COLON

Angiogenesis blockade as a new therapeutic approach to experimental colitis

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Background: Neoangiogenesis is a critical component of chronic inflammatory disorders. Inhibition of angiogenesis is an effective treatment in animal models of inflammation, but has not been tested in experimental colitis.
Aim: To investigate the effect of ATN-161, an anti-angiogenic compound, on the course of experimental murine colitis.
Method: Interleukin 10-deficient ($IL10^{-/-}$) mice and wild-type mice were kept in ultra-barrier facilities (UBF) or conventional housing, and used for experimental conditions. Dextran sodium sulphate (DSS)-treated mice were used as a model of acute colitis. Mice were treated with ATN-161 or its scrambled peptide ATN-163. Mucosal neoangiogenesis and mean vascular density (MVD) were assessed by CD31 staining. A Disease Activity Index (DAI) was determined, and the severity of colitis was determined by a histological score. Colonic cytokine production was measured by ELISA, and lamina propria mononuclear cell proliferation by thymidine incorporation.
Result: MVD increased in parallel with disease progression in $IL10^{-/-}$ mice kept in conventional housing, but not in $IL10^{-/-}$ mice kept in UBF. Angiogenesis also occurred in DSS-treated animals. $IL10^{-/-}$ mice with established disease treated with ATN-161, but not with ATN-163, showed a significant and progressive decrease in DAI. The histological colitis score was significantly lower in ATN-161-treated mice than in scrambled peptide-treated mice. Inhibition of angiogenesis was confirmed by a significant decrease of MVD in ATN-161-treated mice than in ATN-163-treated mice. No therapeutic effects were observed in the DSS model of colitis. ATN-161 showed no direct immunomodulatory activity in vitro.
Conclusion: Active angiogenesis occurs in the gut of $IL10^{-/-}$ and DSS-treated colitic mice and parallels disease progression. ATN-161 effectively decreases angiogenesis as well as clinical severity and histological inflammation in $IL10^{-/-}$ mice but not in the DDS model of inflammatory bowel disease (IBD). The results provide the rational basis for considering anti-angiogenic strategies in the treatment of IBD in humans.

Angiogenesis, the process of formation of new capillaries from pre-existing vasculature in adult tissues, is a fundamental constituent of many complex biological processes, including growth, development and repair.[1] In the last three decades, angiogenesis has emerged as a phenomenon essential for the growth of tumours, and its inhibition has been hailed as a major paradigm shift in the treatment of cancer.[2] However, the importance of angiogenesis extends well beyond cancer biology, and has been shown to be an integral component of non-neoplastic chronic inflammatory and autoimmune diseases as diverse as atherosclerosis, rheumatoid arthritis, diabetic retinopathy, psoriasis, airway inflammation, peptic ulcers and Alzheimer's disease.[1 3 4]

The growth of new blood vessels is a phenomenon intrinsic to inflammation and is associated with morphological and functional changes, including the activation and proliferation of endothelial cells as well as capillary and venule remodelling, all of which result in an expansion of the tissue microvascular bed.[5–7] A crucial functional consequence of this expansion is the promotion of further inflammation through various inter-related mechanisms. There is an increased influx of inflammatory cells, an increased nutrient supply that keeps feeding a metabolically active immune process, and the production of cytokines, chemokines and matrix metalloproteinases by the locally activated endothelium.[8 9] Consequently, the anatomical expansion of the microvascular bed, combined with its increased functional activation, foster recruitment of additional inflammatory cells, and angiogenesis and inflammation become codependent processes.[5 7 9 10]

We have recently reported that angiogenesis is a new component of the pathogenesis of inflammatory bowel disease (IBD).[11] The intestinal microvasculature undergoes intense angiogenesis in both Crohn's disease and ulcerative colitis, as demonstrated by increased microvascular density, upregulation and activation of angiogenic integrins (eg, $\alpha_v\beta_3$), and overexpression of functionally active angiogenic mediators in the inflamed mucosal milieu.[11] The importance of pathological angiogenesis resides in its therapeutic implications. Indeed, the ''disease-feeding'' tissue microvasculature has recently emerged as a prime target in neoplastic conditions and also in inflammatory conditions because of the pathogenic importance of immune-driven angiogenesis in autoimmune and inflammatory conditions.[1 3] In particular, the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ have been implicated in several inflammatory conditions that include arthritis, atherosclerosis and retinal neovascularisation.[8 12–17] Many of the pathogenic pathways involved in experimental angiogenesis are also relevant to inflammation, and therefore it is reasonable to hypothesise that anti-angiogenic treatment may also be effective in animal models of IBD. The present study investigated whether angiogenesis

