), which is consistent with a restricted expres-

Table 1. Clinical, Macroscopic, Histological, and Biochemical Changes in Control and DSS-Treated Mice

Group	Δ Body weight (g)	Stool consistency	Rectal bleeding	DAI	Colon weight (mg)	Colon length (mm)	Colon weight/ length (mg/mm)	Microscopic score	MPO activity (U/g tissue)
Control	0.2 ± 0.2	0 ± 0	0 ± 0	0 ± 0	297.3 ± 7.7	95.6 ± 1.4	3.1 ± 0	0 ± 0	0.4 ± 0.07
DSS	−2.2 ± 0.3[a]	1.0 ± 0.1[b]	1.3 ± 0[b]	1.7 ± 0.1[b]	340 ± 11.8[a]	73.7 ± 1.7[b]	4.6 ± 0.1[b]	52.1 ± 6.7[b]	1.9 ± 0.3[a]

[a] $p < 0.05$ vs control.

[b] $p < 0.0001$ vs control.

Table 3. Endothelial CAMs Expression in ng MAb per Gram of Tissue (ng/g) in Different Vascular Beds in Control and DSS-Treated Mice

	Liver	Spleen	Pancreas	Mesentery	Stomach	Jejunum	Ileum	Cecum
ICAM-1								
Control	7679 ± 662.9	7614 ± 777.9	372 ± 20.7	440.8 ± 38.9	305 ± 22.3	436.3 ± 46.1	356.5 ± 29.1	300.5 ± 23.3
DSS	8469 ± 440.7	6721 ± 531.3	493.7 ± 100.9	523.7 ± 95.2	438 ± 43[a]	437.8 ± 51.5	350.8 ± 27.9	306.2 ± 27.4
ICAM-2								
Control	846.2 ± 239.8	747.7 ± 249.7	320.7 ± 48.8	311.7 ± 20	485.5 ± 40.6	468.2 ± 52.8	410.2 ± 54.6	281.5 ± 32.8
DSS	722.2 ± 143.2	0 ± 0[a]	443.7 ± 28.3	385.2 ± 68.1	493.5 ± 31.5	497.7 ± 45.6	421.2 ± 43.5	551 ± 188
VCAM-1								
Control	976.7 ± 104	7336.7 ± 666.8	56.7 ± 6.8	81.7 ± 3.4	64.7 ± 7.6	91 ± 21.9	41.7 ± 4.3	31 ± 1.6
DSS	1149.8 ± 188.3	5782.5 ± 601.2	79.4 ± 13.1	168 ± 32.1[a]	77.6 ± 7.2	110.2 ± 11.7	59.6 ± 5.3[a]	59.2 ± 6.6[a]
MAdCAM-1								
Control	0 ± 0	0 ± 0	19.6 ± 15.3	58.3 ± 16.7	13.6 ± 10.3	268.6 ± 32.5	302.3 ± 49.8	85 ± 24.8
DSS	0 ± 0	0 ± 0	28.4 ± 3.8	158.4 ± 30.3[a]	40 ± 4.9[a]	253.2 ± 7.6	325.8 ± 16.1	220.2 ± 36.4[a]

[a] $p < 0.05$ vs control.

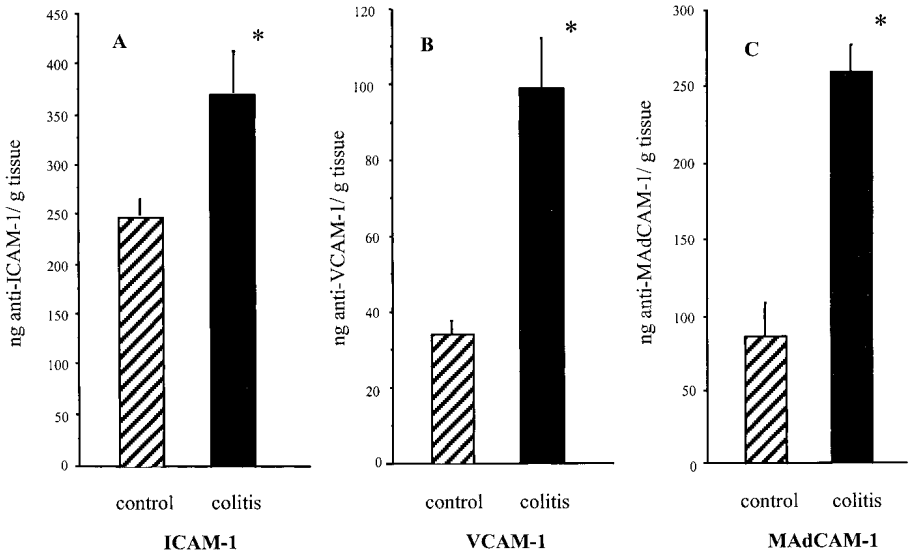

Figure 1.

A, Expression of colonic ICAM-1; *B*, VCAM-1; and *C*, MAdCAM-1 in control mice (hatched) and colitic animals (solid). ICAM-1 expression was estimated by binding of the anti-ICAM-1 MAb YN1/1.7.4, VCAM-1 expression by binding of the anti-VCAM-1 MAb MK1.91, and MAdCAM-1 expression by binding of anti-MAdCAM-1 MAb MECA-367. Note the different scales. Results are calculated as ng MAbs/g tissue. *$p < 0.05$ vs control.

sion of this adhesion molecule to gastrointestinal organs.

Study 3: Leukocyte-Endothelial Cell Interactions in Colonic Venules

When compared with control animals, colitic mice showed a pronounced increase in leukocyte-endothelial cell interactions. There was a 3-fold increase in the flux of rolling leukocytes 10 days after induction of colitis (Fig. 2). Although few adherent leukocytes were present in venules from control mice, a 4-fold increase in leukocyte adhesion was observed in colitic animals (Fig. 2). No differences in leukocyte rolling velocity, venular blood flow, or venular wall shear rate were observed between control and colitic mice (not shown).

Treatment of colitic mice with anti-ICAM-1 MAb did not modify leukocyte adhesion to colonic venules, whereas VCAM-1 and MAdCAM-1 blockade decreased the number of adherent leukocytes to levels close to those of control animals (Fig. 3). Treatment of colitic mice with a nonbinding MAb (UPC-10) did not affect leukocyte adhesion to colonic venules. None of these MAbs had any effect on the flux of rolling leukocytes (Fig. 3), leukocyte rolling velocity, venular blood flow, or venular shear rate (not shown).

In view of the apparent discrepancy between the similar ability of anti-VCAM-1 and anti-MAdCAM-1 antibodies to prevent leukocyte adhesion in colitic animals studied at Day 10 after induction of colitis and the lack of therapeutic effect of anti-MAdCAM-1 antibodies (see below), we hypothesized that these two antibodies may have differential effects on leukocyte-endothelial cell interactions at an earlier time point. To test this hypothesis, we performed additional intravital microscopy studies in groups of colitic animals receiving the same antibodies, at Day 5 after induction of colitis. Comparison of colitic animals studied at Days 5 or 10 after induction of colitis revealed that the former group had a

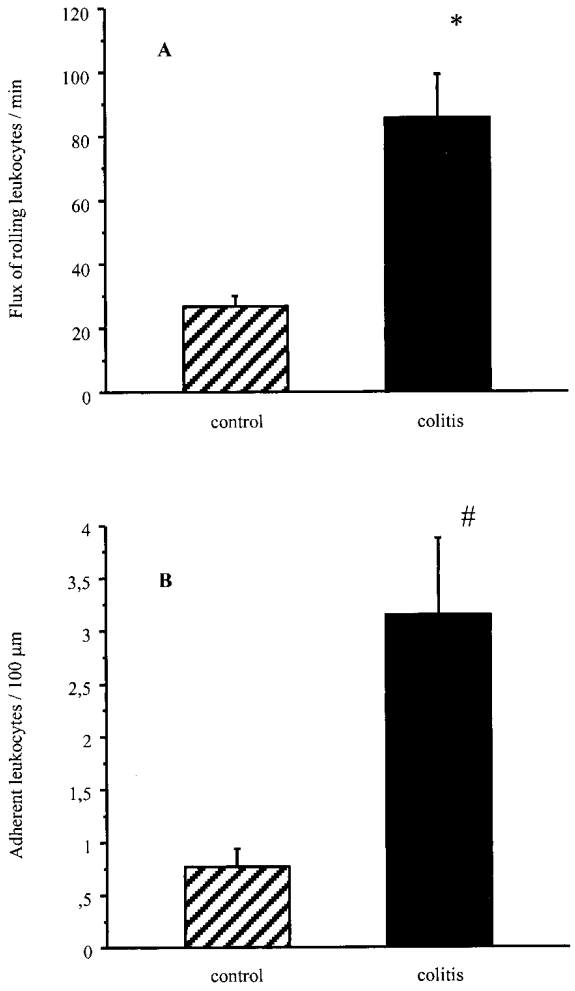

Figure 2.

A, Flux of rolling leukocytes and *B*, leukocyte adhesion in colonic venules. Control animals had a very low number of rolling or adherent leukocytes. A significant increment in leukocyte-endothelial cell interactions was observed at Day 10 after induction of colitis. $^{*}p < 0.01$ vs control; $^{\#}p < 0.001$ vs control.

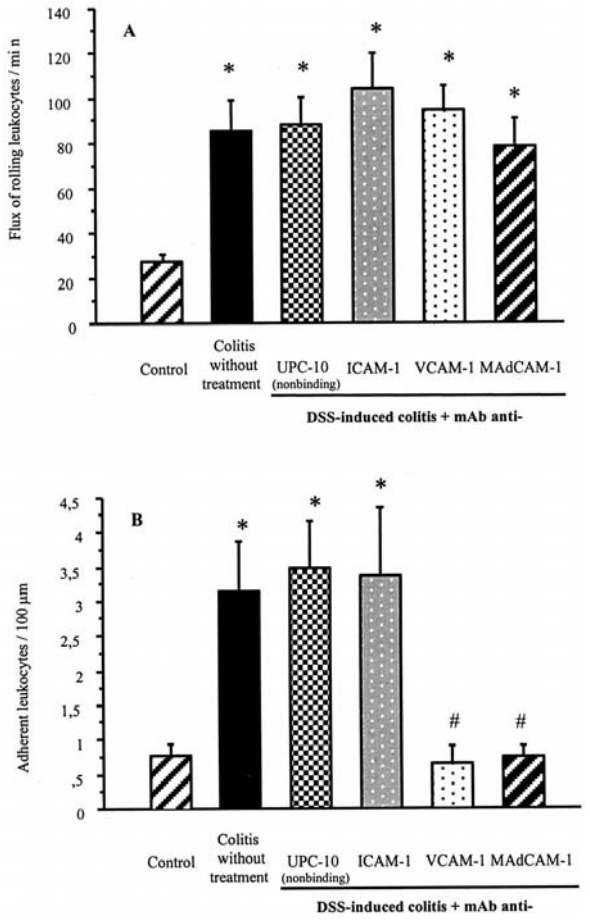

Figure 3.

Effects of immunoneutralization of ICAM-1, VCAM-1, and MAdCAM-1 on *A*, flux of rolling leukocytes and *B*, leukocyte adhesion. Immunoneutralization of either ICAM-1, VCAM-1, or MAdCAM-1 had no effect on leukocyte rolling. Blocking of VCAM-1 or MAdCAM-1 abrogated leukocyte adhesion in colonic venules of colitic mice, whereas ICAM-1 blockade did not have any effect. Control MAb UPC-10 had no effect. $^{*}p < 0.02$ vs control; $^{\#}p < 0.02$ vs colitis without treatment.

significantly lower flux of rolling leukocytes (59.0 $\pm$ 9.4 vs. 85.3 $\pm$ 14.0 leukocytes/minute; $p < 0.05$), a higher number of adherent leukocytes (5.8 $\pm$ 0.4 vs. 3.1 $\pm$ 0.7 cells/100 μm venule; $p < 0.05$), and a higher venular shear rate (330.7 $\pm$ 18.6 vs. 175.6 $\pm$ 17.5 seconds^{-1}; $p <$ 0.05). The increased flux of rolling leukocytes observed in colitic mice at Day 5 after induction of colitis relative to control mice was abrogated by treatment with anti-VCAM-1 MAb, but not by immunoneutralization of ICAM-1 or MAdCAM-1 (Table 4). Although treatment with anti-VCAM-1 and anti-MAdCAM-1 MAbs reduced leukocyte adhesion in colitic animals at Day 5, this reduction only reached statistical significance for the group treated with anti-VCAM-1 (Table 4).

Study 4: Effects of Chronic Treatment with Anti-ICAM-1, Anti-VCAM-1, and Anti-MAdCAM-1 MAb on DSS-Induced Colitis

Daily treatment with anti-VCAM-1 MAb MK1.91 significantly attenuated the loss in body weight as compared with the group of animals treated with the nonbinding MAb UPC-10, from Day 7 to Day 10 after induction of colitis (Fig. 4). In addition, animals treated with MK1.91 rapidly regained weight, whereas in those receiving the control MAb the decrease in body weight persisted as far as Day 9. DAI at Day 10 was significantly lower in mice treated with anti-VCAM-1 MAb than in the control group (Fig. 4). Compared with mice treated with the control MAb, MK1.91-treated animals had a significantly reduced colon weight (252.4 $\pm$ 14.6 vs. 302.4 $\pm$ 9.6 mg; $p < 0.05$), lower reduction in colon length (Fig. 5), lower ratio of colon weight to length (Fig. 5), and lower colonic MPO activity (Fig. 6). Although the global microscopic damage score tended to be lower in MK 1.91-treated animals compared with those receiving the nonbinding MAb UPC-10 (34.8 $\pm$ 14.9 vs. 69.2 $\pm$ 23.1), these results did not reach statistical significance. Neither administration of anti-ICAM-1 nor anti-MAdCAM-1 MAbs had significant effects on body weight loss, DAI, macroscopic, histological, or biochemical alterations associated with DSS-induced colitis as shown in Figures 4 to 6. Differences in the severity of colitis among treatment

Table 4. Leukocyte-Endothelial Cell Interactions at Day 5 after Colitis Induction

Group	Control	Colitis without treatment	DSS-induced colitis + MAb anti- UPC-10 (nonbinding)	ICAM-1	VCAM-1	MAdCAM-1
Flux of rolling leukocytes/min	27.1 ± 3.1	59.0 ± 9.4[a]	55.4 ± 11.7[a]	61.2 ± 14.1[a]	30.8 ± 5.9[b]	51.6 ± 4.2[a]
Adherent leukocytes/100 μm	0.8 ± 0.1	5.8 ± 0.4[a]	5.5 ± 1.1[a]	4.6 ± 2.2[a]	3.1 ± 0.6[a,b]	3.6 ± 0.6[a]

[a] $p < 0.05$ vs control.
[b] $p < 0.05$ vs colitis without treatment.

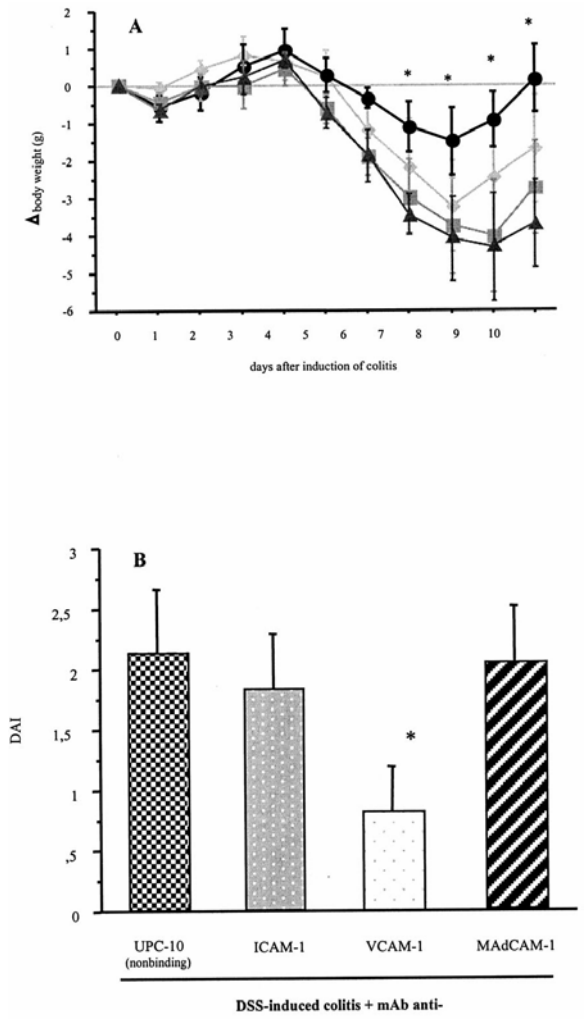

Figure 4.
Effect of treatment with anti-CAM MAbs or a nonbinding MAb (UPC-10) on *A*, body weight loss and *B*, DAI. Treatment with MK1.91 significantly attenuated the loss in body weight from Days 7 to 10 after induction of colitis, induced a more rapid recovery, and resulted in a lower DAI. ◆, YN1/1.7.4 (anti-ICAM-1 MAb); ●, MK1.91 (anti-VCAM-1 MAb); ▨, MECA-367 (anti-MAdCAM-1 MAb); △, UPC-10 (control MAb). *$p < 0.05$ vs UPC-10 treated colitic mice.

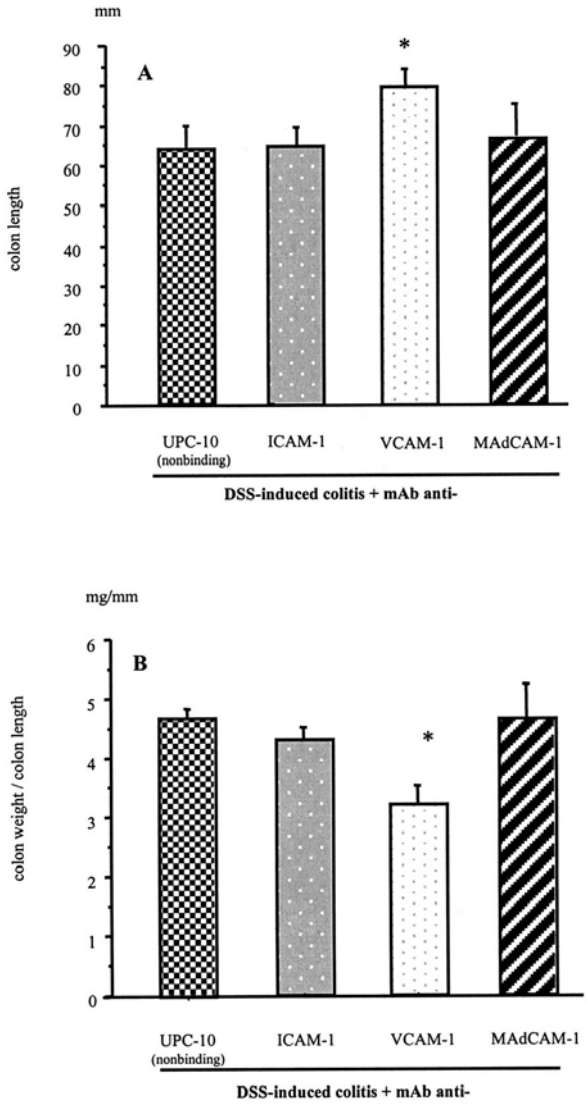

Figure 5.
A, Colon length and *B*, ratio of colon weight to length after treatment with the different anti-CAM MAbs. Treatment of DSS-induced colitis with MK1.91 resulted in a marked reduction of macroscopic damage. *$p < 0.05$ vs UPC-10 treated colitic mice.

groups cannot be attributed to variations in exposure to DSS because the volume of drinking water with DSS consumed by the different groups was similar (not shown).

Discussion

In the present study we assessed the expression and functional role of three inducible endothelial CAMs, namely ICAM-1, VCAM-1, and MAdCAM-1, in a well-characterized model of murine colitis. Likewise, we compared the therapeutic effectiveness of selective blockade of these molecules. Our results demonstrate a marked increase of endothelial VCAM-1 and MAdCAM-1 in the colon of colitic mice, a key functional role for these molecules as mediators of leuko-

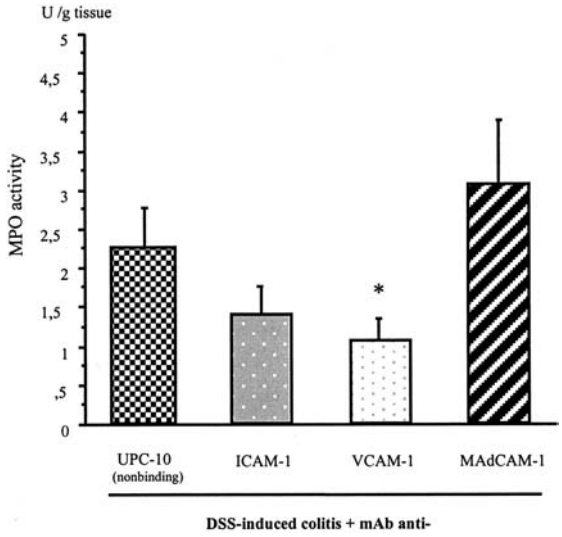

Figure 6.

