Curcumin attenuates DNB-induced murine colitis

B. Salh, K. Assi, V. Templeman, K. Parhar, D. Owen, A. Gómez-Muñoz, and K. Jacobson. Curcumin attenuates DNB-induced murine colitis. Am J Physiol Gastrointest Liver Physiol 285: G235–G243, 2003. First published March 13, 2003; 10.1152/ajpgi.00449.2002.—Numerous therapies used for inflammatory bowel disease (IBD) target the transcription factor NF-κB, which is involved in the production of cytokines and chemokines integral for inflammation. Here we show that curcumin, a component of the spice turmeric, is able to attenuate colitis in the dinitrobenzene sulfonic acid (DNB)-induced model of colitis. When given before the induction of colitis it reduced macroscopic damage scores and NF-κB activation. This was accompanied by a reduction of the DNB-induced message for IL-1β and switch on their expression (22, 45). Curcumin is a component of the spice turmeric (Curcuma longa) used in curries and mustard, whose anti-inflammatory properties have been recognized for years (38). These effects are related, in part, to inhibition of the activities of the cyclooxygenase, lipoxygenase, and NF-κB in several cell systems (17). Furthermore, its role in the attenuation of colonic cancer in animal models has also been established (37).

Several cytokines including TNF-α and IL-1β, have been shown to be upregulated in IBD and serve to amplify and perpetuate tissue damage. Furthermore, chemokines are also upregulated, thus providing a continuous signal for the influx of leukocytes (35, 42). The production of chemokines is normally dependent on the coordinated activation of a number of signaling pathways that converge on the transcription factor NF-κB. This is an inducible heterodimeric transcription factor composed most commonly of the Rel A (p65) and NF-κB1 (p50) subunits. Much has been learned about the activation of this molecule, and key steps in its activation by a variety of stimuli are known to include the activation of IKK followed by the phosphorylation and ubiquitin-mediated degradation of inhibitor κBα. This enables the Rel A subunit to translocate to the nucleus and bind to the promoter region of genes and switch on their expression (22, 45).

Regulation of NF-κB function has been documented by several agents used in the management of IBD, such as corticosteroids, sulfasalazine, and 5-aminosalicylates (5-ASA) (51, 55). Furthermore, antisense oligonucleotides directed against the p65 subunit have been shown to attenuate disease activity in an animal model of colitis (32). Recent work has shown that dietary constituents such as curcumin, may also potently inhibit NF-κB (44) and attenuate proinflammatory molecule expression (5, 6, 26). Curcumin is a component of the spice turmeric (Curcuma longa) used in curries and mustard, whose anti-inflammatory properties have been recognized for years (38). These effects are related, in part, to inhibition of the activities of the cyclooxygenase, lipoxygenase, and NF-κB in several cell systems (17). Furthermore, its role in the attenuation of colonic cancer in animal models has also been established (37).

Recognition that the stress-responsive signaling pathways also play a role in the expression of proinflammatory molecules is of critical importance, for a more complete understanding of inflammation (48). In this regard it is well known that the p38 MAPK can modulate a number of different steps in the inflammatory cascade. These include production of γ-interferon by lymphocytes, degranulation of neutrophils, as well as the expression of cyclooxygenase, inhibitory nitric...
oxide synthase, IL-1, and TNF by monocytes (4, 36, 39).

Despite a recent report concerning the potential role of p38 MAPK as a target in IBD (16), its role in IBD pathogenesis remains unclear.

Management of IBD involves the use of 5-ASA and immunosuppressives such as corticosteroids and 6-mercaptopurine as well as its precursor azathioprine (14). Novel agents such as monoclonal antibodies against TNF-α have been developed and demonstrate clinical efficacy (46). However, these agents are expensive and not without side effects. Consequently, there is a need for alternative agents that may be equally or more effective as well as being cheaper. It is interesting to note that both curcumin and sulfasalazine target IKK molecules (21, 53). However, the relevance of inhibition of IKK by curcumin has never been tested in IBD. Here we show that this compound has beneficial effects in a murine model of IBD, and may warrant further scrutiny in human IBD.