Abbreviations: DAI, Disease Activity Index; DSS, dextran sodium sulphate; IBD, inflammatory bowel disease; IL, interleukin; IP, intraperitoneal; LPMC, lamina propria mononuclear cells; LPS, lipopolysaccharide; MVD, mean vascular density; PBS, phosphate-buffered saline; UBF, ultra-barrier facility; VEGF, vascular endothelial growth factor; WT, wild type

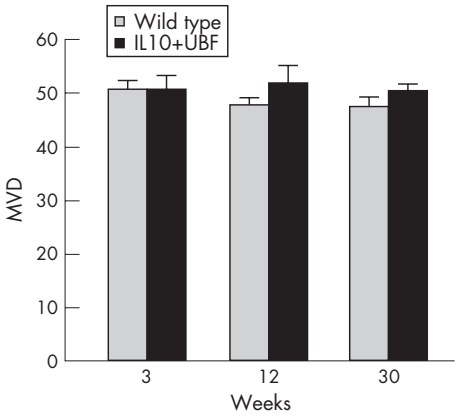

Figure 1 Lack of effect of interleukin 10-deficient (IL $10^{-/-}$) status on intestinal vascularisation. $IL10^{-/-}$ mice kept in ultra-barrier facility (UBF; n = 4 for each time point) and wild-type littermates kept in conventional housing (n = 5 for each time point) were killed at the indicated time points and their colons removed. Colonic tissue sections were immunostained for CD31 to detect the mucosal microvasculature, and vascular density was measured by computerised morphometry. MVD, mean vascular density.

occurs in experimental murine colitis, and whether the inhibition of angiogenesis using an $\alpha_{v\beta3}/\alpha_{5\beta1}$-binding peptide (ATN-161), which is currently in phase II trials in patients with cancer, can block experimental intestinal inflammation.[18 19]

MATERIALS AND METHODS

Animals

Interleukin 10-deficient ($IL10^{-/-}$) mice on a C57BL/10 background and wild-type (WT; $IL10^{+/+}$) mice were obtained from Dr Alan Sher (Bethesda, Maryland, USA) with permission from Dr Werner Muller (Frankfurt, Germany).[20] For the dextran sodium sulphate (DSS) model of colitis,[21] 10-week-old C57B/BL/19 mice were used. All mice, negative for intestinal *Helicobacter hepaticus*, were kept in specific pathogen-free housing and fed autoclaved mouse chow and water. The Institutional Animal Care and Use Committee approved all experimental protocols.

Induction and evaluation of colonic inflammation

As reported previously,[20] 3–4-week-old $IL10^{-/-}$ mice consistently develop colitis at 10–12 weeks of age when transported from the ultra-barrier facility (UBF) to conventional housing. A previously described Disease Activity Index (DAI) was calculated by scoring 1 point for the appearance of each of the following: ruffled fur, occult faecal blood as determined on a Hemoccult Sensa Card (Smith Kline Diagnostics, San Jose, California, USA), rectal prolapse <1 mm and soft stool. The mice were scored an additional point for diarrhoea or severe rectal prolapse >1 mm.[20]

For the DSS-induced colitis model, colonic inflammation was induced in C57B/BL/19 mice by the administration of 3% DSS (molecular mass 40 kDa; ICN Biomedicals, Aurora, Ohio, USA) in filter-purified (Millipore, Bedford, Maryland, USA) drinking water for 8 days, as described previously.[21] Grading of intestinal inflammation was determined in a blinded fashion, as described previously,[21] by three readers: 0, no inflammation; 1, modest numbers of infiltrating cells in the lamina propria; 2, infiltration of mononuclear cells leading to separation of crypts and mild mucosal hyperplasia; 3, massive infiltration with inflammatory cells accompanied by disrupted mucosal architecture, loss of goblet cells, and marked mucosal hyperplasia; and 4, all of the above and crypt abscesses or ulceration, the histological score varying from 0 to 15 points.