Effect of treatment with anti-CAM MAbs or UPC-10 MAb on MPO activity. $*p < 0.05$ vs UPC-10 treated colitic mice.

cyte adhesion, and a potent therapeutic effect for VCAM-1 immunoneutralization in DSS-induced colitis.

The dual-radiolabeled MAb technique has previously been employed to assess expression of different CAMs in vivo, in normal and inflamed tissues. Using this technique we demonstrated that expression of CAMs of the immunoglobulin superfamily is up-regulated in DSS-induced colitis, although the magnitude of the increase varies and is related to the level of basal expression. Hence, whereas ICAM-1 has a high constitutive expression and a weak, although significant, up-regulation in colitic animals, VCAM-1 and MAdCAM-1, which have a considerably lower basal expression than ICAM-1 (one-eighth and one-third, respectively), show a robust up-regulation in colitic animals. These data are consistent with evidence showing that whereas in vivo constitutive level of VCAM-1 expression in a variety of organs is significantly lower than that of ICAM-1, cytokine stimulation results in a higher increase in VCAM-1 than in ICAM-1 expression (Henninger et al, 1997). Likewise, a previous study in the TNBS-induced colitis in the rat showed a marginal, not significant, increase in ICAM-1 expression along with a marked up-regulation of VCAM-1 in the colonic endothelium (Sans et al, 1999). As for MAdCAM-1, our findings are in keeping with the results of previous studies showing that IL-10-deficient mice with active colitis exhibit a profound up-regulation of MAdCAM-1 in the colon (Kawachi et al, 2000) and that human tissue sections of patients with active ulcerative colitis or Crohn's disease have increased endothelial MAdCAM-1 expression as compared with normal subjects (Briskin et al, 1997). Although DSS-induced colitis affects predominantly the distal colon (Okayasu et al, 1990), we also found increased VCAM-1 expression in ileum and cecum and up-regulation of endothelial MAdCAM-1 in stomach and cecum. Up-regulation of CAMs in these vascular beds probably results from DSS-induced mucosal lesions in these upper segments of the gut, whereas increased expression of these molecules in the mesentery might be induced by cytokines released from the inflamed bowel.

We took advantage of fluorescence intravital microscopy to characterize changes in leukocyte-endothelial cell interactions in colonic submucosal and lamina propria venules and to define the functional relevance of alterations in ICAM-1, VCAM-1, and MAdCAM-1 expression by means of immunoneutralization of these CAMs. The current study demonstrates that acute immunoneutralization of ICAM-1 in DSS-induced colitis does not modify leukocyte adhesion, whereas blockade of VCAM-1 or MAdCAM-1 decreases leukocyte adhesion to levels similar to those of control animals. We ruled out a nonspecific effect of injection of the immunoglobulins, because treatment with a nonbinding MAb did not modify leukocyte-endothelial cell adhesion. It has been shown that VCAM-1/$\alpha_4\beta_1$ interactions have the potential to mediate rolling (Alon et al, 1995) and our results, showing that VCAM-1 immunoneutralization in colitic animals studied at Day 5 after induction of colitis abrogates the flux of rolling leukocytes, are in keeping with previous in vitro evidence. However, rolling interactions at Day 10 after induction of colitis seem to be VCAM-1-independent as long as blockade of this adhesion molecule did not affect rolling interactions at this time point. In vitro studies have also suggested that MAdCAM-1/L-selectin interactions (Berg et al, 1993) have the potential to mediate leukocyte rolling; however, we did not observe any effect of immunoneutralization of MAdCAM-1 on rolling interactions at any time point. Collectively, these observations suggest that VCAM-1 and MAdCAM-1 act as mediators of leukocyte adhesion in colonic venules of colitic mice, whereas ICAM-1 does not seem to be a molecular determinant of leukocyte adhesion in this experimental model of colitis.

To date, some studies have demonstrated the potential of CAMs as targets for therapeutic interventions, although information on the relative value of selective blockade of different CAMs is scarce. Podolsky et al (1993) showed a potent therapeutic effect of α_4 integrin blockade in the cotton-top tamarin model of colitis, whereas an anti E-selectin MAb did not provide any amelioration in this model. In the current study, the comparison of the therapeutic value of selective immunoneutralization of ICAM-1, VCAM-1, or MAdCAM-1 clearly demonstrates that VCAM-1 blockade affords a significant amelioration of colitis, superior to that of ICAM-1 or MAdCAM-1 blockade. This observation is in keeping with the aforementioned study and with the findings by Hesterberg et al (1996) showing that immunoneutralization of the VCAM-1 counterreceptor $\alpha_4\beta_7$ significantly ameliorates colitis in the cotton-top tamarin model. It also extends previous evidence from our group (Sans et al, 1999) showing a key role for VCAM-1 in the pathogenesis of colitis in a different experimental model. It should be pointed out that the concept that blocking VCAM-1/α_4 interactions is superior to targeting other molecular determinants of leukocyte recruitment may not apply to all models of colitis (including human IBD), because

the relative role of the different CAMs in leukocyte recruitment into the intestine may depend on the type of colonic inflammation, and might even change over time, as we demonstrate in the current study. Therefore, selective CAM blockade may be differentially effective for different forms of IBD.

The findings of the present work contrast with previous evidence showing a beneficial effect of ICAM-1 blockade in experimental models of colitis. Several studies reported that treatment with an anti-ICAM-1 MAb ameliorates various forms of experimental colitis, including acetic acid-induced (Wong et al, 1995) and DSS-induced (Hamamoto et al, 1999; Taniguchi et al, 1998) models. It has also been shown that sustained treatment for one week with an ICAM-1 antisense oligonucleotide significantly attenuates experimental DSS-induced colitis (Bennett et al, 1997). In a recent study, Bendjelloul and colleagues observed that ICAM-1 deficiency protects mice against DSS-induced colitis (Bendjelloul et al, 2000). The discrepancy between these observations and the results of the current study showing no effect of ICAM-1 blockade on leukocyte adhesion and inflammatory changes in DSS-induced colitis may have several reasons. Wong et al (1995) treated the rats with a single dose of an anti-ICAM-1 MAb 24 hours after exposing the colon to acetic acid, a time point when mucosal damage is nonimmunologic in nature but dependent on the reaction of the mucosa to organic acids and ischemia, a process that might potentially be affected by ICAM-1 blockade (Kurose et al, 1994). The reasons for the disagreement between our observations and other experimental studies using the DSS-induced colitis model are less straightforward. They might be related to species or mouse strain differences or to special features related to experimental conditions, including the concentration of DSS used (3% in studies by Taniguchi et al and Bendjelloul et al) and duration of exposure to this toxic compound, namely continuous administration of DSS for 5 to 7 days versus administration of 5% DSS for only the initial 4 days and study at Day 10. These methodological differences might lead to a different response in up-regulation of ICAM-1, which in our model was mild, but in the models of continuous DSS administration seems to be more robust (Bennett et al, 1997).

The lack of therapeutic effect of anti-MAdCAM-1 MAb in our study was surprising taking into account the potent inhibitory effect of acute administration of an anti-MAdCAM-1 MAb on leukocyte adhesion in colonic venules in animals studied at Day 10 after induction of colitis, which is similar to that of VCAM-1. Assessment of the effects of treatment with anti-VCAM-1 and anti-MAdCAM-1 in animals at Day 5 after induction of colitis revealed a possible explanation, showing a significant reduction in leukocyte rolling and adhesion in response to anti-VCAM-1 blockade, whereas MAdCAM-1 immunoneutralization had no significant effect on leukocyte-endothelial cell interactions. Taken together, these results support the notion that VCAM-1 plays a key role in both the early and late phases of the inflammatory response as a molecular determinant of leukocyte rolling and adhesion, whereas MAdCAM-1 contributes to leukocyte adhesion only at a later time point. Another possibility may be that the differential effect of VCAM-1 and MAdCAM-1 chronic blockade might be related to the ability of the anti-VCAM-1 MAb to interfere with other processes of the inflammatory response in addition to leukocyte adhesion, such as cell activation or binding to nonimmune cells that may retain the lymphocytes on the inflamed organ (Meng et al, 1995). Picarella et al (1997), in a colitic model of *scid* mice reconstituted with $CD45RB^{high}$ $CD4^+$ T cells, demonstrated that treatment with MAbs specific for MAdCAM-1 and/or β_7 integrin (β chain of lymphocyte MAdCAM-1 counterreceptor $\alpha_4\beta_7$) blocks recruitment of lymphocytes into the inflamed colon and reduces the severity of colonic inflammation. This beneficial effect of MAdCAM-1 blockade in the reconstituted *scid* mice may be related to the fact that reconstitution was performed with T cells that express β_7. Therefore, a MAb directed against this molecule could block all lymphocytes. Interestingly, histological improvement was superior in animals treated with anti-β_7 (which interferes with VCAM-1 and MAdCAM-1-mediated adhesion) than in animals that were treated only with MAdCAM-1 MAb.

In conclusion, our work clearly demonstrates the superiority of VCAM-1 blockade over that of ICAM-1 or MAdCAM-1 in inducing remission of colonic inflammation. Therefore, VCAM-1 blockade by means of MAbs represents a potential novel approach for the treatment of inflammatory bowel conditions such as ulcerative colitis. Inhibition of VCAM-1 could have the advantage of preserving physiological leukocyte trafficking because this CAM is only up-regulated by inflammatory stimuli.

Materials and Methods

Colitis Induction

Male CD_1 mice weighing 28–30 g were obtained from Iffa Credo (Lyon, France) and maintained on conventional housing conditions. Experimental colitis was induced in mice by giving 5% (w/v) DSS (mol wt 40 kd; ICN Biomedicals, Aurora, Ohio) in drinking water ad libitum for four days. DSS administration was then stopped and mice received drinking water alone for 6 days until study at Day 10. The volume of drinking water was monitored in control and colitic mice in all treatment groups. Given the variable sensitivity of different mouse strains to DSS-induced colitis (Mahler et al, 1998), the dose of DSS to be used in our CD_1 mice was empirically established to induce moderate to severe colitis while minimizing mortality. Control animals received drinking water without DSS. The type of colonic inflammatory infiltrate and presence of irreversible macroscopic colonic alterations such as shortening of colon length are indicative of a chronic type of colitis. The *Guide for the Care and Use of Laboratory Animals* (NIH publication 86–23, revised 1985) as well as the document "Regulation of proce-

dures for animal experiments" (ref. 5/1995) from the Generalitat de Catalunya were followed.

Study 1: Characterization of Inflammatory Changes

Clinical parameters, including body weight, stool consistency (score: 0, normal stools; 1, soft stools; and 2, liquid stools), and rectal bleeding (score: 0, negative fecal occult blood test, HemoFEC; Boehringer Mannheim, Barcelona, Spain; 1, positive fecal occult blood test; and 2, visible rectal bleeding) were daily assessed in 20 animals (10 mice with DSS-induced colitis and 10 controls). The DAI was determined as a combination of the above parameters according to the scoring criteria detailed in Table 2 (Murthy et al, 1993). Mice were killed on Day 10 after the onset of colitis induction by an overdose of subcutaneous (sc) ketamine (Ketolar, Parke-Davis, Morris Klein, New Jersey) and xylazine (Sigma Chemical, St. Louis, Missouri). The colon was excised and opened by a longitudinal incision, rinsed with saline, weighed, and its length was measured after exclusion of the cecum. Distal colon samples (approximately 20 mg) were then excised, snap-frozen in liquid nitrogen, and stored at −80° C for later assay of MPO activity. MPO activity was assessed according to a technique described by Bradley et al (1982); results are expressed as units per gram of tissue. To evaluate histological damage colonic samples were fixed in 4% formalin, embedded in paraffin, and sections (5–7 μm) were stained with hematoxylin and eosin following standard procedures. Stains were assessed by a single pathologist in a blinded fashion (A Salas). Colitis was graded quantitatively according to previously defined criteria, which takes into account the percentage of mucosal injury, extension of total colon length, and lesion depth (Cooper et al, 1993).

Study 2: Endothelial ICAM-1, VCAM-1, and MAdCAM-1 Expression

ICAM-2, which is constitutively expressed on endothelial cells and is not up-regulated in response to cellular activation (de Fougerolles et al, 1991; Nortamo et al, 1991), was measured as an estimation of endothelial surface area relative to tissue weight. Forty mice (10 mice per group) were used to characterize endothelial expression of ICAM-1, ICAM-2, VCAM-1, and MAdCAM-1 in controls and animals with DSS-induced colitis at Day 10.

Monoclonal Antibodies. The MAbs used to quantify endothelial expression were YN1/1.7.4, a rat IgG_{2b} directed against mouse ICAM-1 (Kumasaka et al, 1996); 3C4, a rat IgG_{2a} against mouse ICAM-2 (Xu et al, 1996); MK1.91, a rat IgG_1 against mouse VCAM-1 (Gerritsen et al, 1995); MECA-367, a rat IgG_{2a} against mouse MAdCAM-1 (Mebius et al, 1998), and UPC-10, a nonbinding IgG (Bickel et al, 1994). YN1/1.7.4 and MK1.91 were scaled up and purified by protein A or G chromatography at Pharmacia Upjohn Laboratories (Kalamazoo, Michigan). 3C4 and MECA367 were purchased from PharMingen (San Diego, California). UPC-10 was purchased from Sigma (Saint Louis, Missouri) and dialyzed to remove sodium azide. The binding MAbs directed against ICAM-1, ICAM-2, VCAM-1, and MAdCAM-1 were labeled with ^{125}I, whereas the nonbinding MAb UPC-10 was labeled with ^{131}I (Amersham Ibérica, Madrid, Spain). Radioiodination of the MAbs was performed by the iodogen method as previously described (Fraker and Speck, 1978). Labeled MAbs were stored at 4° C and used within 3 weeks after the labeling procedure. The specific activity of labeled MAbs was approximately 0.5 mCi/mg.

Endothelial Expression of ICAM-1, ICAM-2, VCAM-1, and MAdCAM-1. Animals were anesthetized with sc ketamine (150 mg/kg body weight) and xylazine (7.5 mg/kg body weight) and the left carotid artery and a tail vein were cannulated with PE-10 tubing (Portex, Hythe, United Kingdom). For assessment of endothelial expression of ICAM-1, ICAM-2, VCAM-1, and MAdCAM-1, a mixture of 10 μg ^{125}I-YN1/1.7.4 and 40 μg unlabeled YN1/1.7.4; 10 μg ^{125}I-3C4 and 60 μg unlabeled 3C4; 10 μg ^{125}I-MK1.91 and 20 μg unlabeled MK1.91; and 10 μg ^{125}I-MECA-367 without additional unlabeled MECA-367 were administered, respectively. In all cases 3 μg ^{131}I-UPC-10 was added to the injection mixture. Doses of anti-ICAM-1, anti-ICAM-2, anti-VCAM-1, and anti-MAdCAM-1 MAbs proved to be saturating in previous assays (Henninger et al, 1997; Langley et al, 1999; Panés et al, 1995; Panés et al, 1996). The mixture of binding and nonbinding MAbs was administered through the tail vein catheter. Blood samples were obtained through the carotid artery catheter 5 minutes after injection of the MAb mixture. Thereafter, the animals were heparinized (1 mg/kg sodium heparin iv) and rapidly exsanguinated. Entire organs were then harvested and weighed. ^{125}I (binding MAb) and ^{131}I (nonbinding MAb) activities in each organ and 100 μl aliquots of cell-free plasma were counted in a Cobra II gamma-counter (Packard, Canberra, Australia) with automatic correction for background activity and spillover. The injected activity in each experiment was calculated by counting a 3-μl sample of the 300-μl injection mixture containing the radiolabeled MAbs. The accumulated activity of each MAb in an organ was expressed as nanograms of binding MAb per gram of tissue. The formula used to calculate either ICAM-1, ICAM-2, VCAM-1, or MAdCAM-1 expression was as follows: Endothelial expression = [(cpm ^{125}I organ $\times$ g^{-1} $\times$ cpm ^{125}I injected^{-1}) − (cpm ^{131}I organ $\times$ g^{-1} $\times$ cpm ^{131}I injected^{-1}) $\times$ (cpm ^{125}I in plasma)/(cpm ^{131}I in plasma)] $\times$ ng injected MAb. This formula was modified from the original method (Eppihimer et al, 1996) to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of both binding and nonbinding MAbs (Sans et al, 1999).

Study 3: In Vivo Assessment of Leukocyte-Endothelial Cell Interactions in Colonic Venules

Leukocyte-endothelial cell interactions in colonic submucosal and lamina propria venules were characterized using intravital microscopy in control animals and

in mice at Days 5 and 10 after induction of colitis (n = 10 animals per group).

Intravital Microscopy. Mice were anesthetized with sc ketamine (150 mg/kg) and xylazine (7.5 mg/kg) and a tail vein was cannulated. Throughout the experiments, rectal temperature was monitored using an electrothermometer and was maintained between 36.5 and 37.5° C with an infrared heat lamp. The abdomen was opened via a midline incision and a segment of the distal colon was chosen for microscopy examination, exteriorized, and covered with a cotton gauze soaked with bicarbonate buffer. Mice were then placed on an adjustable microscope stage, and the colon was extended over a non-autofluorescent coverslip that allowed observation of a 2-cm^2 segment of tissue. An inverted microscope (Diaphot 300; Nikon, Tokyo, Japan) with a CF Fluor 40× objective lens (Nikon) was used. A charge-coupled device (CCD) camera (model XC-77; Hamamatsu Photonics, Hamamatsu, Japan) with a C2400 CCD camera control unit and a C2400–68 intensifier head (Hamamatsu Photonics), mounted on the microscope, projected the image onto a monitor (Trinitron KX-14CP1; Sony, Tokyo, Japan), and the images were recorded using a videocassette recorder (SR-S368E; JVC, Tokyo, Japan) for off-line analysis. A video date-time generator (Panasonic Digital AV Mixer WJ-AVE55; Matsushita Communication Industrial, Tokyo, Japan) displayed these parameters on recorded and live images. Leukocytes were in vivo labeled by iv injection of rhodamine-6G (Molecular Probes, Leiden, The Netherlands) as previously described (Horie et al, 1997). Rhodamine-6G-associated fluorescence was visualized by epi-illumination at 510–560 nm, using a 590 nm emission filter. Single unbranched submucosal and lamina propria venules with internal diameters (ID) of 25–40 μm were selected for observation. Venular ID was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas). The flux of rolling leukocytes, leukocyte rolling velocity, number of adherent leukocytes, venular blood flow (Vbf), and venular wall shear rate (γ) were determined off-line after playback of the videotapes. Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of free-flowing leukocytes in the same vessel. The flux of rolling leukocytes was measured as the number of rolling leukocytes that passed a fixed point within a small (10 μm) viewing area of the vessel in a 1-minute period. Leukocyte rolling velocity was calculated as the mean of 10 rolling leukocyte velocities and expressed in μm/s. Leukocytes were considered adherent to venular endothelium when stationary for 30 seconds or longer, and expressed as the number per 100 μm length of venule. Venular blood flow was estimated from the mean of the velocity of three free-flowing leukocytes (ffv), using the empirical relationship of Vbf = ffv/1.6 (Davis, 1987). Venular wall shear rate was calculated, assuming cylindrical geometry, using the Newtonian definition γ = 8 (Vbf/ID) (Lipowsky et al, 1978). In each animal, three to six random venules were examined and results were calculated as the mean of each parameter in all venules examined.

Role of ICAM-1, VCAM-1, and MAdCAM-1 in Colonic Leukocyte Recruitment. Groups of colitic mice were studied at Days 5 and 10 after induction of colitis. Animals received two doses of the following MAbs: anti-ICAM-1 (YN1/1.7.4, 50 μg), anti-VCAM-1 (MK1.91, 30 μg), anti-MAdCAM-1 (MECA-367, 10 μg), or a nonbinding control MAb (UPC-10, 10 μg). Each treatment group included five mice. The first dose of MAb was injected through the tail vein (volume 0.3 ml) in animals immobilized in a restrainer. Three hours later they were anesthetized with sc ketamine (150 mg/kg) and xylazine (7.5 mg/kg) and a second dose of the corresponding MAb was injected through a tail vein catheter. After a 30-minute equilibration period, leukocyte-endothelial cell interactions were assessed as described above.