MATERIALS AND METHODS

Animal studies. We elected to use the dinitrobenzene sulfonic acid (DNB) murine model of colitis, which has been previously validated (10, 31). Seven-week-old C3H mice were obtained from Charles River, Montreal. Five to six animals were used in each limb of the study and the experiments were repeated at least five times. Inflammation was induced by the intrarectal instillation of 100 µl of DNB (60 mg/ml) in 50% ethanol with control animals receiving 100 µl of 50% ethanol alone. After this, they were kept in a vertical position for 30 s before being returned to their cages. The animals were killed on day 5, postinduction of colitis for evaluation of the colitis. Test groups of five mice had curcumin added to their diet at a concentration of 0.25%, beginning 5 days before the DNB instillation. After death of the animals, several parameters were determined in the inflamed mucosa. These comprised evaluation of macroscopic damage scores, histological evaluation of inflammation by hematoxylin and eosin staining, processing of tissue for Western analysis, and extraction of RNA for RT-PCR analysis. Tissue used for these determinations was processed as previously described (19).

Macroscopic assessment of disease activity was scored by a modification of the Appleyard system (3) as follows: 1 = no damage; 2 = focal hyperemia or ulcer; 3 = ulceration without hyperemia or bowel wall thickening; 4 = ulceration with inflammation at one site, but not more than two sites of ulceration and inflammation and not more than two major sites of ulceration and inflammation, or one site of ulceration extending >1 cm length along the length of colon; 5 = if damage covered >2 cm length along length of colon. Microscopic scoring was performed according to the Ameho criteria (1). After removal from the animal, colon was fixed in 5% formalin and embedded in paraffin before being cut into 4-µm thick sections. They were then stained with hematoxylin and eosin and scored by two different pathologists blinded to the treatment. This score grades the severity of the lesion from 0 to 6 on the basis of the extent of inflammatory infiltrate (superficial mucosal to deeper lesions into the muscularis propria), extent of ulceration, and the presence or absence of necrosis. Neutrophils were counted by examining at least five high-power fields in 10 animals within each of the treatment groups.

Immunohistochemistry. Paraffin-embedded colonic tissue samples were dewaxed in xylene twice for 5 min, rehydrated in a series of ethanol (100–70%) for 3 min each followed by rehydration in PBS for 30 min. After rehydration the endogenous peroxidase was blocked with 0.3% hydrogen peroxide followed by antigen retrieval by microwaving sections in citrate buffer pH 6.0 (10 mM Na citrate). After antigen retrieval, the sections were stained using the above-mentioned kit according to manufacturer’s recommendations but with the following modifications. Sections were incubated with the primary antibody at 4°C overnight. The following antibodies were used at the indicated dilutions: p-38 (1:500), p-p38 (1:100). Sections were stained with Vectastain ABC elite kit and DAB secondary detection kit (Vector Laboratories). Phospho-p38 antibody was purchased from New England Biolabs (Mississauga, ON, Canada). Antibodies to p38 protein were purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Each section had its own control using the secondary antibody only. Preimmune serum was initially used to ensure specificity of the signal with each of the antibodies.

Measurement of MPO activity. MPO activity was measured according to the method described by Wallace (52). Tissue was homogenized in hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer. Aliquots were then added to O-dianisidine hydrochloride solution. Absorbance was read at 450 nm using a microplate reader.

Western analysis. Tissue was placed in homogenization buffer (in mM: 20 MOPS, 50 β-glycerophosphate, 5 EGTA, 1 DTT, 1 sodium vanadate, 1 PMSF, and 50 NaF) and sonicated for 15 s (×2) and centrifuged at 14,000 rpm for 15 min. The protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Mississauga, ON, Canada). Twenty-five micrograms protein from each sample were resolved using 10% SDS-PAGE before transferring to nitrocellulose membranes (Bio-Rad). The blots were blocked in 5% skim milk in 20 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.05% Tween 20 (TBST) for 1 h before probing for 2 h using the appropriate primary antibody. The blots were washed with TBST for 10 min three times, before being incubated with the appropriate secondary antibody for 1 h. After three further washes in TBS-T, they were developed using the enhanced chemiluminescence detection system (Amersham, Montreal, PQ, Canada).