Angiogenesis blockade by ATN-161 administration

ATN-161 (Ac-PHSCN-NH_2), a five-amino acid peptide with documented anti-angiogenic activity, was manufactured under Good Manufacturing Practices as described previously.[19] Fifteen-week-old $IL10^{-/-}$ mice with fully established colitis, as determined by weight loss, diarrhoea, bloody stools and rectal prolapse, were given intra-peritoneal (IP) injections of ATN-161 (1 mg/kg), its scramble peptide ATN-163 (Ac-HSPNC-NH_2; 1 mg/kg) or only phosphate-buffered saline (PBS) on alternate days, for a period of 6 weeks. Another group of animals was submitted to prophylactic treatment with ATN-161. Three-week-old $IL10^{-/-}$ mice were moved from UBF to conventional facilities and, starting at week 8, given IP injections of ATN-161, ATN-163 or PBS on alternate days for the duration of the period necessary to develop colitis (until 10–12 weeks). In the DSS model of colitis, ATN-161, ATN-163 or PBS were given IP either every day or on alternate days for a period of 10 days.

Immunostaining and morphometric analysis of the intestinal microvasculature

Immunostaining was performed as described previously.[22] Paraffin-wax-embedded intestinal sections were cut at 3 μm thickness, deparaffinised, hydrated, blocked for endogenous peroxidase using 3% H_2O_2/H_2O and subsequently subjected to microwave epitope retrieval using a Dako target retrieval solution (BD PharMingen, California, USA) at pH 10.00. Incubation with a primary antibody cocktail containing anti-CD31 (BD PharMingen) was carried out at 1:200 dilution for 30 min at room temperature. Detection was achieved using a standard streptavidin–biotin system (BD PharMingen), and antigen localisation was visualised with 3′,3-diaminobenzidene (BD PharMingen).

Computerised morphometric analysis was carried out as reported recently,[11] using an international consensus method for quantification of angiogenesis.[23 24] Immunostained colonic sections were screened at low power (×40) to detect the most vascularised area, after which at least five microphotographs at high magnification (×200) of the mucosa were taken, the mucosa being defined as the area above the muscularis mucosae. The number of vessels/field (measured as mean vascular density (MVD)) was obtained using an Optronics Color digital camera (Olympus Corporation, Tokyo, Japan). Quantitative analysis was performed using Image Pro Plus (Media Cybernetics, Silver Spring, Maryland, USA).

Measurement of mucosal and cell-derived cytokines and proliferation assay

Murine colonic fragments were collected and maintained in organ culture. After 24 h, supernatants were collected, centrifuged at 900 *g* for 15 min, assayed for protein concentration (Bio-Rad Laboratories, Hercules, California, USA) and then frozen until analysis.[25] Lamina propria mononuclear cells (LPMCs) were isolated as reported previously.[20] LPMCs were seeded in 96-well round-bottom culture plates at a density of 3×10^6 cells/ml in 200 μl of complete medium, and stimulated with lipopolysaccharide (LPS; 10 μg/ml) or anti-CD3+CD28 (1 μg/ml), in the presence or absence of ATN-161 (10 μg/ml). After 48 h, supernatants were collected for ELISA. IL12 was measured as reported previously.[20] Briefly, 96-well ELISA plates (Dynatech, Chantilly, Virginia, USA) were coated for 1 h at 37°C with 4 μg/ml of C15.6 monoclonal antibody against mouse IL12 (grown as an ascites fluid in nude mice and purified by protein G Sepharose; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). The plate was washed five times with PBS/0.05% Tween wash buffer and blocked at room temperature for 2 h with 200 μl 5% bovine serum albumin in