Study 4: Effect of Treatment with Anti-ICAM-1, Anti-VCAM-1, and Anti-MAdCAM-1 MAbs on DSS-Induced Colitis

To assess the relative importance of ICAM-1, VCAM-1, and MAdCAM-1 on colonic inflammatory response to DSS, the effect of chronic treatment with YN1/1.7.4, MK1.91, MECA-365, and UPC-10, an irrelevant control MAb, was determined. Initially, a pharmacokinetic study of these MAbs was performed after intraperitoneal (ip) administration of different doses (1, 2, or 3 mg/kg) of ^{125}I-labeled MAbs to colitic mice. Based on plasma concentrations, the MAb doses selected for daily ip administration were: 3 mg/kg for anti-ICAM-1, 2 mg/kg for anti-VCAM-1, and 1 mg/kg for anti-MAdCAM-1 and UPC-10 (control group). The calculated half-life was 24–30 hours. In all cases circulating levels 5 hours after ip injection were 4–12 μg/ml, whereas trough concentrations at 24 hours were less than 3 μg/ml. Four groups of colitic mice (n = 5 per group) were treated just before induction of colitis and during the following 10 days with the aforementioned MAbs. Body weight and clinical signs of colitis were recorded daily, and animals were sacrificed at Day 10 after induction of colitis. Colonic microscopic damage was evaluated in a blinded fashion using the validated index described above. MPO activity was also measured in colonic samples.

Statistical Analyses

Data were analyzed using standard statistical methods: analysis of variance with the Bonferroni (post-hoc) test and Student's unpaired *t* test, when appropriate. All values are expressed as mean ± SEM. Statistical significance was set at $p < 0.05$.

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Artículo 2

Soriano-Izquierdo A, Gironella M, Massaguer A, May FEB, Salas A, Sans M, Poulsom R, Thim L, Piqué JM, Panés J. Trefoil peptide TFF2 treatment reduces VCAM-1 expression and leukocyte recruitment in experimental intestinal inflammation. **Journal of Leukocyte Biology** 2004; 75: 214-223.

Trefoil peptide TFF2 treatment reduces VCAM-1 expression and leukocyte recruitment in experimental intestinal inflammation

Antonio Soriano-Izquierdo,* Meritxell Gironella,* Anna Massaguer,† Felicity E. B. May,‡ Antonio Salas,§ Miquel Sans,* Richard Poulsom,¶ Lars Thim,|| Josep M. Piqué,* and Julián Panés*,[1]

*Department of Gastroenterology and †Liver Unit, Institut de Malalties Digestives, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Spain; ‡Department of Pathology, School of Clinical and Laboratory Sciences, University of Newcastle-upon-Tyne, Royal Victoria Infirmary, United Kingdom; §Department of Pathology, Hospital Mutua of Terrassa, Barcelona, Spain; ¶Histopathology Unit, Cancer Research UK, London Research Institute, United Kingdom; and ||Novo Nordisk A/S, Bagsvaerd, Denmark

Abstract: There is evidence for a beneficial effect of trefoil peptides in animal models of gastric damage and intestinal inflammation, but the optimal treatment strategy and the mechanistic basis have not been explored thoroughly. It has been suggested that these proteins may modulate the inflammatory response. The aims of this study were to compare the protective and curative value of systemic and topical trefoil factor family (TFF)2 administration in dextran sulfate sodium-induced experimental colitis and to investigate the relationship between the therapeutic effects of TFF2 and modulation of leukocyte recruitment and expression of cell adhesion molecules. Clinical and morphologic severity of colitis was evaluated at the end of the study (Day 10). Leukocyte–endothelial cell interactions were determined in colonic venules by fluorescence intravital microscopy. The expression of cell adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) was measured by the dual radiolabeled monoclonal antibody technique. Pretreatment with TFF2 by subcutaneous or intracolonic (ic) route ameliorated the clinical course of colitis, and the luminal route had a significantly superior effect. This beneficial effect was correlated with significant reductions in endothelial VCAM-1 but not MAdCAM-1 expression and leukocyte adhesion to intestinal venules, which returned to levels similar to those of controls. In established colitis, ic TFF2 treatment did not modify the severity of colonic lesions. In conclusion, TFF2 is useful in the treatment of colitis, and topical administration is superior to the systemic route. Reduction in adhesion molecule expression and leukocyte recruitment into the inflamed intestine contributes to the beneficial effect of this treatment. *J. Leukoc. Biol.* 75: 214–223; 2004.

Key Words: *trefoil peptides · TFF2 · intravital microscopy · cell adhesion molecule · MAdCAM-1 · VCAM-1*

INTRODUCTION

Trefoil peptides are members of the trefoil factor family (TFF), and all contain a conserved trefoil domain that folds to form a unique three-loop structural motif. They are synthesized and secreted by mucin-secreting epithelial cells that line the gastrointestinal tract and have a close association with mucins. Three mammalian trefoil proteins have been identified: TFF1 and TFF2 are expressed primarily by the stomach, and TFF3, by the small intestine and colon. Their abundant expression in a site-specific pattern in the normal physiological state and ectopic expression in various ulcerative conditions suggests an important role in mucosal defense and repair. The underlying molecular mechanisms of trefoil peptide action are unknown, but their physical properties and ability to act cooperatively with mucin glycoproteins [1] and as motogens, which stimulate the migration of epithelial cells [2, 3], suggest that they may prove useful as therapeutic agents in ulcerative conditions, including inflammatory bowel disease (IBD), for which present treatment is far from ideal.

The potential reparative properties of these peptides have been demonstrated in recent studies using transgenic animals in which expression of individual trefoil protein genes has been ablated or augmented [4–6]. Studies using TFF3 knockout mice revealed a normal phenotype but increased sensitivity to damaging agents such as dextran sulfate sodium (DSS) [6]. In addition, intracolonic (ic) administration of TFF2 has been shown to enhance mucosal repair in a model of dinitrobenzenesulphonic acid (DNBS)-induced colitis in rats [7]. However, the potential, preventive effect and the relative therapeutic value of trefoil proteins by diverse routes of administration (topical vs. systemic) have not been assessed directly in experimental colitis.

[1] Correspondence: Gastroenterology Department, Hospital Clínic, IDIBAPS, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain. E-mail: panes@medicina.ub.es

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Central to the pathology of IBD is the role of molecules that regulate the recruitment of leukocytes such as cell adhesion molecules (CAMs) [8]. Leukocyte and endothelial CAMs participate in transmigration of leukocytes from the vascular compartment to sites of inflammation or immune reaction. This process results from a complex series of events involving rolling, activation, firm adhesion, and subsequent migration of leukocytes across the vascular endothelium. Vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) are endothelial CAMs of the immunoglobulin (Ig) superfamily, with a critical role in mediating the firm adhesion of leukocytes to endothelial cells in IBD [9].

Trefoil peptides are thought to have a major role in the maintenance of epithelial integrity, but potential interactions between these proteins and nonepithelial glycoprotein-secreting tissues or cells have not been investigated fully. CAMs expressed by endothelial cells, such as VCAM-1 and MAdCAM-1, and leukocyte integrins, are glycoproteins that may be classed as mucins on the basis of their O-glycosylation pattern and proline-, serine-, and threonine-rich domains [10]. TFF3 has been shown to reduce the expression of several families of adhesion molecules such as E-cadherin, α- and β-catenin, and associated proteins in colorectal carcinoma cell lines to modulate epithelial cell adhesion and to facilitate cell migration [11]. It is possible that trefoil proteins may also influence migration of inflammatory cells through modulation of adhesion molecule function or expression in endothelial cells. A recent study, showing that TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response induced by the bacterial lipopolysaccharide in which monocyte migration is stimulated, supports this hypothesis [12].

In the present investigation, we have used a well-established model of experimental colitis induced by oral administration of DSS in mice. This model is characterized by morphological changes that mimic human ulcerative colitis [13]. The overall aims of the present work were to study the preventive and therapeutic efficacy of TFF2 in experimental colitis, to compare the efficacy of different routes of administration of TFF2, and to examine the possible effects of trefoil peptide administration on the expression of endothelial CAMs and leukocyte recruitment in the context of the intestinal inflammatory response.

MATERIALS AND METHODS

Induction of colitis

Male CD1 mice weighing 28–30 g were obtained from Iffa Credo (Lyon, France) and were maintained in conventional housing conditions. Experimental colitis was induced by giving 5% (w/v) DSS (molecular weight, 40 kDa; ICN Biomedicals, Aurora, OH) in drinking water ad libitum for 4 days. DSS administration was then stopped, and mice received drinking water alone for 6 days until study at Day 10. The volume of drinking water was monitored in control and colitic mice in all treatment groups. Given the variable sensitivity of different mouse strains to DSS-induced colitis [14], the dose of DSS used in our CD1 mice was established empirically to induce moderate-to-severe colitis while minimizing mortality. Control animals received drinking water without DSS. The Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1985) as well as the document "Regulation of Procedures for Animal Experiments" (ref. 5/1995) from the Generalitat de Catalunya were followed.

Study groups

Study 1. ic TFF2 pretreatment

Colitic mice were pretreated with ic administration of recombinant, glycosylated, human TFF2, a 14-kDa protein, at a dose of 2.0 mg/kg in 1% carboxymethylcellulose (MC; n=10), once daily for 10 days, starting 2 h before induction of colitis and until sacrifice at Day 10. Recombinant TFF2 was expressed in yeast (*Saccharomyces cerevisiae*) and purified, as described previously [15]. The control group consisted of mice (n=10) treated with vehicle (0.1 ml MC). For treatment administration, a flexible, plastic cannula (PE-10, Portex, Hythe, UK) with an outside diameter of 1 mm was inserted into the colon so that the tip was 5 cm proximal to the anus.

Study 2. Subcutaneous (sc) TFF2 pretreatment

To evaluate the effects of systemic administration of trefoil peptide, mice were treated once daily, starting 2 h before induction of colitis and during 10 days with sc recombinant, glycosylated TFF2 at doses 2.0 mg/kg in vehicle, 0.1 ml 1% MC (n=10). These doses were based on previous in vivo studies showing therapeutic effectiveness in models of gastric injury [16]. A colitic control group (n=10) was treated with sc vehicle.

Study 3. ic TFF2 treatment of established colitis

Twenty colitic mice were treated with ic recombinant, glycosylated TFF2 after removing toxic DSS, which is from Day 4 to Day 9 after induction of colitis. The daily dose of TFF2 was 2.0 mg/kg.

Assessment of inflammatory damage

Clinical parameters including body weight, stool consistency (score: 0, normal stools; 1, soft stools; 2, liquid stools), and rectal bleeding (score: 0, negative fecal occult blood test; 1, positive fecal occult blood test; 2, visible rectal bleeding) were assessed daily. For assessment of rectal bleeding, HemoFEC (Boehringer Mannheim, Barcelona, Spain) was used. The disease activity index (DAI) was determined at Day 10 and combines the aforementioned parameters as described by Murthy et al. [17]. Mice were killed by cervical dislocation at Day 10 after the induction of colitis. The colon was excised and opened by a longitudinal incision, rinsed with saline, and weighed, and its length was measured after exclusion of the cecum. To evaluate histological damage, colonic samples were fixed in 4% formalin and embedded in paraffin, and sections (5–7 μm) were stained with hematoxylin and eosin following standard procedures. A single pathologist, unaware of the treatment group, assessed sections. Colitis was graded quantitatively according to previously defined criteria, which take into account the lesion depth and the percentage of colon with mucosal injury over the total colon length [18]. This histological score grades the degree of colon inflammation, and higher values indicate more severe lesions.

In vivo assessment of leukocyte–endothelial cell interactions

Leukocyte–endothelial cell interactions in colonic submucosal and lamina propria venules were characterized using intravital microscopy in control and colitic animals and in mice pretreated or treated with ic TFF2. Studies were performed at Day 10 after induction of colitis (n=6 animals per group).

Fluorescence intravital microscopy

Mice were anesthetized with sc ketamine (150 mg/kg body weight; Ketolar, Parke-Davies, Morris Klein, NJ) and xylazine (7.5 mg/kg body weight; Sigma Chemical Co., St. Louis, MO), and a tail vein was cannulated. Throughout the experiments, rectal temperature was monitored using an electrothermometer and was maintained between 36.5°C and 37.5°C with an infrared heat lamp. The abdomen was opened via a midline incision, and a segment of the distal colon was chosen for microscopy examination, exteriorized, and covered with cotton gauze soaked with bicarbonate buffer. Mice were then placed on an adjustable microscope stage, and the colon was extended over a nonautofluorescent plastic coverslip that allowed observation of a 2-cm^2 segment of tissue. An inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) with a CF Fluor

40× objective lens (Nikon) was used. A charge-coupled device (CCD) camera (Model XC-77, Hamamatsu Photonics, Hamamatsu, Japan) with a C2400 CCD camera control unit and a C2400-68 intensifier head (Hamamatsu Photonics) mounted on the microscope projected the image onto a monitor (Trinitron KX-14CP1, Sony, Tokyo, Japan), and the images were recorded using a videocassette recorder (SR-S368E, JVC, Tokyo, Japan) for off-line analysis. A video date–time generator (Panasonic Digital AV Mixer WJ-AVE55, Matsushita Communication Industrial, Tokyo, Japan) displayed these parameters on recorded and live images. Leukocytes were in vivo-labeled by intravenous (iv) injection of rhodamine-6G (Molecular Probes, Leiden, The Netherlands) as described previously [19]. Rhodamine-6G-associated fluorescence was visualized by epi-illumination at 510–560 nm using a 590-nm emission filter. Single, unbranched submucosal and lamina propria venules with internal diameters (ID) of 25–40 μm were selected for observation. Venular ID was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station). The flux of rolling leukocytes, leukocyte rolling velocity, number of adherent leukocytes, venular blood flow (Vbf), and venular wall shear rate (γ) were determined off-line after playback of the videotapes. Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of free-flowing leukocytes in the same vessel. The flux of rolling leukocytes was measured as the number of rolling leukocytes that passed a fixed point within a small (10 μm) viewing area of the vessel in a 1-min period. Leukocyte rolling velocity was calculated as the mean of 10 rolling leukocyte velocities and expressed in μm/s. Leukocytes were considered adherent to venular endothelium when stationary for 30 s or longer and expressed as the number per 100 μm length of venule. Vbf was estimated from the mean of the velocity of three free-flowing leukocytes (ffv), using the empirical relationship of Vbf = ffv/1.6. γ was calculated, assuming cylindrical geometry, using the Newtonian definition $\gamma = 8$ (Vbf/ID). In each animal, three to six random venules were examined, and results were calculated as the mean of each parameter in all venules examined.

Effects of TFF2 on adhesion molecule expression in colitis

Thirty mice were used to characterize endothelial expression of intercellular adhesion molecule 2 (ICAM-2; see below), VCAM-1, and MAdCAM-1 in the colon under baseline conditions (n=15) and after induction of colitis (n=15) at Day 10. Additional groups of animals were studied to assess the effect of topical TFF2 pretreatment (n=10) or treatment (n=10) on endothelial expression of VCAM-1 and MAdCAM-1.

Dual radiolabeled monoclonal antibody (mAb) technique

Expression of CAMs was characterized using this technique [20]. ICAM-2, which is constitutively expressed on endothelial cells and is not increased in response to cellular activation [21], was measured to provide an estimation of endothelial surface area relative to tissue weight with which to correct CAM expression.

The mAb used to quantify endothelial expression of CAMs were 3C4, a rat $IgG_{2a\kappa}$ against mouse ICAM-2 [22]; MK1.91, a rat IgG_1 against mouse VCAM-1 [23]; MECA-367, a rat IgG_{2a} against mouse MAdCAM-1 [24]; and UPC-10, a nonbinding IgG [25]. MK1.91 was purified by protein G chromatography at Pharmacia Upjohn Laboratories (Kalamazoo, MI). 3C4 and MECA367 were purchased from PharMingen (San Diego, CA). UPC-10 was purchased from Sigma Chemical Co. and was dialyzed to remove sodium azide. The binding mAb directed against ICAM-2, VCAM-1, and MAdCAM-1 were labeled with ^{125}I, whereas the nonbinding mAb UPC-10 was labeled with ^{131}I (Amersham Ibérica, Madrid, Spain). Radioiodination of the mAb was performed by the iodogen method as described previously [26]. Labeled mAb were stored at 4°C and used within 3 weeks after the labeling procedure. The specific activity of labeled mAb was ~0.5 mCi/mg.

Animals were anesthetized with sc ketamine (150 mg/kg) and xylazine (7.5 mg/kg), and the left carotid artery and a tail vein were cannulated with PE-10 tubing. For assessment of endothelial expression of ICAM-2, VCAM-1, and MAdCAM-1, a mixture of 10 μg ^{125}I-3C4 and 60 μg unlabeled 3C4, 10 μg ^{125}I-MK1.91 and 20 μg unlabeled MK1.91, and 10 μg ^{125}I-MECA-367 without additional unlabeled MECA-367 was administered, respectively. In all cases, 10 μg ^{131}I-UPC-10 was added to the injection mixture. Doses of anti-ICAM-2, anti-VCAM-1, and anti-MAdCAM-1 mAb proved to be saturating in previous assays [27, 28]. The mixture of binding and nonbinding mAb was administered through the tail vein catheter. Blood samples were obtained through the carotid artery catheter 5 min after injection of the mAb mixture. Thereafter, animals were heparinized (1 mg/kg sodium heparin iv) and rapidly exsanguinated. Entire organs were then harvested and weighed. ^{125}I (binding mAb) and ^{131}I (nonbinding mAb) activities in each organ and 100 μl aliquots of cell-free plasma were counted in a Cobra II γ-counter (Packard, Canberra, Australia) with automatic correction for background activity and spillover. The injected activity in each experiment was calculated by counting a 3-μl sample of the 300-μl injection mixture containing the radiolabeled mAb. The accumulated activity of each mAb in an organ was expressed as nanograms of binding mAb per gram of tissue. The formula used to calculate ICAM-2, VCAM-1, or MAdCAM-1 expression was as follows: Endothelial expression = [(cpm ^{125}I organ$\times g^{-1}\times$cpm ^{125}I injected^{-1})–(cpm ^{131}I organ$\times g^{-1}\times$cpm ^{131}I injected^{-1})$\times$(cpm ^{125}I in plasma)/(cpm ^{131}I in plasma)] $\times$ ng injected mAb. This formula was modified from the original method [20] to correct the tissue accumulation of nonbinding mAb for the relative plasma levels of binding and nonbinding mAb [29].

Effects of TFF2 on endothelial cell adhesion molecule expression in vitro

To determine whether changes in adhesion molecule expression in response to TFF2 treatment were a direct effect of the peptide, primary cultures of human umbilical vein endothelial cells (HUVEC) under basal or stimulated conditions were exposed to different concentrations of TFF2. HUVEC were cultured in Endothelial Cell Basal Medium-2 (Bio-Whittaker, Verviers, Belgium) in 96-well plates. When endothelial monolayers were confluent, they were incubated for 20 h at 37°C and 5% CO_2 with different doses of TFF2 (10 nM, 100 nM, and 1000 nM) with or without tumor necrosis factor α (TNF-α; 10 ng/ml; Sigma Chemical Co.). This cytokine was chosen because of the central role it has in the pathogenesis of IBD. The concentration of TFF2 used was based on previous studies showing efficacy as proangiogenic factors [30]. Thereafter, cells were washed and incubated for 45 min at room temperature (RT) with a mouse anti-VCAM-1 antibody (Hae2d) or a mouse anti-E-selectin antibody (E83), as a positive control for endothelial activation. Dr. Pablo Engel (Liver Unit, Institut de Malalties Digestives, Hospital Clínic, Barcelona, Spain) provided antibodies. After several washes, cells were incubated with a 1/1000 dilution of rabbit anti-mouse peroxidase-conjugated antibody for 30 min at RT and added a developing solution of O-phenylenediamine dihydrochloride (Sigma Chemical Co.). After 30 min, absorbance was read at a wavelength of 450 nm. Results were expressed as optical density units. Given that MAdCAM-1 is not expressed by HUVEC, this CAM could not be studied.