Isolation of RNA and PCR. Isolated colon was snap frozen in liquid nitrogen. RNA was isolated using the TRIzol method (Life Technologies, Burlington, ON, Canada). The purity of the RNA was determined by running 1 µg of RNA on a 1% agarose gel for 1.5 h at 75 V. One microgram of RNA was reverse transcribed using 0.5 µg of oligo(dT)₁₂₋₁₅ (Amersham), 1 µl of 10 mM 2-deoxyribonucleotide 5′-triphosphate (dNTP), 2 µl of 0.1 M DTT, 40 units of RNA-guard (Amersham) in 1 × first-strand buffer (Life Technologies) using 200 units of Moloney murine leukaemia virus RT, by incubating the reaction mixture for 50 min at 37°C. Two microliters of cDNA were used in each subsequent PCR reaction. For each 50 µl PCR reaction, 2 units of Thermus aquatilis DNA polymerase (PE Biosystems, Branchburg, New Jersey), 1× PCR Buffer (PE Biosystems), 10 pmol of each primer, 1 µl of 10 mM dNTP, and 3 µl of 25 mM MgCl₂ were used. The PCR temperatures used were 94°C denaturing for 45 s, 56°C annealing for 45 s, and 72°C extension for 1 min. Ten microliters aliquots of the reaction were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Negative controls for cDNA synthesis were run without template, and also without RT. Linearity of PCR reactions was determined in the range between 20 and 30 cycles. Sensitometry was performed by using Bio-Rad Quantity-One software. The sizes of the PCR products were 563 and 851 bp for IL-1β and...
curcumin, compared with the DNB group (Fig. 1). From the accompanying animal weight data loading dose, animals had colitis induced by using the intestinal lumen for DNB treatment, whereas Fig. 2 illustrates macroscopic damage induced by the hapten DNB (*P < 0.001).

Disease severity was evaluated based on a macroscopic score. As the data indicate (Fig. 1B), there was a predictable increase in inflammation in response to the induction of colitis with DNB. This was significantly attenuated in those animals, which had been prefed curcumin-containing chow (P < 0.001). Notably there was no increase in basal inflammation due to the diet alone. Thus, from this data, it would appear that animals are able to tolerate curcumin at these concentrations and that there was a beneficial response observed in the attenuation of DNB-induced colitis.

**Histological improvement in response to curcumin.** The removed colons were sectioned, fixed, and stained with hematoxylin and eosin. As the representative sections show (Fig. 2), there is a reduction in the inflammatory cellular infiltrate, mucosal and muscle damage, as well as wall thickening, observed with pretreatment with curcumin before DNB-induced colitis. Figure 2, A and C represent cross sections through the intestinal lumen for DNB treatment, whereas Fig. 2, B and D demonstrate longitudinal sections. Fig. 2, E and G are the corresponding sections for curcumin pretreatment, and Fig. 2, F and H are the longitudinal sections for comparison. Curcumin alone did not result

**RESULTS**

**DNB attenuates macroscopic tissue damage.** Although curcumin has been shown to be safe ≤100,000 ppm (10%), we elected to use a concentration of the same order of magnitude previously shown to be effective in murine cancer models: 2,500 ppm or 0.25% (2, 37). The curcumin was accurately weighed out and blended into the chow. Assessment of daily weights and evaluation of feeding behavior indicated that there was no effect on food intake at this dose. After a 5-day loading dose, animals had colitis induced by using DNB. From the accompanying animal weight data (Fig. 1A) there appears to be a significant reduction in weight loss in the diseased animals that were given curcumin, compared with the DNB group (P = 0.005).

**Real-time PCR for IL-1β.** Real-time PCR was performed by using an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), as previously described (28, 29). This relies on the SYBRgreen I dye binding to the dsDNA directly in the reaction tube. The software detects the threshold cycle number (Ct) when signals reach 10-fold the standard deviation of the baseline. It has been previously reported that the Ct values are a quantitative measurement for the mRNA being tested (13). Each reaction contained 25 μl of 2 × SYBR Green Master Mix (containing 200 μM deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; 400 μM deoxyuridine triphosphate; 2.5 mmol/l MgCl2; and 0.625 U AmpliTaq Gold DNA polymerase). Primers were as described above, and actin primers were as described below, and actin primers were as described below.
in any dramatic changes in the intestinal mucosa other than a mild increase in the lymphocytic infiltrate (data not shown). A time course study was performed to assess the characteristics of the curcumin-induced response. The data indicate that there is a significant reduction in the histological damage score at both days 2 and 4 postinduction of colitis. At 10 days postinduction of colitis, there appears to be a similar trend toward improvement; however, it is also apparent that the disease resolves spontaneously. This data support the macroscopic damage scores in validating an anti-inflammatory role for curcumin in this model of colitis.

Reduction in inflammatory markers by curcumin pretreatment. Myeloperoxidase activity is an established marker for inflammatory cell (mainly neutrophils) infiltration and activity in murine models of colitis and was thus examined. Data clearly indicate (Fig. 3A) a significant reduction in this parameter on day 5 postinduction of colitis ($P = 0.005$). This was accompanied by a reduction in the number of neutrophils within the histology samples. We counted an average of $8.4 \pm 0.9$ (means $\pm$ SE) cells/high-power field in the DNB-treated animals, whereas there were only $4.33 \pm 0.8$ cells/high-power field in the curcumin pretreated animals ($P = 0.0007$). This compared with only $0.7 \pm 0.21$ cells/high-power field in the control (PBS treated) animals.