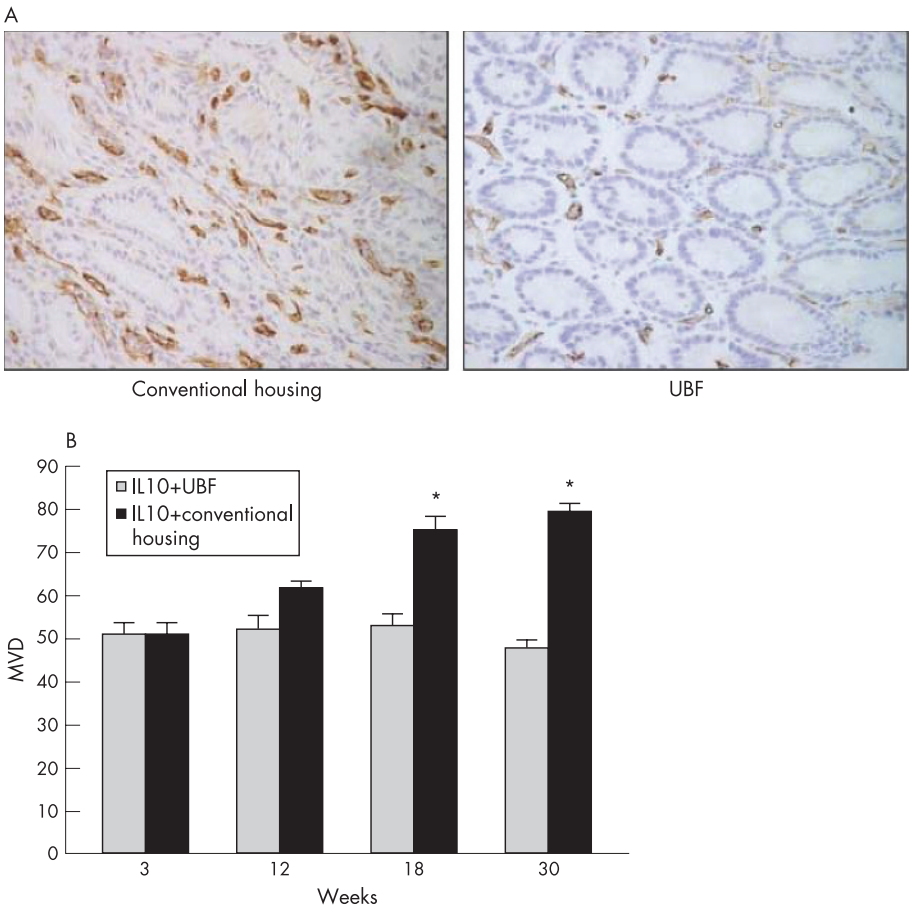

Figure 2 Increased vascularisation of the colonic mucosa in colitic interleukin 10-deficient (IL $10^{-/-}$) mice. $IL10^{-/-}$ mice kept in ultra-barrier facility (UBF) or conventional housing (n = 5 for each time point) were killed at different time points. Their colons were removed and immunostained for CD31 to detect the mucosal microvasculature, and vascular density was measured by computerised morphometry. (A) Increased vascularisation in the colon of an $IL10^{-/-}$ mouse kept in conventional housing (week 18) compared with the colon of a mouse kept in the UBF facility. (B) Progressive increase of the colonic microvasculature paralleling the evolution of colitis in $IL10^{-/-}$ mice kept in conventional housing. *$p<0.05$. MVD, mean vascular density.

PBS (Sigma, St Louis, Missouri, USA). After five washes with PBS/Tween, 40 µl of supernatant or murine recombinant IL12 standard (5000–78 pg/ml in twofold serial dilutions; R&D Systems, Minneapolis, Minnesota, USA) was added to the plate in duplicate at room temperature for 2 h. The plate was washed five times with PBS/Tween, followed by 1 µg/ml of biotinylated C17.8 (PharMingen, San Diego, California, USA) for 1 h at room temperature. After washing five times, a 1:4000 dilution of a horseradish peroxidase–streptavidin conjugate (Zymed, San Francisco, California, USA) was added to each well for 30 min at room temperature. The plate was washed seven times and developed with 1:1 vol/vol of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonate) peroxidase substrate and H_2O_2 (Kirkegaard and Perry Labs, Gaithersburg, Maryland, USA) for 30 min at room temperature. Absorbance was read at 405 nm using a microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA). IL6 was measured using a commercially available ELISA (R&D Systems).