Measurement of cytokine production

Levels of murine TNF-α and interleukin-6 (IL-6) were quantified in protein extracts from colon homogenates by using commercially available enzyme-linked immunosorbent assay (ELISA) kits supplied by R&D Systems (Abingdon, UK) and Diaclone (Besançon, France), respectively. Results were expressed in pg/mg protein. Moreover, plasma samples were taken after centrifugation at 1000 *g* during 10 min at 4°C of 1 ml blood, obtained from each mouse by cardiac puncture with a heparinized syringe for measurements of circulating TNF-α (R&D Systems) and IL-6 (Diaclone) by ELISA. Results were expressed in pg/ml serum. Assessment of cytokine production was performed in controls, colitic-untreated animals, and colitic animals pretreated with ic TFF2, which induced significant amelioration of colitis (n=7–8 per group).

Statistical analyses

Statistical analysis was performed by using the nonparametric tests Kruskal-Wallis and the Mann-Whitney tests, ANOVA with the Bonferroni (post-hoc) test, and Student's unpaired *t*-test when appropriate. All values are expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

Inflammatory changes in DSS-induced colitis

Administration of DSS induced a significant loss in body weight that was maximum at Days 7 and 8 after induction of

colitis. From 24 h after the induction of colitis, all animals had a positive fecal occult blood test and diminished stool consistency when compared with control animals. Clinical, macroscopic, and histological changes in control and colitic mice are summarized in **Table 1**. At Day 10 after induction of colitis, a significant reduction in colon length and an increase in colon weight, with a corresponding increase in the colon weight-to-length ratio, were observed in colitic animals relative to controls. Histological, colonic damage was present only in colitic animals.

Study 1. Preventive effect of ic TFF2

Pretreatment with TFF2 by the ic route ameliorated the severity of colitis. When compared with the group of colitic animals treated with vehicle, ic TFF2 significantly improved stool consistency score and diminished rectal bleeding, DAI score, colon weight, colon shortening, and colon weight-to-length ratio. There was also a trend to amelioration of histological lesions and a reduction in body weight loss, although these differences did not reach statistical significance (Table 1). Comparison of the animals in which colitis was induced after treatment with ic TFF2 with control noncolitic mice shows that some of the parameters are within the normal range. Notably, the body weight, stool consistency, and colon weight-to-length ratio are not significantly different in the two groups of mice.

Study 2. Preventive effect of sc TFF2

Pretreatment with sc-administered TFF2 also reduced the severity of colitis induced in mice by DSS. Compared with sc vehicle-treated mice, daily pretreatment with sc TFF2 significantly diminished rectal hemorrhage score (1.1±0.1 vs. 1.4±0.1; $P<0.05$) and colon weight (257±18 vs. 354±8 mg; $P<0.0001$) as well as the colon weight-to-length ratio (3.6±0 vs. 4.7±0 mg/mm; $P<0.0001$). By the end of the study (Day 10), sc administration of TFF2 had no significant effects on body weight loss (TFF2 –1.7±0.8, vehicle –1.4±0.7 g), stool consistency (TFF2 1.3±0.4, vehicle 1.1±0.1), DAI (TFF2 2±0.5, vehicle 1.9±0.1), colon length (TFF2 74±6, vehicle 72±2 mm), or the histological damage score (TFF2 71±17, vehicle 71±11).

Colitis was less severe in animals treated with ic TFF2 than in those treated by the sc route. Compared with the latter group, topical administration of TFF2 significantly decreased the DAI score and improved stool consistency at Day 10 (Table 1). Although there also was a trend for reduction of other parameters of disease severity examined in animals receiving TFF2 by ic route, no significant differences were observed between this group and animals pretreated sc in body weight loss, rectal bleeding, colon weight, colon length, ratio of colon weight to length, or histological damage score (Table 1).

Study 3. Curative effect of ic TFF2

Treatment with ic TFF2 from Day 4 to Day 9 after induction of colitis did not have any beneficial effect for any of the parameters analyzed. Comparison of TFF2-treated colitic mice with vehicle-treated colitic mice showed that body weight loss (–1.7±1.0 vs. –1.0±0.5 g), stool consistency (1.1±0.1 vs. 1.0±0.1), rectal bleeding (1.1±0.1 vs. 1.2±0.1), DAI (1.7±0.2 vs. 2.0±0.3), colon weight (339.4±14.8 vs. 359.5±9.5 mg), colon length (65.4±1.7 vs. 73.9±2.0 mm), colon weight-to-length ratio (5.2±0.2 vs. 4.9±0.1 mg/mm), and the histological damage score (97.2±24.6 vs. 57.2±10.9) was not significantly different in the two treatment groups.

Leukocyte–endothelial cell interactions in colonic venules

When compared with control animals, colitic mice showed a pronounced increase in leukocyte–endothelial cell interactions. There was a 2.5-fold increase in the flux of rolling leukocytes at Day 10 after induction of colitis (**Figs. 1A** and **2, A** and **B**). Few adherent leukocytes were present in venules of control mice, and a 19-fold increase in leukocyte adhesion was observed in colitic animals (Figs. 1B and 2, A and B). No differences in leukocyte rolling velocity, venular blood flow, or venular wall shear rate were observed between control and colitic mice (not shown).

Pretreatment of colitic mice with ic TFF2 did not reduce the flux of rolling leukocytes below the level observed in vehicle-treated, colitic mice ($P=0.30$; Figs. 1A and 2C) but decreased significantly the number of adherent leukocytes to levels close

TABLE 1. Clinical, Macroscopic, and Histological Changes in Noncolitic Control Animals and Colitic Mice Pretreated with Vehicle, ic TFF2, or sc TFF2

	Control (n = 10)	Vehicle (n = 10)	ic TFF2 (n = 10)	sc TFF2 (n = 10)
Δ Body weight (g)	0.2 ± 0.2	−1.6 ± 0.4*	−1.2 ± 0.8	−1.7 ± 0.8
Stool consistency	0 ± 0	1.1 ± 0.1*	0.4 ± 0.2†,‡	1.3 ± 0.4*
Rectal bleeding	0 ± 0	1.3 ± 0.1*	0.9 ± 0.1*,†	1.1 ± 0.1*,†
DAI	0 ± 0	1.8 ± 0.1*	1.2 ± 0.3*,†,‡	2 ± 0.5*
Colon weight (mg)	304 ± 9	359 ± 9*	267 ± 11*,†	257 ± 18*,†
Colon length (mm)	96 ± 1	74 ± 2*	82 ± 5*,†	74 ± 6*
Colon weight/length (mg/mm)	3.1 ± 0	4.9 ± 0*	3.3 ± 0†	3.6 ± 0†
Microscopic damage score	0 ± 0	57 ± 11*	52 ± 18*	71 ± 17*

There are four groups of control colitic mice in this study: One group was treated with DSS alone, a second group was pretreated with ic MC prior to induction of colitis with DSS, a third group was treated with sc MC prior to induction of colitis, and the fourth group with ic MC after induction of colitis. The values for the different parameters were similar in all four groups, and for simplicity, only the values obtained in the second group are shown in the table, but the appropriate values are listed in the text. * $P < 0.05$ versus noncolitic control mice. † $P < 0.05$ versus vehicle-treated colitic mice. ‡ $P < 0.05$ versus sc TFF2-pretreated colitic mice.

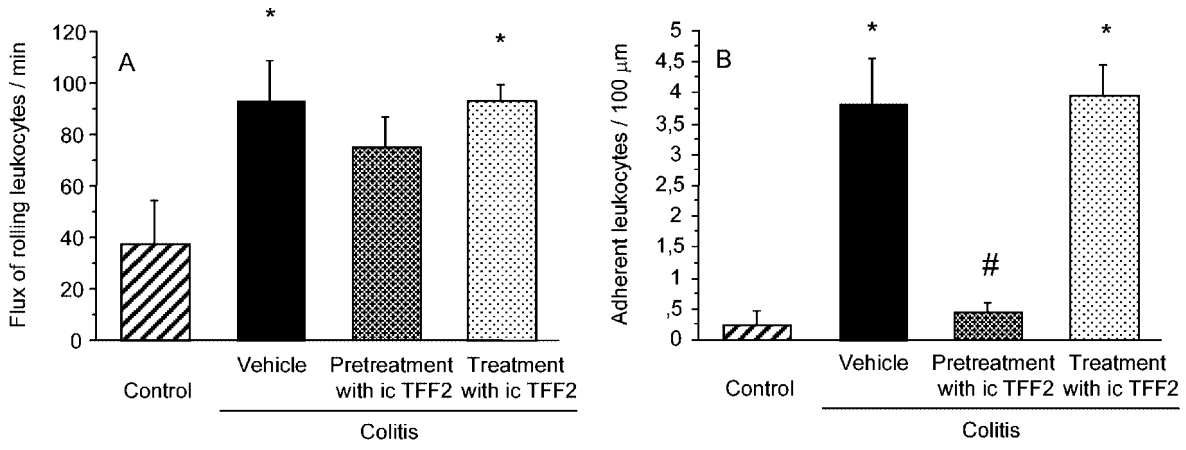

Fig. 1. Flux of rolling leukocytes (A) and leukocyte adhesion in colonic venules (B). A significant increment in leukocyte–endothelial cell interactions was observed at Day 10 after induction of colitis. Pretreatment of colitic mice with ic TFF2 abrogated leukocyte adhesion but had no effect in the flux of rolling leukocytes. Treatment with this peptide did not have any effect when colitis was already established. *, $P < 0.05$, versus noncolitic control mice; #, $P < 0.001$, versus ic vehicle-treated colitic mice.

to those of control animals ($P=0.0001$; Figs. 1B and 2C). In contrast, TFF2 treatment of animals with established colitis did not modify significantly leukocyte rolling or adhesion in intestinal venules (Figs. 1, A and B, and 2D).

Effects of TFF2 on adhesion molecule expression in colitis

The endothelial surface area for the colon per gram of tissue was estimated from endothelial binding of the anti-ICAM-2 mAb 3C4. Values were 350.0 ± 92.0 ng mAb/g tissue in colitic animals and 277.2 ± 27.8 ng mAb/g tissue in control animals; this difference was not significant ($P=0.47$). Binding of the anti-ICAM-2 mAb was also similar in control and colitic mice in other organs including liver, pancreas, mesentery, stomach, small intestine, and cecum (data not shown). Values for endothelial anti-VCAM-1 and anti-MAdCAM-1 binding were not, therefore, corrected for changes in endothelial surface area relative to organ weight, which might result from edema or capillary recruitment.

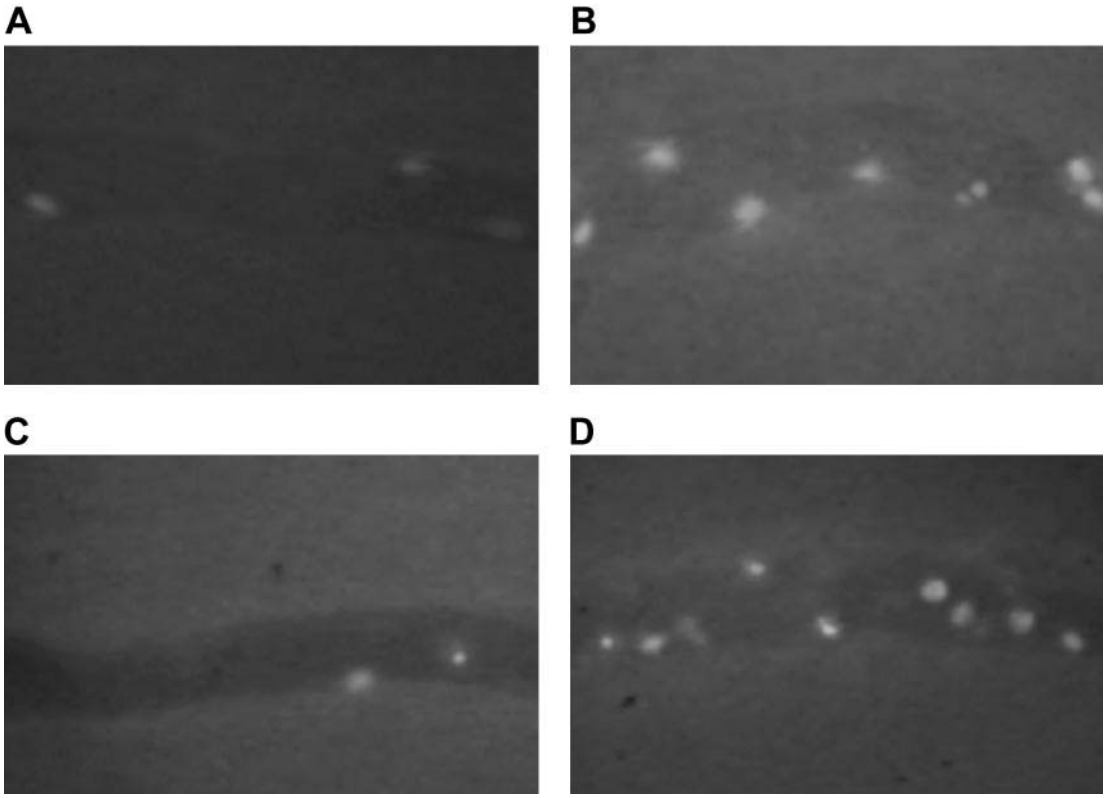

Fig. 2. Photographs of intravital microscopy. Flux of rolling leukocytes and cellular adhesion in noncolitic animals (A), vehicle-treated colitic mice (B), ic TFF2-pretreated colitic mice (C), and ic TFF2-treated colitic animals (D). Pretreatment of colitic mice with ic TFF2 decreased significantly the number of adherent leukocytes to levels close to those of control animals.

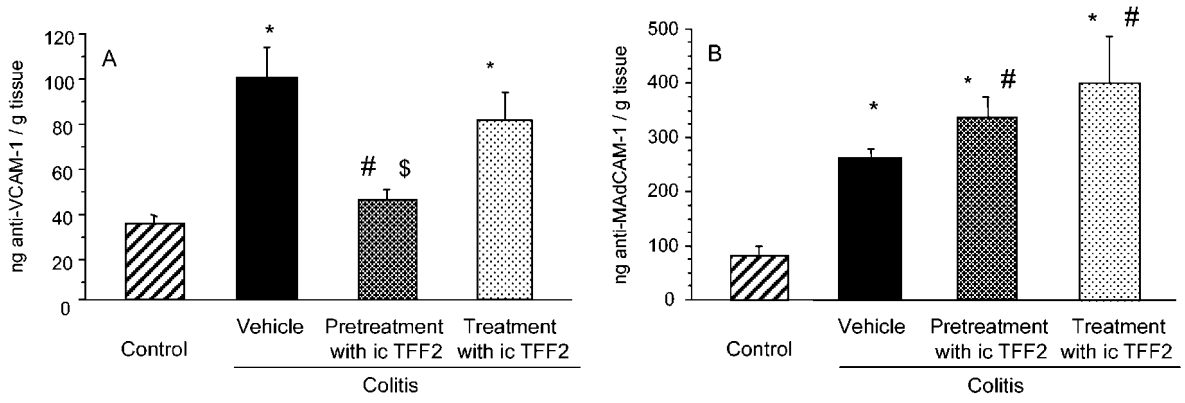

Fig. 3. Endothelial expression of VCAM-1 (A) and MAdCAM-1 (B) in colonic vessels of colitic mice pretreated or treated with ic vehicle or ic TFF2. When compared with control animals, colitic mice showed a pronounced increase in leukocyte–endothelial cell interactions. Pretreatment with TFF2 significantly decreased VCAM-1 expression and increased MAdCAM-1 as compared with vehicle-treated colitic mice. Treatment with TFF2 from Day 4 to Day 9 after induction of colitis did not modify endothelial VCAM-1 expression, whereas MAdCAM-1 expression was augmented as compared with vehicle-treated colitis mice. *, $P < 0.05$, versus noncolitic control mice; #, $P < 0.05$, versus vehicle-treated colitic mice; $, $P < 0.05$, versus ic TFF2-treated colitic mice.

Endothelial VCAM-1 and MAdCAM-1 expression in the colons of colitic mice increased significantly (threefold; $P=0.01$) compared with noncolitic mice (**Fig. 3**). VCAM-1 expression was also increased significantly in the mesentery, ileum, and cecum, and MAdCAM-1 expression was higher in the mesentery, stomach, and cecum (**Table 2**). No accumulation of the anti-MAdCAM-1 antibody was observed in liver, spleen, or other extra-abdominal organs such as heart or lung, which is consistent with the restricted expression of this adhesion molecule in gastrointestinal organs (data not shown). The increase in endothelial expression of VCAM-1 and MAdCAM-1 in colonic vessels correlated significantly with the severity of DSS-induced colitis measured using DAI score and pathological alterations such as the colon weight-to-length ratio (**Fig. 4**).

In TFF2-pretreated animals, the increase in colonic endothelial VCAM-1 expression was significantly attenuated as compared with vehicle-pretreated colitic mice ($P=0.001$; Fig. 3). Similar reductions were observed in the mesentery, jejunum, and ileum (Table 2). In contrast, TFF2 induced a small but significant increase in MAdCAM-1 expression in the colon (Fig. 3). TFF2 pretreatment did not affect MAdCAM-1 expression in other gastrointestinal organs (Table 2).

Treatment of established colitis with TFF2, which did not cure intestinal inflammation, did not reduce significantly endothelial VCAM-1 expression in the colon (Fig. 3) or in other gastrointestinal organs (Table 2). An increase in MAdCAM-1 expression in response to TFF2 administration was again observed in the colon (Fig. 3), jejunum, and ileum (Table 2) as compared with vehicle-treated colitic animals.

Effects of TFF2 on endothelial cell adhesion molecule expression in vitro

Resting HUVEC expressed low levels of E-selectin and VCAM-1 (**Fig. 5**). Following activation with TNF-α, there was a dramatic increase in the cell-surface expression of these adhesion molecules. Coincubation with TFF2 at concentrations from 10 to 1000 nM did not modify TNF-induced VCAM-1 expression (Fig. 5A). As for E-selectin expression, a minor, although significant, reduction of TNF-α-induced expression was observed when cells were incubated with 10 nM TFF2 (Fig. 5B).

Cytokine production

Levels of TNF-α and IL-6 quantified in protein extracts of colon homogenates were increased in colitic animals with respect to control mice, fivefold and 18-fold, respectively (**Fig. 6**). Treatment with ic TFF2 significantly reduced the levels of both cytokines in colonic tissue (Fig. 6).

TABLE 2. Expression of Endothelial VCAM-1 and MAdCAM-1 in Different Vascular Beds in Noncolitic Control Mice and in Vehicle-Treated, ic TFF2-Pretreated, and ic TFF2-Treated Colitic Mice

	VCAM-1				MAdCAM-1			
	Control ($n = 5$)	Vehicle ($n = 5$)	Pretreatment TFF2 ($n = 5$)	Treatment TFF2 ($n = 5$)	Control ($n = 5$)	Vehicle ($n = 5$)	Pretreatment TFF2 ($n = 5$)	Treatment TFF2 ($n = 5$)
Mesentery	82 ± 3	168 ± 32*	80 ± 5†	119 ± 15	58 ± 17	158 ± 30*	205 ± 32*	232 ± 48*
Stomach	65 ± 8	78 ± 7	72 ± 6	66 ± 7	14 ± 10	40 ± 5*	57 ± 8*	50 ± 9*
Jejunum	91 ± 22	110 ± 12	39 ± 1†	115 ± 24‡	269 ± 33	253 ± 8	240 ± 33	403 ± 61*,†,‡
Ileum	42 ± 4	60 ± 5*	30 ± 2†	47 ± 8	302 ± 50	326 ± 16	329 ± 37	484 ± 51*,†,‡
Cecum	31 ± 2	59 ± 7*	45 ± 8	43 ± 5	85 ± 25	220 ± 36*	332 ± 52*	244 ± 55*

* $P < 0.05$ versus noncolitic control mice. † $P < 0.05$ versus vehicle-treated colitic mice. ‡ $P < 0.05$ versus ic TFF2-pretreated colitic mice.