Another potential mechanism whereby curcumin achieves its effects within the intestine was then examined, expression of proinflammatory cytokines. The message for one of the best-characterized cytokines in IBD, IL-1β, (11, 30) was determined by using semiquantitative RT-PCR with GAPDH acting as an internal control (54). The data show (Fig. 3, B and C) that there was a significant reduction in the DNB-induced IL-1β message after pretreatment with curcumin. This
Finding was validated by using real-time PCR, which revealed CT values of 31.67/H110060.11, 29.26/H110060.35, and 32.97/H110060.34 for the control, DNB-treated, and curcumin-pretreated DNB animal groups, respectively (means/H11006SD). The corresponding CT values were obtained by subtracting the CT for -actin from these values and were 16.21, 14.06, and 17.38, respectively. Collectively, these findings confirm an anti-inflammatory effect for curcumin in murine colitis.

Reduced NF-κB DNA binding in vivo. The next step was to determine whether or not curcumin altered NF-κB DNA binding, because this has been widely documented to occur in various cell culture models. Five micrograms aliquots of total protein lysate were incubated with the probe and resolved on nondenaturing polyacrylamide gels. A representative autoradiogram (Fig. 4) indicates that there is minimal basal binding within the intestine, and that this is dramatically increased on exposure of the animals to DNB (a control using CaCo-2 cells stimulated with IL-1β is shown with the actual band and a nonspecific band shown for comparison). The curcumin-treated control animals show little change in this parameter. When animals were pretreated with curcumin, however, there was a clear reduction in DNA binding, thus verifying that curcumin does indeed inhibit NF-κB activation in the colon in vivo. These findings indicate for the first time that curcumin is able to impact on an important transcriptional mechanism in the gastrointestinal tract.

Curcumin attenuates activation of p38 MAPK. In previous work curcumin has been reported to influence MAPK signaling, in addition to its effects on IKK regulation (7, 21). Therefore, we chose to investigate this signaling nexus in the DNB model. For this work, we carried out a time course in which the expression and activation of the p38 MAPK were examined by Western analysis. We made use of phosphospecific MAPK antibodies, which correlate with the activation of the respective family members. The data indicate (Fig. 5M) that there is an early activation of p38 MAPK as well as p42/44 ERKs, however, whereas curcumin led to an activation of only p38 MAPK, DNB activated both MAPKs. Intriguingly, whereas the p38 MAPK is
inhibited in the curcumin-treated group, the p44/42 ERK activity is unaffected. It should be emphasized that the control samples depicted were those treated with ethanol and not PBS. The latter animals exhibited lower levels of p38 MAPK activity (data not shown). Remarkably, p38 MAPK inhibition was also observed at the 48-h time point (Fig. 5N).

**Site-specific curcumin attenuation of p38 MAPK activation.** Because a Western immunoblot gives no indication of the location of the observed changes in p38 MAPK activity, this component was investigated in tissue sections by using the same antibody. There was an impressive increase in the active p38 MAPK signal in the nuclei of epithelial cells (Fig. 5I, red arrows). Other structures such as the smooth muscle and inflammatory cells also revealed evidence of p38 MAPK activity (data not shown). When the curcumin pre-treated DNB sections were examined, in direct agreement with the Western data, there was a dramatic reduction in the activity of p38 MAPK, especially at the

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**Fig. 5.** Specific modulation of p38 MAPK and not p42/44 MAPK in vivo by curcumin. A–L: site-specific modulation of p38 MAPK activity. The p38 MAPK antibodies were used in immunohistochemical studies to determine the site of change in p38 MAPK activity. Specimens were counterstained with hematoxylin and eosin. Each vertical panel represents the sample probed with isotypic control (A, D, G, and J), the protein antibody (p38, B, E, H, and K), and the activation-specific antibody (P-p38, C, F, I, and L). A–C: control (ethanol-treated animals), D–F: curcumin-treated, G–I: DNB-treated; J–L: curcumin-pretreated animals subsequently given DNB. Red arrows indicate the sites of significant signal intensity. Data obtained is representative of the experiment performed on 3 separate occasions. M: tissue lysates from 2 animals/group were resolved by 10% SDS-PAGE, and samples were transferred to nitrocellulose membranes. After blocking, membranes were incubated with the following antibodies: p38 MAPK, p-p38MAPK, p44 Erk, and p42/44 p-Erk (phosphospecific MAPK antibodies were obtained from New England Biolabs). Membranes were subsequently washed extensively and developed as described in MATERIALS AND METHODS. N: one representative experiment using 4 animals/group. Time points at which samples were analyzed are indicated on the left.
DISCUSSION