LPMC proliferation was assessed as reported previously.[26] LPMCs isolated from the normal colon of $IL10^{-/-}$ mice kept under UBF conditions were cultured for 3 days in 96-well round-bottom culture plates (Falcon) at a density of 3×10^6 cells/ml in 200 µl of complete medium, and stimulated with LPS (10 µg/ml) or anti-CD3 (1 µg/ml), in the presence or absence of ATN-161 (10 µg/ml). Eighteen hours before the end of the culture, 0.5 µCi of [^{3}H] thymidine/well (DuPont NEN, Boston, Massachusetts, USA) was added, and proliferation measured in a scintillation counter.

Statistical analysis

Data were analysed by analysis of variance, followed by the appropriate post hoc test (GraphPad Software, San Diego, California, USA) and expressed as mean (SEM). Significance was set at $p<0.05$.

RESULTS

Evidence of increased angiogenesis during the progression of experimental colitis

To obtain objective evidence that angiogenesis is a component of experimental colitis, we initially performed a quantitative analysis of the mucosal microvasculature in the $IL10^{-/-}$ and the DSS models of colitis. The analysis was performed by immunohistochemical staining of murine colonic tissues with antibodies specifically recognising CD31, a well-known endothelial cell marker, and by measuring microvascular density by quantitative morphometry.[11] First, to investigate whether the $IL10^{-/-}$ status alone would affect the mucosal microvasculature, age-matched $IL10^{-/-}$ mice kept in UBF and WT mice kept in conventional housing (both of which failed to develop colitis) were evaluated at several time points during the course of experimental colitis. At 3, 12 and 30 weeks of age, no differences were found in MVD between $IL10^{-/-}$ mice and their