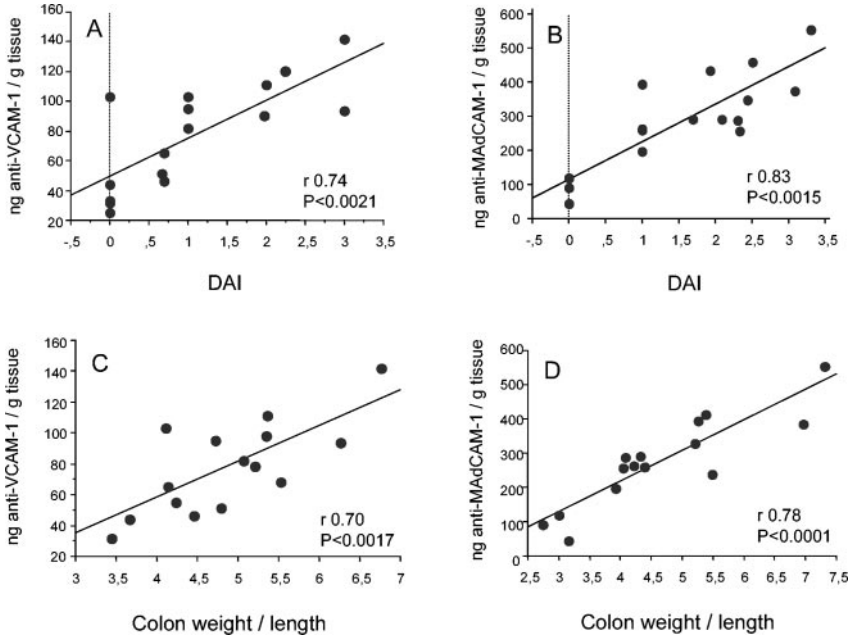

Fig. 4. Endothelial expression of VCAM-1 (A, C) and MAdCAM-1 (B, D) in colonic vessels of colitic mice and correlation with DAI and ratio of colon weight to length. A higher endothelial expression of CAMs was correlated with a higher severity of colitis in terms of DAI and colon weight-to-length ratio increased.

Plasma IL-6 increased significantly in vehicle-treated colitic mice (3.96±2.52 pg/ml) in comparison with control mice (0.00±0.00; $P<0.05$). ic TFF2 significantly decreased plasma levels of IL-6 in colitic mice (0.08±0.07; $P<0.05$) relative to vehicle-treated colitic mice. Plasma TNF-α levels remained under the detection limits in control and colitic mice.

DISCUSSION

The results presented in the current study demonstrate a protective effect for TFF2 in DSS-induced colitis. This protective effect appears to be greater when the peptide is administered topically as compared with systemically. In contrast, ic administration of TFF2 had no significant curative effect on established colitis. The protective effect of TFF2 against colitis was accompanied by a dramatic reduction in leukocyte adhesion. We also found that the protective effect was paralleled by a reduction in VCAM-1 expression in colonic endothelium, which is known to be a key factor in the pathogenesis of this model of colitis.

There have been only two previous therapeutic studies using trefoil peptides in experimental colitis. Mashimo et al. [6] demonstrated that mice lacking TFF3 had impaired mucosal healing and died from extensive colitis after oral administration of DSS. The failure in healing was shown to be a result of the absence of TFF3. After rectal application of acetic acid, TFF3-deficient mice treated by luminal instillation of rat TFF3 showed normal healing with enhanced epithelial migration and marked attenuation of gross injury [6]. A recent study showed

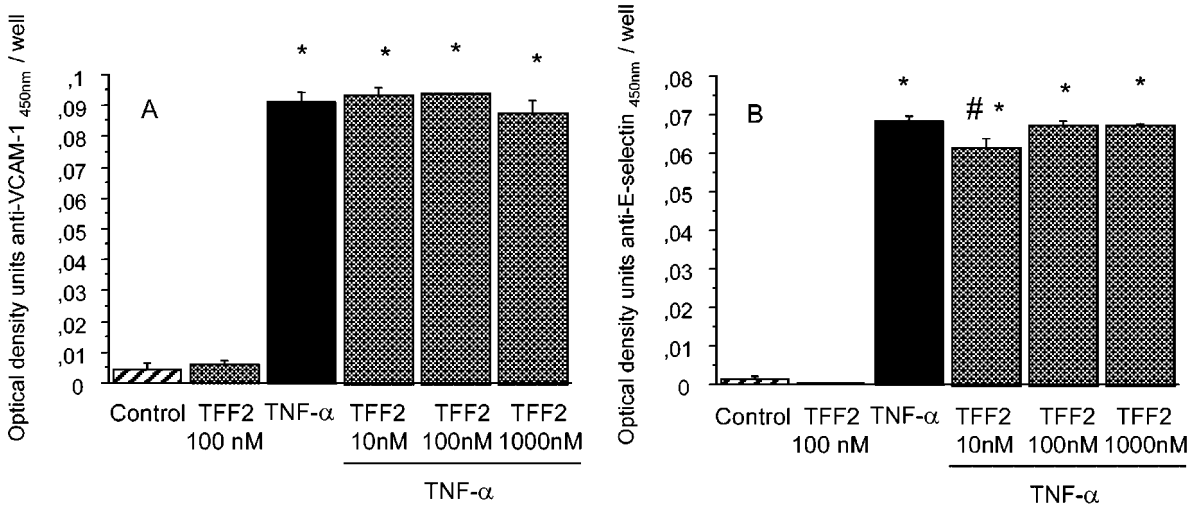

Fig. 5. Effect of TFF2 treatment on VCAM-1 expression (A) and E-selectin expression (B) in HUVEC. Cultures were stimulated with 10 ng/ml TNF-α for 20 h in the presence or absence of TFF2 at different concentrations (10 nM, 100 nM, and 1000 nM). Data are expressed as the mean of triplicate wells ± SD. There was a significant increase in the density of cell adhesion molecule expression in activated HUVEC. Treatment with TFF2 did not significantly modify VCAM-1 expression. TFF2, 10 nM, slightly diminished E-selectin expression with respect to the TNF-α group. *, $P < 0.05$, versus control; #, $P < 0.05$, versus TNF-α.

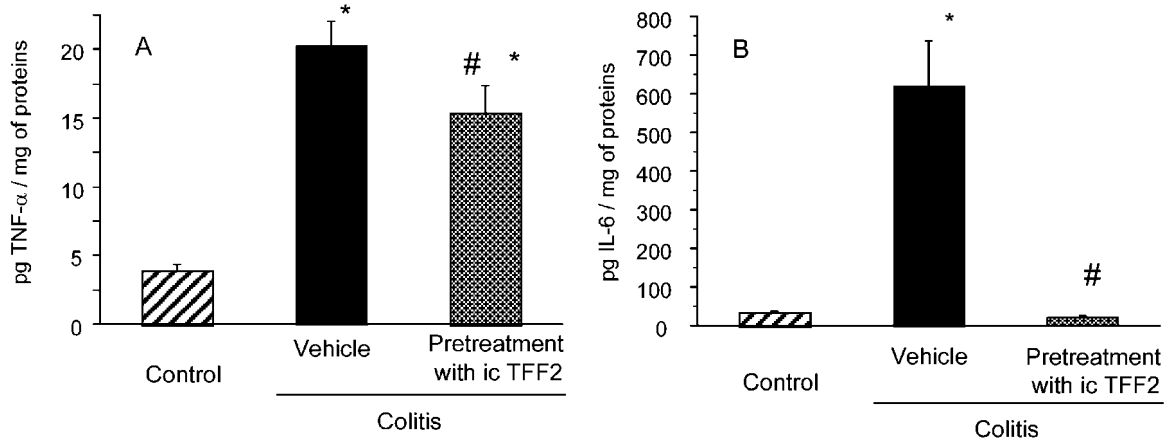

Figure 6. Levels of TNF-α (A) and IL-6 (B) in colon homogenates of control animals, vehicle-treated colitic mice, and TFF2-pretreated colitic mice. Levels of TNF-α and IL-6 were increased in colitic animals with respect to noncolitic control mice. Pretreatment with TFF2 reduced the levels of cytokines significantly. *, $P < 0.05$, versus noncolitic control mice; #, $P < 0.05$, versus vehicle-treated colitic mice.

that ic TFF2 enhances the rate of colonic epithelial repair in the DNBS/ethanol-induced colitis model in the rat [7], which resembles human Crohn's disease. There are no published data on the potential therapeutic value of TFF2 in DSS-induced colitis, which resembles human ulcerative colitis, or on the relative efficacy of different routes of administration of TFF2. Further, the possibility that trefoil peptides might act by modulation of inflammatory cell migration pathways has not been investigated previously.

In the current study, we demonstrate that ic TFF2 ameliorates DSS-induced colitis when administered in a preventative mode. The clinical course and colonic, pathological changes such as colon weight-to-length ratio were reduced in response to TFF2 treatment. In contrast to the study by Tran et al. [7], we did not observe any beneficial effect of TFF2 when this treatment was administered after induction of colitis. Although the doses of TFF2 used in both studies are very similar, a number of factors may explain this apparent discrepancy, including the different experimental models of colitis (DNBS/ethanol vs. DSS) and different animal species studied (rat vs. mouse).

Studies comparing the efficacy of different routes of administration of TFF2 in the prevention of gastric mucosal damage induced by ethanol or indomethacin have produced conflicting results. Some authors found greater protection with topical administration [31], whereas others found greater protection with systemic administration [3], and others reported that both routes of administration were equally effective [32, 33]. A possible endocrine-mediated effect, which may imply the presence of a receptor for the peptides, has been proposed. A putative receptor for TFF3 has been reported [34], and there is evidence that parenterally administered porcine TFF2 is taken up by mucus-secreting cells in the stomach and secreted into the mucus layer in the same way as endogenous TFF2 [35]. To determine whether the protective effects of the TFF2 are dependent on the route of administration, we compared systemic (sc) and luminal TFF2 administration. The results show that both routes of administration can afford amelioration of various parameters of colitis severity, but the luminal route was superior for reduction of the DAI, a very important clinical index. This difference may be related to the ability of trefoil peptides to bind mucins and hence, to improve organization of the mucus layer that protects the apical side of the mucosa from deleterious luminal agents.

We used fluorescence intravital microscopy to characterize changes in leukocyte–endothelial cell interactions in colonic submucosal and lamina propria venules. We have shown that pretreatment with ic TFF2, which improves clinical and pathological scores in DSS-induced colitis, decreased leukocyte adhesion to levels observed in control animals. Administration of TFF2 after onset of colitis, which was clinically ineffective, did not modify leukocyte adhesion in intestinal venules.

To explore whether the reduced adhesion of leukocytes to venular endothelium in the inflamed intestine was related to differences of the known determinants of leukocyte recruitment in this pathological condition, we measured VCAM-1 and MAdCAM-1 expression using the dual radiolabeled mAb technique. We corroborated previous findings that expression of VCAM-1 and MAdCAM-1 is increased significantly in colitic animals; studies in the trinitrobenzene sulfonic acid [29] and DSS models of colitis [9] have shown a marked increase of VCAM-1 in the colonic endothelium. For MAdCAM-1, previous studies have shown that IL-10-deficient mice with active colitis have much higher levels of MAdCAM-1 in the colon [36], and tissue sections from patients with active ulcerative colitis or Crohn's disease have increased endothelial MAdCAM-1 expression as compared with those from normal subjects [37]. Although DSS-induced colitis affects predominantly the distal colon [13], we also found increased VCAM-1 expression in the ileum and cecum and increased endothelial MAdCAM-1 in the stomach and cecum. Higher levels of CAMs in these vascular beds probably result from DSS-induced mucosal lesions in these upper segments of the gut.

It is interesting that we have found a strong positive correlation between endothelial expression of VCAM-1 and MAdCAM-1 in colonic vessels and severity of DSS-induced colitis, evaluated by DAI and the colon weight-to-length ratio. Measurement of the expression of these CAMs may prove,

therefore, a useful parameter with which to evaluate the severity of colitis and the effects of therapeutic interventions in experimental models. It could also provide a rational method for monitoring the severity of IBD in patients.

VCAM-1-mediated leukocyte recruitment seems to be a key factor in the pathogenesis of DSS-induced colitis, as immunoneutralization of VCAM-1 significantly ameliorates the severity of the experimental colitis [9]. TFF2 pretreatment decreased endothelial VCAM-1 expression in the colon in association with an amelioration of colitis and a decrease in leukocyte adhesion. This is consistent with the notion that pretreatment with TFF2 significantly improves colonic inflammation. To our surprise, MAdCAM-1 expression was increased in the colon, jejunum, and ileum in colitic animals receiving TFF2. The explanation for the paradoxical increase in MAdCAM-1 expression after pretreatment or treatment with TFF2 is not readily apparent. It is possible that TFF2 may stabilize MAdCAM-1 molecules on the surface of endothelial cells through interactions with their mucin-like regions.

Data derived from our in vitro experiments assessing the effects of TFF2 on TNF-α-stimulated endothelial cells indicate that the observed reduction of VCAM-1 expression in colitic animals in response to ic TFF2 was probably an indirect effect resulting from modulation of other factors involved in the inflammatory cascade, as incubation of HUVEC in the presence or absence of different concentrations of TFF2 did not modify TNF-induced VCAM-1 up-regulation. Among the other factors that may be affected by treatment with TFF2, activation of the immune cells and the resulting cytokine production are probably relevant, as concentrations of TNF-α and IL-6 in colonic tissue as well as circulating levels of IL-6 were significantly reduced in response to TFF2 treatment. This may be a consequence of "improved" barrier function of the intestinal epithelium afforded by TFF2, as has been shown previously [1].

In conclusion, our work demonstrates clearly an effect of TFF2 in establishing protection against colonic inflammatory damage, especially after topical application. However, in the model of colitis used in the current study, TFF2 did not show efficacy in the treatment of established colitis. These observations suggest that luminal application of TFF2 might be useful for prevention of disease flares in ulcerative colitis. Further studies assessing the value of these peptides in milder forms of colitis as the sole treatment or as coadjuvant therapy are warranted.

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Artículo 3

Soriano-Izquierdo A, Gironella M, Massaguer A, Salas A, Gil F, Piqué JM, Panés J. Effect of cyclosporin A on cell adhesion molecules and leukocyte-endothelial cell interactions in experimental colitis. **Inflammatory Bowel Diseases** 2004; 10: 789-800.

Effect of Cyclosporin A on Cell Adhesion Molecules and Leukocyte-Endothelial Cell Interactions in Experimental Colitis

Antonio Soriano-Izquierdo, MD, Meritxell Gironella, PhD,* Anna Massaguer, PhD,† Antonio Salas, MD,‡ Félix Gil, BA,* Josep M. Piqué, MD,* and Julián Panés, MD**

Background: Cyclosporin A (CsA) is an immunosuppressive agent that is believed to act primarily through effects on T-helper lymphocyte function and proliferation. The aim of this study was to investigate whether modulation of leukocyte recruitment and expression of cell adhesion molecules contribute to the therapeutic efficacy of CsA in a model of experimental colitis.

Methods: The therapeutic effects of CsA were assessed in mice with dextran sulfate sodium–induced colitis. Leukocyte-endothelial cell interactions were determined in colonic venules by intravital microscopy. The expression of cell adhesion molecules intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) was measured by the radiolabeled antibody technique.

Results: Treatment with CsA (4 mg/kg/day) significantly improved the clinical course of colitis, decreasing weight loss, diarrhea, rectal bleeding, disease activity index, colon weight, and colonic shortening. Microscopic damage score, myeloperoxidase activity, tumor necrosis factor alpha (TNF-α), and interleukin-6 in colonic tissue were significantly diminished by CsA. CsA also significantly reduced ICAM-1 and VCAM-1, but not MAdCAM-1, expression in colitic mice. TNF-α–induced ICAM-1 and VCAM-1 expression in primary cultures of human umbilical vein endothelial cells was reduced by co-incubation with CsA. The reduction in adhesion molecule expression was followed by a marked decrease in leukocyte adhesion in colonic venules of colitic mice.

Conclusions: CsA ameliorates experimental colitis in mice. Reduced adhesion molecule expression resulting from diminished proinflammatory cytokine production and from a direct effect of CsA in endothelial cells decreases leukocyte recruitment into the inflamed intestine, contributing to this protective effect.

Key Words: cell adhesion molecule, cyclosporin A, endothelium, inflammation, inflammatory bowel disease, intercellular adhesion molecule-1, intravital microscopy, mucosal addressin cell adhesion molecule 1, vascular cell adhesion molecule 1

(*Inflamm Bowel Dis* 2004;10:789–800)

Ulcerative colitis is a chronic inflammatory disease of the colonic mucosa. Approximately 6% to 15% of patients have a severe attack requiring hospital admission at some time during their illness.[1,2] Cyclosporin A (CsA) is the first drug (and the only one as yet) to demonstrate efficacy in the treatment of severe colitis unresponsive to corticosteroids, avoiding urgent colectomy, at least short-term.[3] CsA has a more rapid onset of action compared with azathioprine and 6-mercaptopurine, making this drug more suitable for severe or refractory inflammatory bowel disease (IBD). This agent alters the immunoinflammatory cascade by acting as a potent inhibitor of T-cell–mediated responses. Although it acts primarily via inhibition of interleukin (IL)-2 production from T helper cells, it also decreases the number of cytotoxic T cells in the inflamed intestine and blocks the production of other cytokines, including IL-4, γ-interferon, and tumor necrosis factor (TNF)-α.

Central to the pathology of IBD is the role of molecules that regulate the recruitment of leukocytes such as cell adhesion molecules (CAMs).[5,6] Both leukocyte and endothelial CAMs participate in transmigration of leukocytes from the vascular compartment to sites of inflammation or immune reaction. This process results from a complex series of events involving rolling, activation, firm adhesion, and subsequent migration of leukocytes across the vascular endothelium. Intercellular cell adhesion molecule 1 (ICAM-1), mucosal addressin cell adhesion molecule 1 (MAdCAM-1), and vascular cell adhesion molecule 1 (VCAM-1) are endothelial CAMs of the immunoglobulin superfamily with a crucial role in mediating the firm adhesion of leukocytes to endothelial cells in IBD.[7–9]

Recent advances in the study of the molecular basis of inflammation suggest that cell–cell interactions mediated by

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From the *Department of Gastroenterology and †Liver Unit, Institut de Malalties Digestives, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain; and ‡Department of Pathology, Hospital Mutua of Terrassa, Barcelona, Spain.

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Reprints: Julián Panés, MD, Gastroenterology Department, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain (e-mail: panes@medicina.ub.es).

specific CAMs could be new targets for immunosuppressive agents. The potential role of CsA in the regulation of CAMs expression in IBD remains to be clarified. In vitro studies indicate that CsA may directly affect adhesion molecule expression in stimulated endothelial cells, but this point has never been tested in intact animal models of inflammatory bowel disease.[10] The hypothesis that CsA treatment may affect cellular adherence by down-regulating CAMs expression formed the rationale for the current study.

In the current investigation, we have used a well-established model of experimental colitis induced by oral administration of dextran sulfate sodium (DSS) in mice. This model is characterized by morphologic changes that mimic human ulcerative colitis.[11] The overall aims of the current work were (1) to study the efficacy of CsA in the treatment of a model of experimental colitis resembling human ulcerative colitis, (2) to examine the possible effects of CsA administration on the expression of endothelial CAMs and leukocyte recruitment in the context of intestinal inflammation, evaluating whether a possible beneficial effect of CsA in experimental colitis is associated with inhibition of adhesion molecules expression and cytokines production, and (3) to determine whether changes in CAMs expression following treatment with CsA are a primary effect of this drug.

MATERIALS AND METHODS

Evaluation of the Therapeutic Efficacy of CsA in DSS-Induced Colitis

Male CD1 mice weighing 28 to 30 g were obtained from Iffa Credo (Lyon, France) and maintained in conventional housing conditions. Experimental colitis was induced by giving 5% (wt/vol) DSS (MW 40 kDa; ICN Biomedicals, Aurora, OH) in drinking water ad libitum for 4 days. DSS administration was then stopped, and mice received drinking water alone for 6 additional days until study at Day 10. The volume of drinking water was monitored in control and colitic mice in all treatment groups. Given the variable sensitivity of different mouse strains to DSS-induced colitis,[12] the dose of DSS used in our CD1 mice was established empirically to induce moderate to severe colitis while minimizing mortality. Control animals received drinking water without DSS. The document "Regulation of Procedures for Animal Experiments" (ref. 5/1995) from the Generalitat de Catalunya and the 1996 revision of "Guiding Principles in the Care and Use of Animals" (American Physiological Association, Bethesda, MD) were followed.

To evaluate the effects of CsA (Sandimmun Neoral solution; Novartis Farmacéutica SA, Barcelona, Spain) administration, different doses of the drug were tested (1, 4, and 8 mg/kg body weight/d, intraperitoneally [ip]; 10 animals per group). Best results were obtained with the dose of 4 mg/kg/d, which corresponds to the dose used in clinical practice in patients with severe ulcerative colitis. Colitic mice treated with 4 mg/kg/d of CsA showed a minimal body weight loss and the smallest colon weight to length ratio, one of the best parameters in the assessment of the severity of colitis (Fig. 1).

According to these data, for the rest of the study colitic mice were treated with ip CsA at the dose of 4 mg/kg/d, beginning 2 hours before induction of colitis and during 10 days (n = 12). A colitic control group treated with ip vehicle (n = 12), and a noncolitic group of animals receiving vehicle (n = 12) were also studied.