To our knowledge, this is the first evaluation of curcumin and its effects on the MAPKs and NF-κB in an experimental model of IBD. In addition to the demonstration that it is able to attenuate inflammatory activity in IBD, we have also shown that it can reduce NF-κB DNA binding activity as well as inhibiting the activation of p38 MAPK. We have shown that the anti-inflammatory effects of curcumin involve a reduction in p38 MAPK signaling through these structures.

Precisely how curcumin achieves its effects is not clear. It has been shown to possess free radical scavenging (antioxidant) properties and in addition to its known effects on the activation of NF-κB. Its in vivo effects may well rely on a complementation of these two and other activities. Previous work has documented the beneficial effects of compounds that quench free radicals, such as the membrane-permeable radical scavenger tempol in experimental colitis (9). It is well established that generation of free radicals can effect the activation of multiple intracellular signaling pathways (27). Inhibition of this process would be expected to correlate with a reduction in the expression of key inflammatory molecules; indeed, we show that there is a reduction of the expression of the proinflammatory cytokine, IL-1β. Moreover, curcumin has not only been shown to inhibit the proliferation and effect apoptosis of T lymphocytes (43), but also to inhibit the production of IL-12 by LPS-stimulated macrophages (24). Its other properties include inhibition of TNF-induced adhesion molecule expression on endothelial cells (26), as well as TPA-induced lipoxygenase and cyclooxygenase activities in mouse epidermis (17). Thus its effects are likely to occur at multiple sites in vivo, and further work will be required to correlate which of these are important for attenuation of colitis.

Previous work has clearly indicated an involvement of the MAPK in the inflammatory responses of epithelial cells (15, 21). More recent work has demonstrated that use of p38 MAPK inhibitors can be effective for human IBD (16). Moreover, the involvement of p38 MAPK in human IBD has also been addressed (50). Thus, whereas it was anticipated that the inflammatory response may entail an activation of the p38 MAPK in the infiltrating immune cells, it was unexpected and surprising to observe such prominent activity within the other tissue components. This was most obvious in the epithelial cell nuclei and to a lesser extent within the smooth muscle. The exact role that the activated p38 MAPKs play at these sites remains unclear and will require further investigation, but it may involve secretion of chemokines, neuropeptides, trophic factors, or possibly even reflect a response to cell damage (8, 12). Significantly, it has been shown that histone phosphorylation on the promoters of certain proinflammatory genes, is regulated by p38 MAPK and enhances NF-κB recruitment (40). With specific reference to epithelial cells, we have shown a role for p38 MAPK (using pharmacological inhibitors and transfection of the dominant negative construct) in regulation of IL-1β-induced IL-8 promoter activity and IL-8 gene expression (35a). Other mechanisms may also be involved in regulation of IL-8 in intestinal epithelial cells at the posttranscriptional level (20).

Curcumin alone also influences p38 MAPK activation, as seen in the Western immunoblot, as well as on immunohistochemistry (Fig. 5). This will merit further investigation and is not dissimilar to the activation that has been documented with the anti-TNF antibody previously reported (50). This duality of function of p38 MAPK, i.e., activation in response to the disease and its treatment, is important and may offer an explanation of why specific inhibition using SB-203580 was not beneficial in a murine model of colitis (47). The authors of this work indicated that inhibition of IL-10 production by T regulatory cells in the lamina propria may be a potential mechanism (25).

Although curcumin has been shown to be safe up to levels as high as 10% (100,000 ppm), we have shown an effect at a concentration as low as 0.25%. This dose was well tolerated and we observed no reduction in dietary intake and, consequently, weight in these animals. Further work will help to clarify the optimal dose for this and other models of IBD. Additionally, comparison with other standard agents such as 5-ASA and sulfasalazine, which are compounds effective only in mild and moderate disease, will be of obvious importance. Curcumin may prove to be a cheap, well-tolerated, and effective therapy.

In conclusion, we report an intriguing immunomodulatory effect for a dietary component that has for generations been regarded as a potent anti-inflammatory within many eastern civilizations. It is equally intriguing that the same agent is a potent antineoplastic agent. It is proposed that it may hold promise for the treatment of IBD in humans.

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