Clinical Assessment of Inflammatory Damage

Clinical parameters including body weight, stool consistency (score: 0, normal stools; 1, soft stools; and 2, liquid stools), and rectal bleeding (score: 0, negative fecal occult blood test; 1, positive fecal occult blood test; and 2, visible rectal bleeding) were assessed daily. For assessment of rectal bleeding, HemoFEC (Boehringer Mannheim, Barcelona, Spain) was used. The Disease Activity Index (DAI) was determined at day 10 and combines the aforementioned parameters as described by Murthy et al.[13] Mice were killed by cervical dislocation at day 10 after the induction of colitis. The colon was excised and opened by a longitudinal incision, rinsed with saline, weighed, and its length was measured after exclusion of the cecum.

Evaluation of Histologic Damage

To evaluate histologic damage, colonic samples were fixed in 4% formalin, embedded in paraffin, and sections (5–7 μm) were stained with hematoxylin and eosin following standard procedures. A single pathologist unaware of the treatment group assessed the preparations. Colitis was graded quantitatively according to previously defined criteria, which take into account the lesion depth and the percentage of colon with mucosal injury over the total colon length.[14] This histologic score grades the degree of colon inflammation, with higher values indicating more severe lesions.

Myeloperoxidase Assay

Distal colon samples (≃20 mg) were excised, snap-frozen in dry ice, and stored at –80°C for later assay of myeloperoxidase (MPO) activity as a measure of neutrophil infiltration.[15] Results are expressed as units per milligram of protein. Samples were homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0. Homogenates were then disrupted for 30 seconds using a Labsonic sonicator (B. Braun, Melsungen, Germany) and were subsequently snap-frozen in dry ice and thawed on 3 consecutive occasions before a final 30 seconds of sonication. Samples were incubated at 60°C for 2 hours and then spun down at 4000*g* for 12 minutes. Supernatants were collected, and enzyme activity was assessed photometrically at 630 nm using 3,3′,5,5′-tetramethylbenzidine as a substrate.

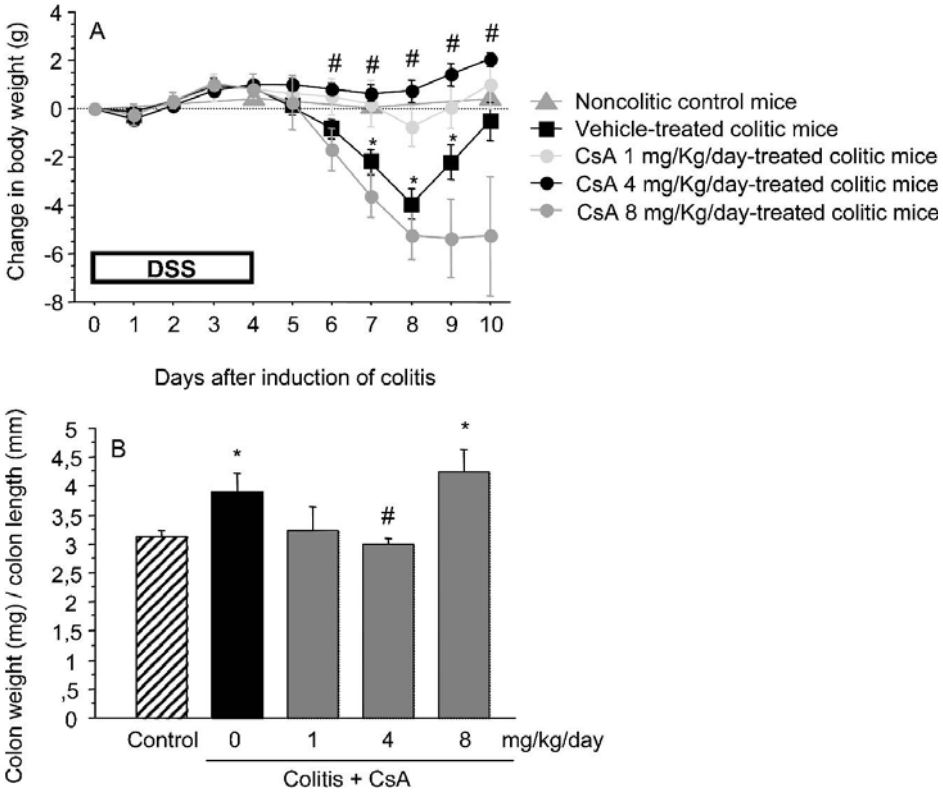

FIGURE 1. Effects of treatment with different doses of intraperitoneal CsA (1, 4, and 8 mg/kg/d) on body weight (A) and on colon weight to length ratio (B) in DSS-induced colitis. Best results were obtained with the dose of 4 mg/kg/d^{-1}. *$P < 0.05$ versus noncolitic control mice; $^{\#}P < 0.05$ versus vehicle-treated colitic mice.

The assay mixture consisted of 20 μL of supernatant, 10 μL of tetramethylbenzidine (final concentration 1.6 mM) dissolved in dimethyl sulfoxide, and 140 μL H_2O_2 (final concentration 3.0 mM) diluted in 80 mM phosphate buffer, pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

Measurement of Cytokine Production

Levels of murine TNF-α and IL-6 were quantified in protein extracts from colon homogenates by using commercially available enzyme-linked immunosorbent assays (ELISA) supplied by R&D Systems (Abingdon, UK) and Diaclone (Besançon, France), respectively. Results were expressed in pg/mg of protein. Moreover, plasma samples were taken after centrifugation at 1000*g* for 10 minutes at 4°C of 1 mL of blood obtained from each mouse by cardiac puncture with a heparinized syringe for measurements of circulating TNF-α and IL-6 (Diaclone) by ELISA. Results were expressed in pg/mL of serum. Assessment of cytokine production was performed in controls, colitic untreated animals, and in colitic animals treated with ip CsA, which induced significant amelioration of colitis (n = 7 to 8 per group).

Blood samples were also used for measurement of leukocyte counts to monitor differences between treatment groups.

In Vivo Assessment of Leukocyte-Endothelial Cell Interactions

Leukocyte–endothelial cell interactions in colonic submucosal and lamina propria venules were characterized using intravital microscopy in control animals and in vehicle-treated and ip CsA-treated colitic mice. Studies were performed at day 10 after induction of colitis (n = 8 animals per group).

Fluorescence Intravital Microscopy

Mice were anesthetized with subcutaneous (sc) ketamine (150 mg/kg body weight; Ketolar, Parke-Davies, Morris Klein, NJ) and xylazine (7.5 mg/kg body weight; Sigma Chemical, St. Louis, MO), and a tail vein was cannulated. Throughout the experiments, rectal temperature was monitored using an electrothermometer and was maintained between 36.5°C and 37.5°C with an infrared heat lamp. The abdomen was opened via a midline incision, and a segment of the distal colon was chosen for microscopy examination, exterior-

ized, and covered with cotton gauze soaked with bicarbonate buffer. Mice were then placed on an adjustable microscope stage, and the colon was extended over a non-autofluorescent plastic coverslip that allowed observation of a 2-cm^2 segment of tissue. An inverted microscope (Diaphot 300; Nikon, Tokyo, Japan) with a CF Fluor 40× objective lens (Nikon) was used. A charge-coupled device (CCD) camera (model XC-77; Hamamatsu Photonics, Hamamatsu, Japan) with a C2400 CCD camera control unit and a C2400-68 intensifier head (Hamamatsu Photonics), mounted on the microscope, projected the image onto a monitor (Trinitron KX-14CP1; Sony, Tokyo, Japan), and the images were recorded using a videocassette recorder (SR-S368E; JVC, Tokyo, Japan) for off-line analysis. A video date-time generator (Panasonic Digital AV Mixer WJ-AVE55; Matsushita Communication Industrial, Tokyo, Japan) displayed these parameters on recorded and live images. Leukocytes were in vivo–labeled by intravenous (iv) injection of rhodamine-6G (Molecular Probes, Leiden, The Netherlands) as previously described.[16] Rhodamine-6G–associated fluorescence was visualized by epi-illumination at 510–560 nm using a 590-nm emission filter. Single unbranched submucosal and lamina propria venules with internal diameters (ID) of 25 to 40 μm were selected for observation. Venular ID was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). The flux of rolling leukocytes, leukocyte rolling velocity, number of adherent leukocytes, venular blood flow (Vbf), and venular wall shear rate (γ) were determined off-line after playback of the videotapes. Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of free-flowing leukocytes in the same vessel. The flux of rolling leukocytes was measured as the number of rolling leukocytes that passed a fixed point within a small (10 μm) viewing area of the vessel in a 1-minute period. Leukocyte rolling velocity was calculated as the mean of 10 rolling leukocyte velocities and expressed in μm/s. Leukocytes were considered adherent to venular endothelium when stationary for 30 seconds or longer and expressed as the number per 100-μm length of venule. Venular blood flow was estimated from the mean of the velocity of 3 free-flowing leukocytes (ffv), using the empirical relationship of Vbf = ffv/1.6. Venular wall shear rate was calculated, assuming cylindrical geometry, using the Newtonian definition $\gamma = 8$ (Vbf/ID). In each animal, 3 to 6 random venules were examined, and results were calculated as the mean of each parameter in all venules examined.

Effects of CsA on Endothelial Cell Adhesion Molecule Expression in Colitis

Thirty mice were used to characterize endothelial expression of ICAM-1, MAdCAM-1, and VCAM-1 in the colon, under baseline conditions (n = 15), and after induction of colitis (n = 15) at Day 10. Additional groups of animals were studied to assess the effect of ip CsA treatment (n = 15) on endothelial expression of ICAM-1, MAdCAM-1, and VCAM-1.

Dual Radiolabeled Monoclonal Antibody Technique

Expression of CAMs was characterized using this technique.[17] We have previously shown that, in this model, endothelial surface area relative to tissue weight is not significantly altered by the development of colitis.[9]

The monoclonal antibodies (MAbs) used to quantify endothelial expression of CAMs were YN1/1.7.4, a rat IgG_{2b} against mouse ICAM-1[18]; MECA-367, a rat IgG_{2a} against mouse MAdCAM-1[19]; MVCAM.A, a rat $IgG_{2a\kappa}$ against mouse VCAM-1[20]; and UPC-10, a nonbinding IgG.[21] MECA367 and MVCAM.A were purchased from BD Pharmingen (Heidelberg, Germany). YN1/1.7.4 was purified by protein G chromatography at Pharmacia Upjohn Laboratories (Kalamazoo, MI). UPC-10 was purchased from Sigma and dialyzed to remove sodium azide. The binding MAbs directed against ICAM-1, MAdCAM-1, and VCAM-1 were labeled with ^{125}I, whereas the nonbinding MAb UPC-10 was labeled with ^{131}I (Amersham Ibérica, Madrid, Spain). Radioiodination of the MAbs was performed by the iodogen method as previously described.[22] Labeled MAbs were stored at 4°C and used within 3 weeks after the labeling procedure. The specific activity of labeled MAbs was approximately 0.5 mCi/mg.

Animals were anesthetized with sc ketamine (150 mg/kg) and xylazine (7.5 mg/kg), and the left carotid artery and a tail vein were cannulated with PE-10 tubing (Portex, Hythe, UK). For assessment of endothelial expression of ICAM-1, MAdCAM-1, and VCAM-1, a mixture of 10 μg of ^{125}I-YN1/1.7.4 and 40 μg of unlabeled YN1/1.7.4; 10 μg of ^{125}I-MECA-367 without additional unlabeled MECA-367; and 10 μg of ^{125}I-MVCAM.A and 20 μg of unlabeled MVCAM.A were administered, respectively. In all cases, 10 μg of ^{131}I-UPC-10 was added to the injection mixture. Doses of anti-ICAM-1, anti-MAdCAM-1, and anti-VCAM-1 MAbs proved to be saturating in previous assays.[23] The mixture of binding and nonbinding MAbs was administered through the tail vein catheter. Blood samples were obtained through the carotid artery catheter 5 minutes after injection of the MAb mixture. Thereafter, animals were heparinized (1 mg/kg sodium heparin iv) and rapidly exsanguinated. Entire organs were then harvested and weighed. ^{125}I (binding MAb) and ^{131}I (nonbinding MAb) activities in each organ and 100-μL aliquots of cell-free plasma were counted in a Cobra II gamma-counter (Packard, Canberra, Australia) with automatic correction for background activity and spillover. The injected activity in each experiment was calculated by counting a 3-μL sample of the 300-μL injection mixture containing the radiolabeled MAbs. The accumulated activity of each MAb in an organ was expressed as nanograms of binding MAb per gram of tissue. The formula used to calculate either ICAM-1, MAdCAM-1, or VCAM-1 expression was as follows:

Endothelial expression = [(cpm ^{125}I organ × g^{-1} × cpm ^{125}I injected^{-1}) − (cpm ^{131}I organ × g^{-1} × cpm ^{131}I injected $^{-1}$) × (cpm ^{125}I in plasma)/(cpm ^{131}I in plasma)] × ng injected MAb. This formula was modified from the original method[17] to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of both binding and nonbinding MAbs.[8]

Effects of CsA on Endothelial Cell Adhesion Molecule Expression in Vitro

To determine whether changes in ICAM-1 and VCAM-1 expression in response to CsA were or were not a direct effect of the drug, primary cultures of human umbilical vein endothelial cells (HUVECs) under basal or stimulated conditions were exposed to different concentrations of CsA. HUVEC were cultured in Endothelial Cell Basal Medium-2 (EBM-2 medium, Bio-Whittaker, Verviers, Belgium) in a 96-well plate. When endothelial monolayers were confluent, they were incubated for 20 hours at 37°C and 5% CO_2 with different doses of CsA (1 μM, 10 μM, 25 μM, and 100 μM) with or without TNF-α (10 ng/mL) (Sigma). This cytokine was chosen because of the central role it has in the pathogenesis of IBD. The concentration of CsA used was based on a previous study showing efficacy to decrease CAMs expression in activated human intestinal microvascular endothelial cells.[10] Thereafter, cells were washed and incubated for 45 minutes at room temperature (RT) with a mouse anti–ICAM-1 antibody (HA58), a mouse anti–VCAM-1 antibody (51-10C9), or a mouse anti–E-selectin antibody (68-5H11), as a positive control for endothelial activation. Antibodies were purchased from BD Pharmingen (Heidelberg, Germany). After several washes, cells were incubated with a 1/1000 dilution of rabbit anti-mouse-peroxidase conjugated antibody for 30 minutes at RT. After that, cells were incubated with a developing solution of *O*-phenylenediamine dihydrochloride (Sigma). After 30 minutes, absorbance was read at a wavelength 450 nm. Results were expressed as optical density units. Given that CsA might be directly toxic to HUVEC cells, additional experiments were performed to determine whether overall protein in cell culture medium was altered as a result of incubation with CsA, and expression of each adhesion molecule of interest in relation to total protein was calculated.

Statistical Analyses

Statistical analysis was performed by using the Kruskal-Wallis and Mann-Whitney non-parametric tests, analysis of variance with the Bonferroni (post-hoc) test, and Student's unpaired *t* test, when appropriate. All values are expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

Evaluation of the Therapeutic Efficacy of CsA in DSS-Induced Colitis

Administration of DSS induced a significant loss in body weight that was maximum at Days 7 and 8 after induction of colitis (Fig. 1A). From 24 hours after the induction of colitis, all animals had a positive fecal occult blood test and diminished stool consistency when compared with control animals. Clinical and macroscopic changes in control and colitic mice at the end of the study period are summarized in Table 1. At Day 10 after induction of colitis, a significant reduction in colon length and an increase in colon weight, with a corresponding increase in the colon weight to length ratio, was observed in colitic animals relative to controls (Fig. 1B). Histologic colonic damage was present only in colitic animals (Figs. 2 and 3A).

Treatment with ip CsA at the dose of 4 mg/kg/d significantly ameliorated the severity of colitis. When compared with the group of colitic animals treated with vehicle, CsA treatment significantly improved the stool consistency score and diminished body weight loss (Fig. 1A), rectal bleeding, DAI score, colon weight, colon shortening, colon weight to length ratio (Table 1 and Fig. 1B), and the microscopic damage score (Figs. 2 and 3A). CsA completely abrogated the changes in some of the parameters reflecting colitis severity. Notably, the results in body weight and colon weight to length ratio were not significantly different from the results obtained in noncolitic control group. As described in the "Materials and Methods" section, an initial dose-finding experiment demonstrated that doses of 1 or 8 mg/kg/d of CsA were less effective (Fig. 1). Therefore, for the rest of the study the dose of 4 mg/kg/d was used.

Neutrophil infiltration of the colon measured as tissue MPO activity was significantly increased in vehicle-treated colitic animals compared with the control group (Fig. 3B). The

TABLE 1. Clinical and Macroscopic Changes in Noncolitic Control Animals and Colitic Mice Treated with Vehicle or Intraperitoneal CsA

	Control (n = 12)	Vehicle (n = 12)	CsA (n = 12)
Change in body weight (g)	0.2 ± 0.2	−2.2 ± 0.5*	0.6 ± 0.3†
Stool consistency	0 ± 0	1.4 ± 0.2*	0.4 ± 0.1*†
Rectal bleeding	0 ± 0	1.3 ± 0.2*	0.7 ± 0.1*†
Disease Activity Index (DAI)	0 ± 0	3.9 ± 0.8*	1.2 ± 0.2*†
Colon weight (mg)	304 ± 9	340 ± 12*	254 ± 6*†
Colon length (mm)	96 ± 1	71 ± 3*	85 ± 2*†
Colon weight/length (mg/mm)	3.1 ± 0	4.8 ± 0*	3.0 ± 0†

*$P < 0.05$ vs noncolitic control mice.
†$P < 0.05$ vs vehicle-treated colitic mice.

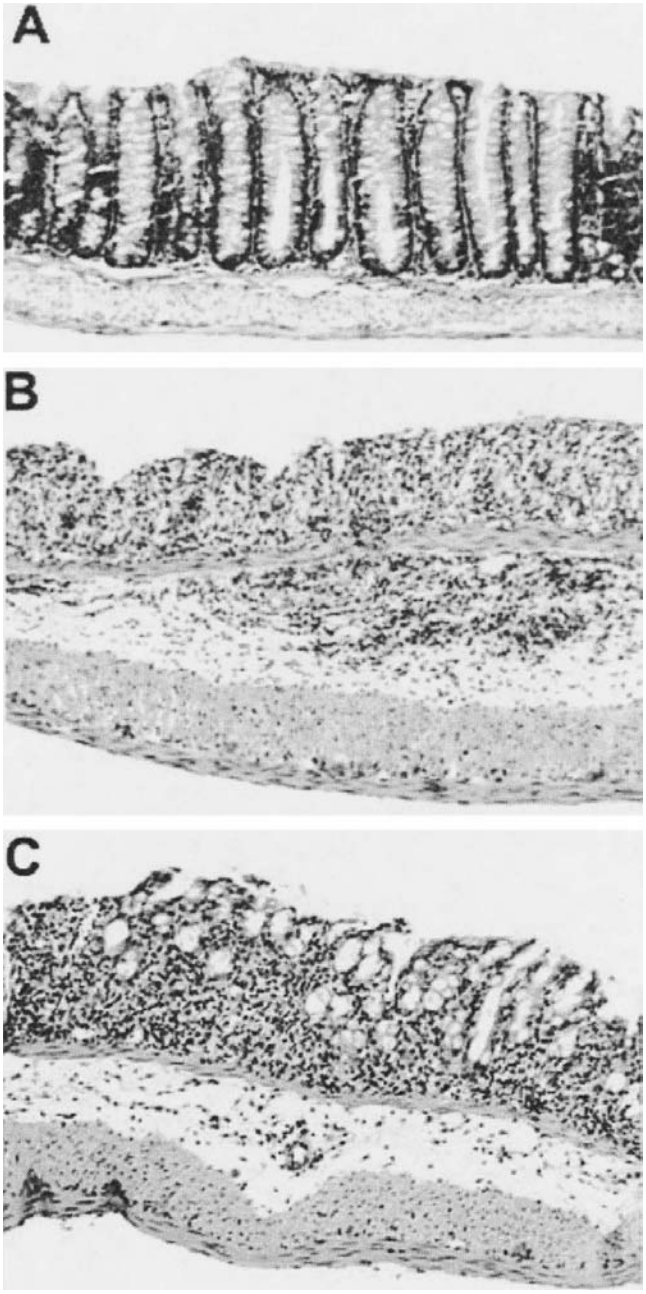

FIGURE 2. Histologic damage evaluated in colonic samples stained with hematoxylin and eosin. In noncolitic control mice (A), epithelial crypts of the mucosal layer are preserved and there is no infiltration of inflammatory cells. In contrast, in vehicle-treated colitic mice (B), epithelial crypts are completely lost, and there is a marked inflammatory cell infiltration affecting the lamina propria and submucosa, along with submucosal edema. In CsA-treated colitic mice (C), epithelial crypts are shortened but still present, and the inflammatory infiltration and edema have decreased (original magnification $\times 60$).

increase in MPO activity associated with the development of colitis was significantly reduced in CsA-treated colitic mice.

Levels of TNF-α and IL-6 quantified in protein extracts of colon homogenates were increased in colitic animals with respect to control mice, 2.5-fold and 17-fold, respectively (Fig. 4). Treatment with CsA significantly reduced the levels of both cytokines in colonic tissue.

Plasma IL-6 concentrations significantly increased in vehicle-treated colitic mice (3.46 ± 2.58 pg/mL) in comparison with control mice (0.00 ± 0.00; $P < 0.05$). Intraperitoneal CsA significantly decreased plasma levels of IL-6 in colitic mice

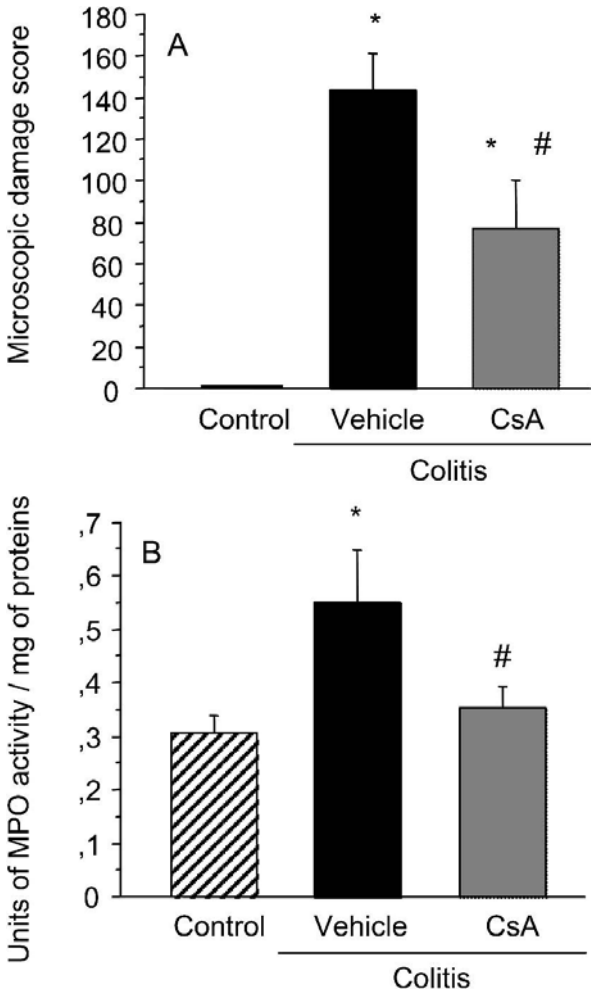

FIGURE 3. Microscopic damage score (A) and MPO activity (B) in the colon of noncolitic control mice, vehicle-treated colitic mice, and CsA-treated colitic mice. Microscopic damage score and MPO activity were increased in colitic animals in respect to noncolitic control mice. Treatment with CsA significantly reduced both markers of colitis severity. $^{*}P < 0.05$ versus noncolitic control mice; $^{\#}P < 0.05$ versus vehicle-treated colitic mice.

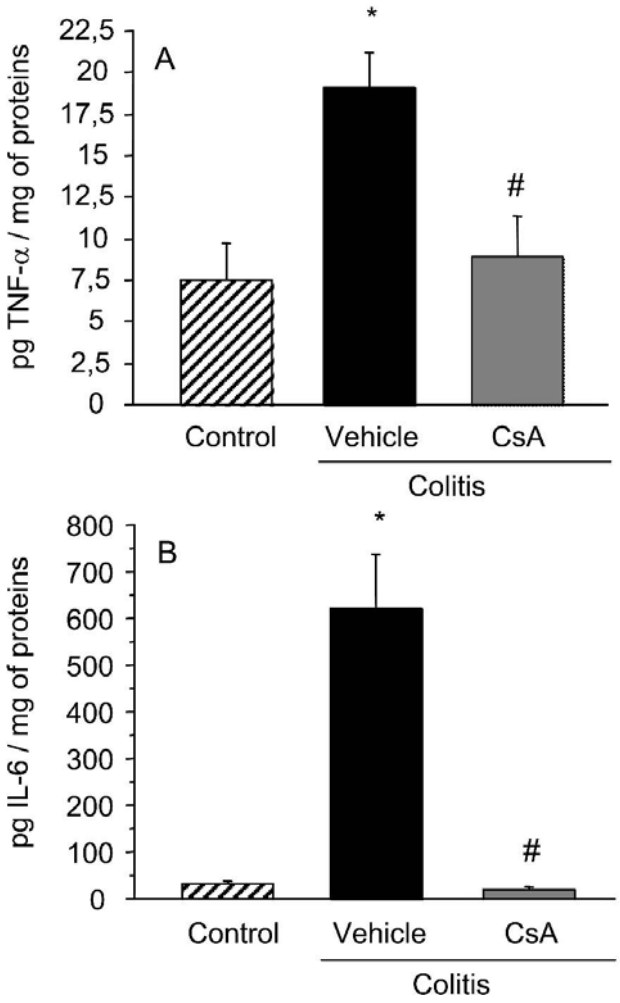

FIGURE 4. Levels of TNF-α (A) and IL-6 (B) in colon homogenates of control animals, vehicle-treated colitic mice, and CsA-treated colitic mice. Levels of TNF-α and IL-6 were increased in colitic animals in respect to noncolitic control mice. Treatment with CsA significantly reduced the levels of the two proinflammatory cytokines. *$P < 0.05$ versus noncolitic control mice; $^{\#}P < 0.05$ versus vehicle-treated colitic mice.

(0.00 ± 0.00; $P < 0.05$) relative to vehicle-treated colitic mice. Plasma TNF-α levels remained under the detection limits in both control and colitic mice.

Treatment with ip CsA did not modify peripheral blood leukocyte counts (vehicle-treated colitic mice: 3.16 ± 0.8 × 10^9/L; CsA-treated colitic mice: 3.94 ± 0.7 × 10^9/L; $P = 0.53$), red blood cell counts (vehicle-treated colitic mice: 6.80 ± 0.3 × 10^{12}/L; CsA-treated colitic mice: 6.47 ± 0.3 × 10^{12}/L; $P = 0.49$), or platelet counts (vehicle-treated colitic mice: 424 ± 45 × 10^9/L; CsA-treated colitic mice: 319 ± 44 × 10^9/L; $P = 0.18$). Likewise, the number of neutrophils and lymphocytes did not change as a result of CsA administration (data not shown).

Leukocyte-Endothelial Cell Interactions in Colonic Venules

When compared with control animals, colitic mice showed a pronounced increase in leukocyte–endothelial cell interactions (Figs. 5 and 6). There was a 3-fold increase in the flux of rolling leukocytes at day 10 after induction of colitis. Few adherent leukocytes were present in venules of control mice, and a 4-fold increase in leukocyte adhesion was observed in colitic animals. No differences in leukocyte rolling velocity, venular blood flow, or venular wall shear rate were observed between control and colitic mice (data not shown).

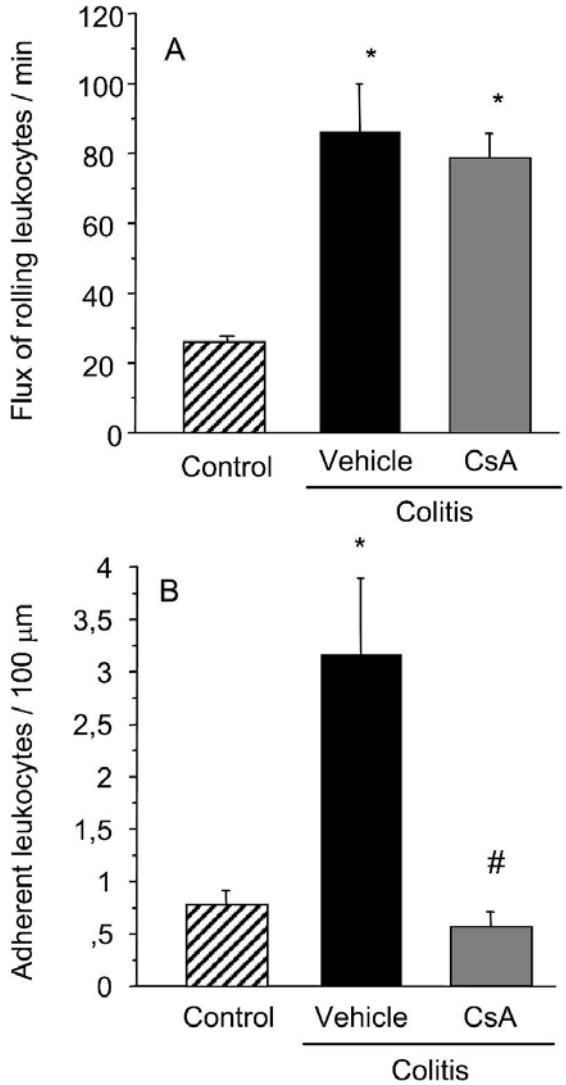

FIGURE 5. Flux of rolling leukocytes (A) and number of adherent leukocytes (B) in colonic venules. A significant increment in leukocyte-endothelial cell interactions was observed at day 10 after induction of colitis. Treatment of colitic mice with intraperitoneal CsA abrogated leukocyte adhesion but had no effect on the flux of rolling leukocytes. *$P < 0.05$ versus noncolitic control mice; $^{\#}P < 0.05$ versus vehicle-treated colitic mice.

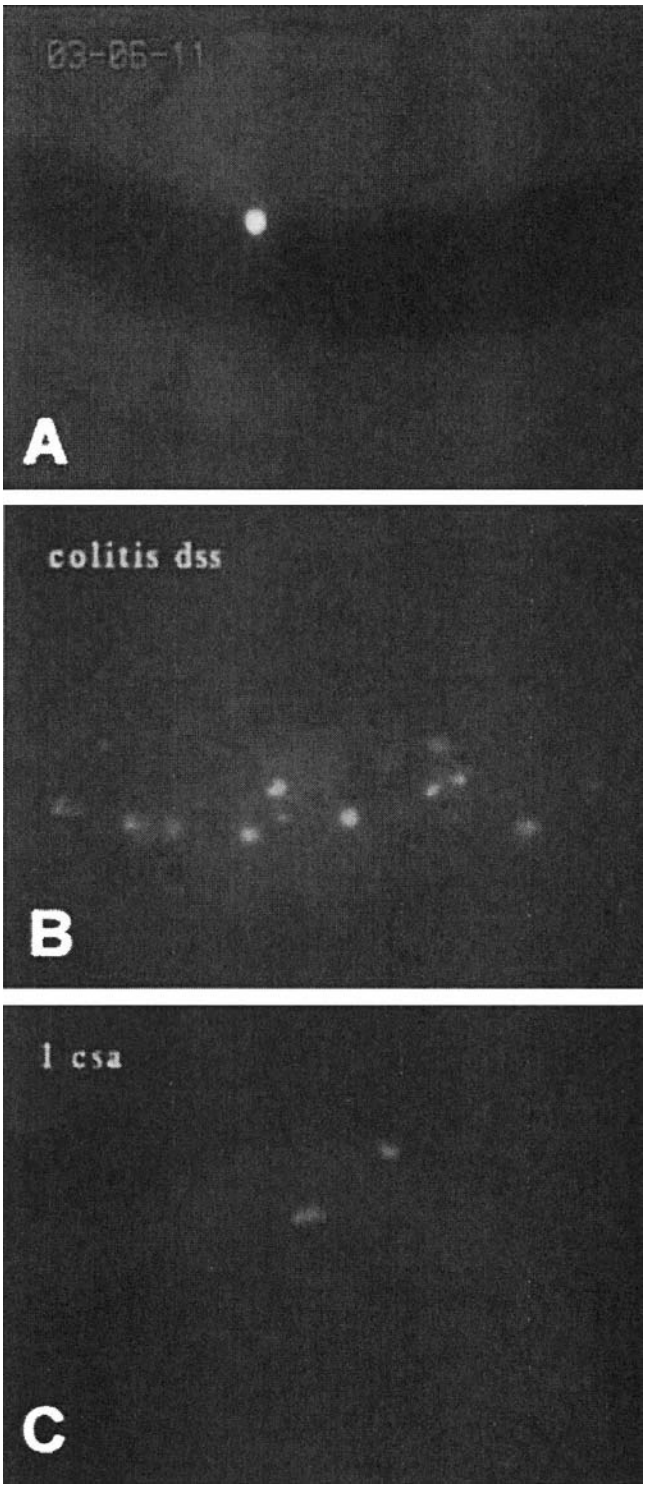

FIGURE 6. Photographs of intravital microscopy. Flux of rolling leukocytes and cellular adhesion in noncolitic animals (A), vehicle-treated colitic mice (B), and CsA-treated colitic animals (C). Treatment of colitic mice with intraperitoneal CsA decreased significantly the number of adherent leukocytes to levels close to those of control animals.

Treatment of colitic mice with ip CsA did not reduce the flux of rolling leukocytes below the level observed in vehicle-treated group of colitic mice ($P = 0.56$) but completely abrogated the increase in the number of adherent leukocytes associated with the presence of colonic inflammation ($P = 0.0023$) (Figs. 5 and 6).

Effects of CsA on Adhesion Molecule Expression in Colitis

Endothelial ICAM-1, MAdCAM-1, and VCAM-1 expression in the colons of colitic mice, increased significantly 1.5-, 3-, and 4.7-fold, respectively, compared with noncolitic mice ($P < 0.05$; Fig. 7). ICAM-1 expression was also increased significantly in the stomach, MAdCAM-1 in the mesentery, and VCAM-1 expression was higher in the mesentery, jejunum, and ileum (Table 2). No expression of MAdCAM-1 was detected in liver, spleen, or other extra-abdominal organs such as heart or lung, which is consistent with the predominant expression of this adhesion molecule in gastrointestinal organs (data not shown).

In CsA-treated animals, the increase in ICAM-1 and VCAM-1 expression in the colon was significantly attenuated as compared with vehicle-treated colitic mice ($P < 0.05$; Figs. 7A and 7C). Similar reductions were observed in the mesentery, jejunum, and ileum with regard to VCAM-1 expression (Table 2). Treatment with CsA had no effect on MAdCAM-1 expression in the colon (Fig. 7B).

Effects of CsA on Endothelial Cell Adhesion Molecule Expression in Vitro

To determine whether the observed effects of CsA in CAMs expression may result from a direct action of the drug on endothelial cells or from modulation of immune cell activation, the effects of CsA on isolated endothelial cells in culture were assessed.

Concentrations of CsA of 1 and 10 μM did not affect cellular viability. However, incubation of HUVECs with CsA at concentrations of 25 or 100 μM led to significant cellular death in the primary cultures. Therefore, in subsequent studies only the concentrations of 1 and 10 μM were used.

Resting HUVECs expressed low levels of ICAM-1, and VCAM-1 (Fig. 8). Following activation with TNF-α, there was a dramatic increase in the cell surface expression of these 2 adhesion molecules. Co-incubation with CsA at concentrations of 1 and 10 μM significantly decreased TNF-induced ICAM-1 and VCAM-1 expression in a dose-dependent manner. Reduction in CAMs expression may not be attributed to a direct toxic effect of CsA affecting overall protein synthesis. Compared with cells cultured in medium alone (76 ± 15 μg of protein/mL), overall protein expression was not different in cell cultures treated with CsA at the doses that significantly reduced ICAM-1 and VCAM-1 expression (CsA 1 μM, 73 ± 1; and CsA 10 μM, 82 ± 8). The ratio ICAM-1 optical

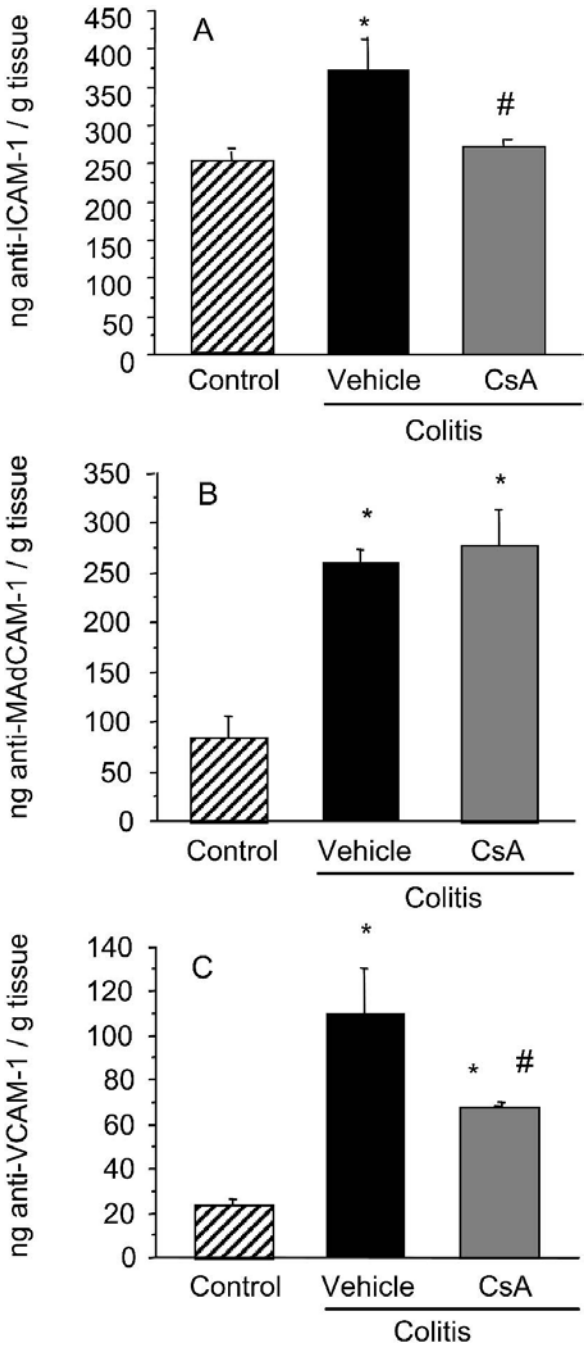

FIGURE 7. Endothelial expression of ICAM-1 (A), MAdCAM-1 (B), and VCAM-1 (C) in colonic vessels of noncolitic animals and colitic mice treated with intraperitoneal vehicle or CsA. When compared with control animals, colitic mice showed a pronounced increase in expression of these adhesion molecules. Treatment with CsA significantly decreased ICAM-1 and VCAM-1 expression but did not modify MAdCAM-1, as compared with vehicle-treated colitic mice. $^{*}P < 0.05$ versus noncolitic control mice; $^{\#}P < 0.05$ versus vehicle-treated colitic mice.

density/total protein (ng/mL) in resting cells (0.7 ± 0.1) was increased by TNF-α stimulation (4 ± 0.4) and significantly reduced by CsA 10 μM (2.9 ± 0.03), but not by CsA 1 μM (3.8 ± 0.1). Similarly, the ratio VCAM-1 optical density/overall protein expression in resting cells (0.3 ± 0.1) was increased by TNF-α stimulation (1.8 ± 0.2) and significantly reduced by CsA treatment at doses of 1 μM (1.1 ± 0.2) and 10 μM (0.4 ± 0.05).

DISCUSSION

The results of the current study demonstrate that treatment with CsA markedly attenuates the up-regulation of endothelial adhesion molecule expression and leukocyte recruitment, normally associated with the presence of colonic inflammation, and affords a significant amelioration of the clinical course of DSS-induced colitis.

Our observations on the clinical effects of CsA are in keeping with previous evidence showing that immunosuppressive therapy with CsA attenuates the severity of different models of experimental colitis, including colitis induced by DSS,[13] peptidoglycan/polysaccharide,[24] oxazolone,[25] or dinitrofluorobenzene.[26] We observed that treatment with ip CsA afforded a marked amelioration of several parameters of colitis severity, including clinical manifestations and microscopic damage score. Likewise, CsA administration diminished neutrophil infiltration, measured as tissue MPO activity, and levels of TNF-α and IL-6 in the colon. The preliminary dose-response studies showed that ip CsA at doses of 4 mg/kg/d is the most effective, whereas increasing the dose to 8 mg/kg/d was associated with a worse clinical course that may be related to toxic effects of the drug, which may adversely affect NO production from the intestinal microvasculature, leading to worsening of chronic intestinal inflammation.[10] In fact, the most effective dose (4 mg/kg/d) corresponds to that used in clinical practice in patients with severe ulcerative colitis. Although a recent paper concludes that there is no additional efficacy of 4 mg/kg iv CsA over 2 mg/kg in the acute management of severe ulcerative colitis attacks,[27] the apparent discrepancy may be related to the fact that small mammals usually require higher doses of a certain drug to achieve the same biological effects as in humans.

In contrast to the well-characterized suppressive effect of CsA on IL-2 gene transcription in T cells, other immunosuppressive effects of CsA have received less attention. Additional effects of CsA that, if present, could contribute to its beneficial action in the treatment of inflammatory conditions include a possible modulation of the expression of adhesion molecules involved in the process of leukocyte recruitment and the production of other proinflammatory cytokines. Studies performed to investigate the effects of CsA on vascular endothelial functions have reported both protective[28] and adverse[29,30] effects. The expression of ICAM-1 and VCAM-1 on endothelial cells is induced in multiple inflammatory condi-

TABLE 2. Expression of Endothelial ICAM-1, MAdCAM-1, and VCAM-1 in Different Vascular Beds in Noncolitic Control Animals and Vehicle-Treated or Intraperitoneal CsA-Treated Colitic Mice

	ICAM-1			MAdCAM-1			VCAM-1		
	Control (n = 5)	Vehicle (n = 5)	CsA (n = 5)	Control (n = 5)	Vehicle (n = 5)	CsA (n = 5)	Control (n = 5)	Vehicle (n = 5)	CsA (n = 5)
Mesentery	441 ± 39	524 ± 95	534 ± 81	72 ± 15	158 ± 30*	180 ± 16*	82 ± 3	168 ± 32*	92 ± 1†
Stomach	305 ± 22	438 ± 43*	425 ± 96	20 ± 13	40 ± 5	58 ± 12*	65 ± 8	78 ± 7	75 ± 2
Jejunum	436 ± 46	438 ± 51	498 ± 91	269 ± 33	253 ± 8	223 ± 24	36 ± 2	83 ± 1*	41 ± 2†
Ileum	356 ± 29	351 ± 28	425 ± 85	302 ± 50	326 ± 16	328 ± 33	29 ± 1	79 ± 9*	29 ± 2†

*$P < 0.05$ vs noncolitic control mice.
†$P < 0.05$ vs vehicle-treated colitic mice.

tions. Clinical studies have shown that CsA reduces ICAM-1 expression in the dermis of patients with actinic prurigo[31] and in the transplanted kidney. Experimental studies documented that CsA also down-regulates ICAM-1 expression in postcapillary venules of rats exposed to platelet activating factor (PAF),[32] in the kidney of rats with chronic serum sickness,[33] and the expression of ICAM-1 and VCAM-1 in myocardial rejection in rabbits.[34] Finally, an in vitro study demonstrated that co-incubation of stimulated HUVECs with CsA results in a significant down-regulation on the surface expression of E-selectin and VCAM-1, but not ICAM-1.[35]

In the current study, we corroborate previous findings showing that expression of ICAM-1, MAdCAM-1, and, to a greater magnitude, VCAM-1 is significantly increased in colitic animals. Previous studies in the trinitrobenzene sulfonic acid (TNBS)[8] and DSS models of colitis[9,36] have shown a marked increase of these CAMs in the colonic endothelium. Although DSS-induced colitis affects predominantly the distal colon,[11] we also found increased VCAM-1 expression in the mesentery, jejunum, and ileum, increased endothelial MAdCAM-1 in the mesentery, and increased ICAM-1 in the stomach. Up-regulation of these CAMs in proximal segments of the gastrointestinal tract probably results from DSS-induced mucosal lesions in these upper segments of the gut.

We provide novel evidence that CsA treatment markedly decreases endothelial expression of ICAM-1 and VCAM-1 in the colon of colitic mice and has no effect on MAdCAM-1 up-regulation. To determine whether the observed effects of CsA in ICAM-1 and VCAM-1 expression may result from a direct action of the drug on endothelial cells or from modulation of immune cell activation, the effects of CsA on TNF-α–stimulated endothelial cells in culture were assessed. Incubation of HUVECs in the presence of different concentrations of CsA did decrease TNF-α–induced ICAM-1 and VCAM-1 up-regulation in a dose-dependent manner. Thus, the observed reduction of ICAM-1 and VCAM-1 expression in colitic animals in response to CsA may be due, at least in part, to a direct effect of this drug on CAMs expression.

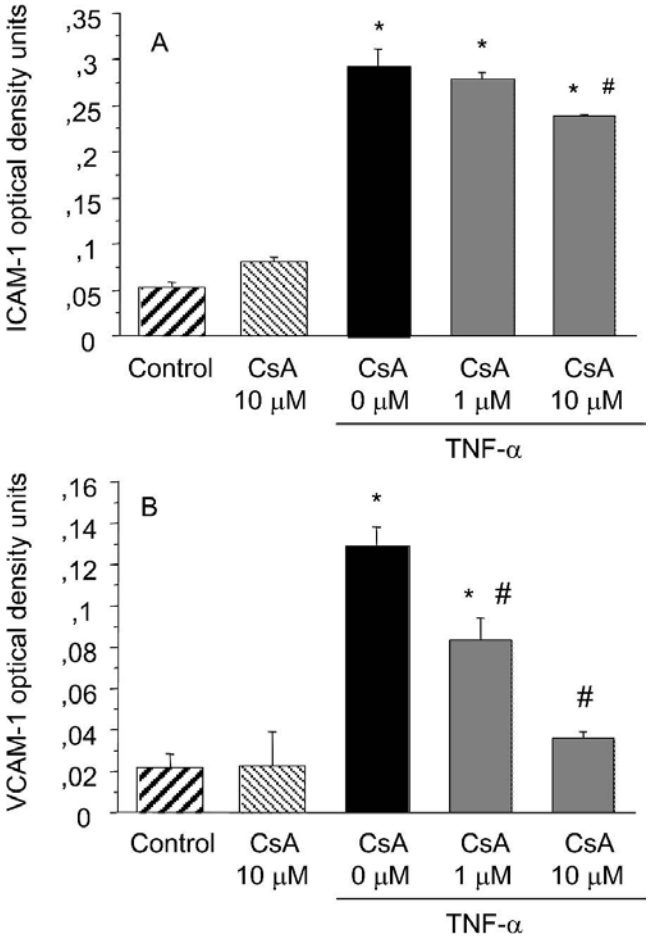

FIGURE 8. Effect of CsA treatment on ICAM-1 expression (A) and VCAM-1 expression (B) in HUVECs. Cultures were stimulated with 10 ng/mL of TNF-α for 20 hours in the presence or absence of CsA at two concentrations (1 μM and 10 μM). Data are expressed as the mean of triplicate wells ± SD. There was a significant increase in the density of cell adhesion molecule expression in activated HUVECs. Treatment with CsA significantly decreased CAMs expression in a dose-dependent manner. *$P < 0.05$ versus control; #$P < 0.05$ versus CsA 0 μM + TNF-α.

Among the other factors that may be affected by treatment with CsA, activation of the immune cells and the resulting cytokine production are probably relevant, as concentrations of TNF-α and IL-6 in colonic tissue, as well as circulating levels of IL-6, were significantly reduced in response to CsA treatment. TNF-α is a well-known potent inducer of endothelial CAMs expression, and reduced production of this cytokine as a result of CsA administration may also contribute to reduce expression of ICAM-1 and VCAM-1 in colitis.

We also demonstrate that in the DSS-induced colitis model, down-regulation of ICAM-1 and VCAM-1 expression following treatment with CsA is accompanied by a marked reduction in leukocyte recruitment into the inflamed intestine. Using intravital microscopy, we observed that CsA treatment markedly decreased the number of firmly adhered leukocytes in postcapillary colonic venules without affecting rolling interactions. The notion that reduced ICAM-1 and VCAM-1 may significantly contribute to the beneficial effects of CsA in colonic inflammation is supported by observations showing that selective immunoneutralization of these adhesion molecules results in a marked improvement in the clinical course and severity of pathologic alterations in experimental colitis.[8,9]

In conclusion, CsA treatment inhibits the expression of adhesion molecules ICAM-1 and VCAM-1 in experimental colitis. This reduction is accompanied by decreased leukocyte-endothelial cell interactions. These findings give an insight into mechanisms that may contribute to the beneficial effect of CsA in ulcerative colitis and underline the importance of factors governing leukocyte recruitment in the pathogenesis of this experimental model. The target of the action of CsA is the immune system rather than the endothelium, although the results of the current study indicate that modulation of leukocyte-endothelial cell adhesive interactions may represent a novel mechanism of action for CsA. This aspect should be taken into account to optimize treatment of severe ulcerative colitis and especially to design adequate synergistic therapeutic associations.

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EDITORIAL

Péptidos trefoil, reparación y progresión celular

A. Soriano

Servicio de Gastroenterología. Institut de Malalties Digestives. Hospital Clínic i Provincial. Barcelona.

La mucosa gastrointestinal tiene la difícil tarea de compatibilizar un alto grado de permeabilidad que le permita secretar y absorber moléculas con una gran resistencia a los múltiples agentes lesivos a los que se ve expuesta. Si resulta dañada, es vital una rápida reparación del defecto mucoso para prevenir el daño adicional que podría causar la secreción ácida, los enzimas proteolíticos y las bacterias. Este proceso reparativo es bifásico: la fase inicial o de restitución tiene lugar durante las primeras horas tras haberse producido el daño mucoso y consiste en una rápida migración celular desde el fondo y la periferia de la lesión hacia el área denudada para restablecer la continuidad epitelial[1]. La proliferación celular y remodelación arquitectural que se ponen en marcha aproximadamente a las 24 horas constituirán la fase final del proceso reparativo tisular.

Cada vez existen más evidencias de que una nueva familia de pequeños péptidos, conocidos como factores o péptidos trefoil (PT), está implicada en la fase de restitución epitelial[2]. Todos ellos comparten una estructura básica que los identifica, conocida como dominio trefoil y que consiste en una triple asa en forma de trébol (trefoil) mantenida por 3 puentes disulfuro entre residuos de cisteína. Los PT se expresan en diversos tejidos epiteliales del organismo, aunque sobre todo lo hacen a nivel gastrointestinal y de manera tejido-específica. En la actualidad se conocen 3 diferentes PT en los mamíferos: el TFF1 (TreFoil Factor 1) o pS2 que se expresa en el estómago, el TFF2 o SP (polipéptido espasmolítico) en el estómago y duodeno, y el TFF3 o ITF (factor trefoil intestinal) expresado en el intestino delgado y grueso. El TFF1 y el TFF2 fueron descubiertos en 1982 y posteriormente, en 1991, se conoció la existencia del TFF3. Son expresados de manera constitutiva por las células secretoras de mucina, liberándose a la luz intestinal junto con ella. Además, su síntesis aumenta en las zonas lesionadas del epitelio y otros tipos celulares adyacentes a las mismas también los pueden sintetizar. Su compacta estructura les protege de la digestión por enzimas proteolíticos y el ácido, permitiéndoles que se mantengan estables en el medio endoluminal.

La función principal conocida de estos péptidos es la de participar activamente en el mantenimiento de la integridad de la mucosa gastrointestinal, tanto en condiciones basales como facilitando su reparación una vez que la inflamación o la ulceración han ocurrido (mediante el proceso de restitución). Como regla general, encontraremos una sobreexpresión de los PT a nivel gastrointestinal en úlceras[3], en la metaplasia gástrica[4], en la enfermedad inflamatoria crónica intestinal[3,5] y en algunos tumores[6]. Recientemente se ha caracterizado un nuevo linaje celular epitelial, conocido como linaje celular asociado a úlcera (UACL)[7], que constituye una específica estructura anatómica que aparece en íntima relación con áreas de ulceración crónica. Originado en las *stem-cells* de las criptas adyacentes a la úlcera, parece jugar un papel clave en la restauración de la integridad mucosa. Hoy se sabe que este tipo celular expresa péptidos de particular interés como los péptidos trefoil TFF1, TFF2 ,TFF3, el factor de crecimiento epidérmico y la lisozima y que, además, puede inducir la expresión de los PT en células próximas como las neuroendocrinas. No deja de ser curioso, por lo inusual, que un péptido como el TFF1 se pueda almacenar junto con el moco en las células caliciformes siendo secretado a la luz intestinal y que al mismo tiempo pueda almacenarse también en los gránulos de las células neuroendocrinas, actuando presumiblemente de manera paracrina o endocrina.

Los mecanismos a través de los cuáles los PT ejercen sus funciones son desconocidos, aunque se barajan algunas hipótesis. Su estabilidad luminal y coexpresión con mucinas, así como las grandes concentraciones que alcanzan dentro de la capa de gel mucoso, sugieren que en condiciones basales estas moléculas llevan a cabo su acción protectora actuando como barrera física al interaccionar directamente con el moco en la superficie mucosa, incrementando su densidad óptica, viscosidad y estabilidad física[8]. Otro mecanismo adicional de acción que podrían utilizar sería a través de la unión a receptores específicos en la superficie celular, hecho que se ha comprobado para el TFF3.

El nivel de actuación de estos péptidos parece sobre todo local, pero algunos trabajos avalan también sus efectos protectores al administrarlos por vía sistémica[9,10].

Los PT son motógenos *in vitro,* es decir, promueven la migración de las células epiteliales en cultivo, observándose llamativos patrones de crecimiento difuso. Esta acción, estrechamente vinculada a su función reparativa de la mucosa al facilitar la movilidad celular hacia las zonas lesionadas, parecen ejercerla induciendo una rápida fosforilación de la β-catenina[11] y disminuyendo la expresión de la E-caderina, principal regulador del contacto entre células

epiteliales[12]. También parece que tienen un débil efecto mitógeno y que se relacionan de manera muy estrecha con los factores de crecimiento.

Para comprobar su importancia en la reparación tisular gastrointestinal han sido decisivos los estudios en animales carentes o que sobreexpresan alguno de los factores trefoil. La producción de ratones que carecen del gen que codifica el TFF3 por manipulación genética permitió comprobar que, aunque el crecimiento de los mismos era normal, tenían alterado el recambio celular colónico, lo que se traducía en un deterioro de la capacidad reparativa de la mucosa[13]. Espontáneamente estos animales no desarrollaron lesiones, pero al ser expuestos a cantidades mínimas de irritantes como el sulfato sódico dextrano la mitad de ellos murió a causa de la colitis química resultante. Mientras que en los ratones normales (con el gen del TFF3) las úlceras eran pequeñas y claramente estaban reepitelizando, en los ratones deficientes eran mucho mayores y mostraban una pobre regeneración epitelial. La administración en enema de la proteína recombinante TFF3 a los animales deficientes en la misma y a los que previamente se les había aplicado ácido acético (otro agente inflamatorio experimental) mejoró la migración de las células desde las criptas hacia la superficie epitelial y aceleró la curación de la colitis.

En un modelo de ratones transgénicos que sobreexpresan TFF1 de manera ectópica a nivel intestinal se observó un aumento de la resistencia al daño inducido por indometacina en el yeyuno, lo que refuerza la hipótesis de los que PT estimulan la reparación gastrointestinal[14].

La creación de ratones con el gen del TFF1 inactivado tuvo resultados inesperados: la mucosa gástrica antral y pilórica de los ratones TFF1-deficientes era disfuncional, no producía mucina y exhibía hiperplasia severa y displasia[15]. Todos los animales mostraron, a las pocas semanas de vida, adenomas antropilóricos y el 30% desarrolló carcinomas gástricos intramucosos o intraepiteliales multifocales. Las vellosidades intestinales se habían agrandado y era evidente un anormal infiltrado de células linfoides en el intestino delgado de los ratones de más edad. Estos hallazgos relacionan el TFF1 con la producción de mucina y, sobre todo, destacan su importante papel en la diferenciación de la mucosa gástrica antral y pilórica, lo que ha llevado a los autores del artículo a considerar el gen que lo codifica como un posible gen supresor de tumores a nivel gástrico. Esta posibilidad, aunque muy atractiva, no nos debe hacer olvidar el hecho de que los tumores pueden ser debidos a la acción directa de la bilis y de otros tóxicos sobre las células al faltarles la protección de la capa de moco.

La relación de los PT con los procesos neoplásicos no acaba en la anterior observación, ya que incrementos en la expresión de estos factores se han observado en lesiones tumorales benignas (pólipos hiperplásicos), preneoplásicas (esófago de Barrett) y en tumores malignos (esófago, mama, páncreas, pulmón, ovario, próstata,...). De hecho, el TFF1 fué descubierto en líneas celulares de cáncer de mama y hoy se sabe que más del 50% de los carcinomas de mama expresan TFF1, lo que está significativamente asociado al estado de portador de receptores estrogénicos, a respuesta a la terapia hormonal y a un pronóstico, por tanto, favorable[16]. Centrándonos en los cánceres originados en el tracto gastrointestinal, se sabe que los niveles de expresión de TFF1 y TFF2 están aumentados en el cáncer de esófago y que el 70% de los carcinomas pancreáticos expresan TFF1. Si este incremento en la expresión de los PT es un epifenómeno de la secuencia displasia-neoplasia o un factor que contribuye al fenotipo maligno tendrá que ser establecido en el futuro. En el adenocarcinoma gástrico parece ocurrir lo contrario y hasta en un 50% de ellos no existe expresión de TFF1[17]. De igual manera, mientras que la pérdida completa del factor TFF3 es rara en los carcinomas colónicos, la disminución de sus niveles está significativamente asociada con necrosis tumoral y estadio de Dukes avanzado[18]. Aunque algunos autores consideran la hipótesis de que los genes codificantes de los PT pudieran ser genes supresores en estos dos últimos tipos tumorales, no hay que olvidar que su expresión parece correlacionarse de manera positiva con la diferenciación histológica tumoral, lo que deberíamos tener muy en cuenta a la hora de establecer relaciones de causalidad. Así, la disminución en los niveles de PT podría deberse sencillamente a la progresiva falta de células con capacidad de sintetizarlos conforme avanza el cáncer.

El creciente conocimiento acumulado en los últimos 5-10 años de la participación de los PT en los procesos de protección y reparación a nivel gástrico e intestinal demuestra la vital importancia de estos péptidos en el normal funcionamiento del aparato digestivo. Aunque sus mecanismos de acción están comenzando a ser conocidos ahora, su estabilidad les hace, *a priori,* buenos candidatos a ser utilizados como agentes terapéuticos orales. La ausencia de toxicidad en los modelos de experimentación animal (incluso en los animales transgénicos que los sobreexpresan) habla a favor de su seguridad de utilización. No parece que vayan a aportar nada nuevo al tratamiento de la úlcera péptica, donde la terapéutica erradicadora de Helicobacter pylori es de elección por su gran eficacia y su relativamente bajo coste, pero sí podrían jugar un papel en el tratamiento de enfermedades para las que no existe aún una terapéutica específica como la enfermedad inflamatoria crónica intestinal y las lesiones gastrointestinales secundarias a la administración de AINE, cirugía, radiación ó quimioterapia. Podrían ayudar a promover en estas circunstancias los mecanismos naturales implicados en la reparación epitelial. Dado el sinergismo de acción que parece existir entre estos péptidos y los factores de crecimiento, su utilización conjunta permitiría potenciar los efectos reparadores y minimizar la proliferación celular indeseable debida a la utilización de dosis altas de factores de crecimiento.

Finalmente, y dado que la relación de estos péptidos con los procesos neoplásicos está aún por definir, resulta difícil aventurar su utilidad en este campo. Quizá pudieran ser empleados como factores pronósticos (el TFF1 parece que ya lo es en el cáncer de mama, como hemos comentado) o como marcadores de progresión tumoral y, por tanto, también pronósticos, en algunos tipos de cánceres (el TFF3 se expresa en las metástasis hepáticas del carcinoma colorrectal)[19]. De confirmarse los argumentos que hablan a favor del gen del TFF1 como gen supresor en el cáncer gástrico y del gen del TFF3 en el cáncer de colon (se sabe que el TFF3 induce apoptosis, lo que refuerza su potencial papel supresor), podrían llegar a ser considerados futuras dianas de la nueva terapia génica.

De lo que no cabe la menor duda es de que son moléculas de gran interés para el gastroenterólogo al estar implicadas en dos campos básicos de la patología digestiva como son la inflamación y la progresión tumoral. Esperemos que la reciente elaboración de anticuerpos monoclonales contra estos péptidos y la producción de proteínas recombinantes trefoil en bacterias, sistemas baculovirales o en levaduras, permita un conocimiento más profundo de estos "curalotodo" gastrointestinales. Los digestólogos lo agradeceremos.

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