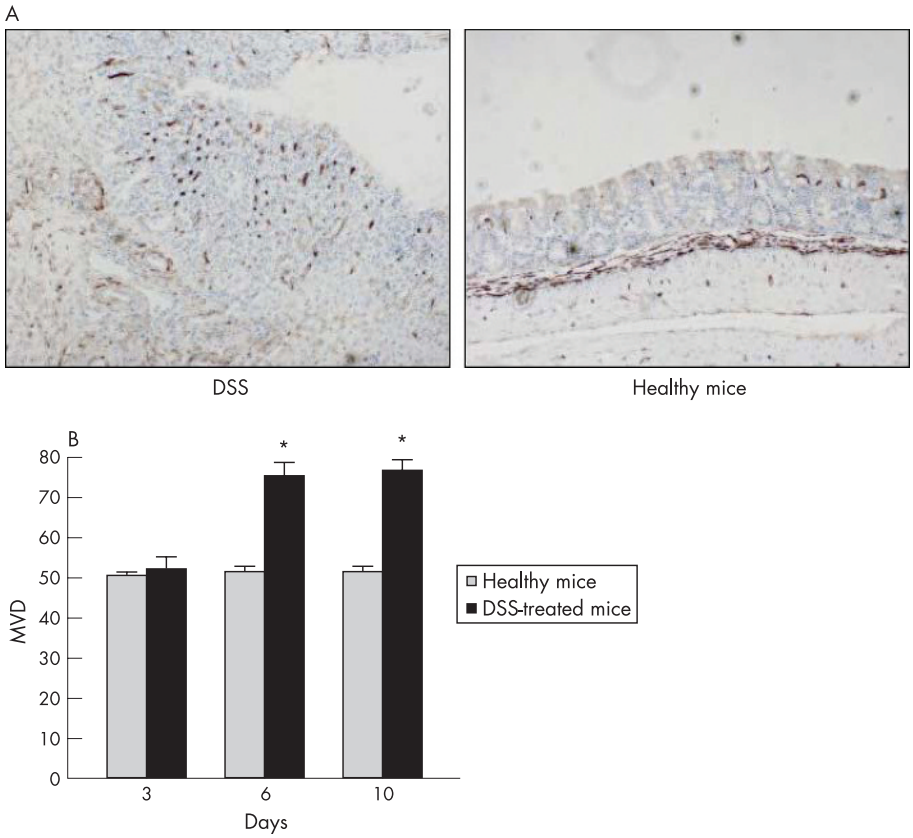

Figure 3 Increased vascularisation of the colonic mucosa in mice with dextran sodium sulphate (DSS)-induced colitis. Wild-type mice treated with DSS (n = 5 for each time point) were killed at different time points. Their colons were removed and stained for CD31 to detect the mucosal microvasculature, and vascular density was measured by computerised morphometry. (A) Increased vascularisation in the colon of a mouse given DSS (day 10) compared with the colon of a normal control mouse. (B) Increase in the colonic microvasculature accompanying the development of DSS-induced colitis. *p<0.05. MVD, mean vascular density.

age-matched WT controls (fig 1). Then, to test whether angiogenesis occurs with the progression of colitis, angiogenesis was assessed in IL10$^{-/-}$ mice kept in UBF (which failed to develop colitis) and conventional housing (which developed colitis). MVD increased in parallel with disease progression in IL10$^{-/-}$ mice kept in conventional housing from 12 (61 (2) MVD) to 18 (74 (3) MVD, p<0.01) and 30 (79 (2) MVD, p<0.01) compared with age-matched UBF mice) (fig 2A,B). In mice that did not develop colitis, there were no significant changes in MVD over time (ie, 3 vs 12 vs 30 weeks). In the DSS model of colitis, a progressive and significant (p<0.01) increase in MVD was also detected after 6 (75 (4) MVD) and 10 (76 (3) MVD) days of induction of colitis compared with healthy mice (50 (1) MVD p<0.01; fig 3A,B).

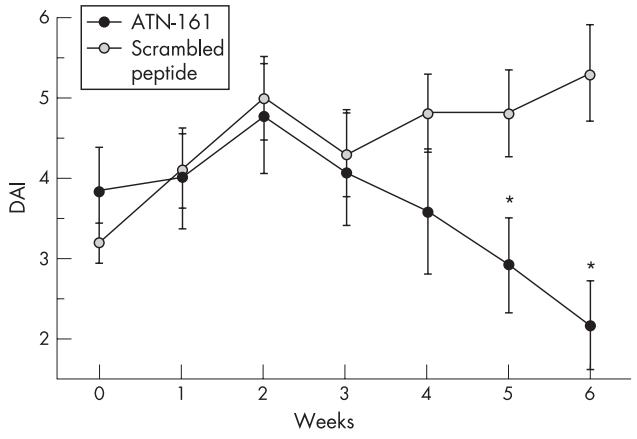

Figure 4 Therapeutic effect of angiogenesis blockade in the interleukin 10-deficient model of colitis. Fifteen-week-old mice with fully established colitis, as determined by weight loss, diarrhoea, bloody stools and rectal prolapse, were given intraperitoneal injections of ATN-161 (n = 12) or its scrambled peptide ATN-163 (n = 10) for 6 weeks on alternate days, and the Disease Activity Index (DAI) was assessed weekly. *p<0.05.

Effect of angiogenesis blockade on clinical parameters in experimental colitis

We have previously demonstrated that intestinal angiogenesis in patients with IBD is associated with a significant upregulation of the integrin $\alpha_V\beta_3$, which is known to promote angiogenesis.[11] We have also observed that integrin $\alpha_5\beta_1$ is expressed in murine normal and inflamed intestinal endothelium. On the basis of these observations, we investigated whether ATN-161, a peptide drug that binds to $\alpha_V\beta_3$ and to $\alpha_5\beta_1$, would inhibit intestinal angiogenesis in experimental murine colitis.

One group of 15-week-old IL10$^{-/-}$ animals with fully established colitis, as determined by weight loss, diarrhoea, bloody stools and rectal prolapse, was given IP injections of ATN-161 (1 mg/kg) on alternate days. As a control, a similar group of animals received the scrambled peptide ATN-163 (1 mg/kg) using the same administration schedule as for ATN-161. Follow-up over a period of 6 weeks showed a significant decline of the DAI in the ATN-161-treated group, which

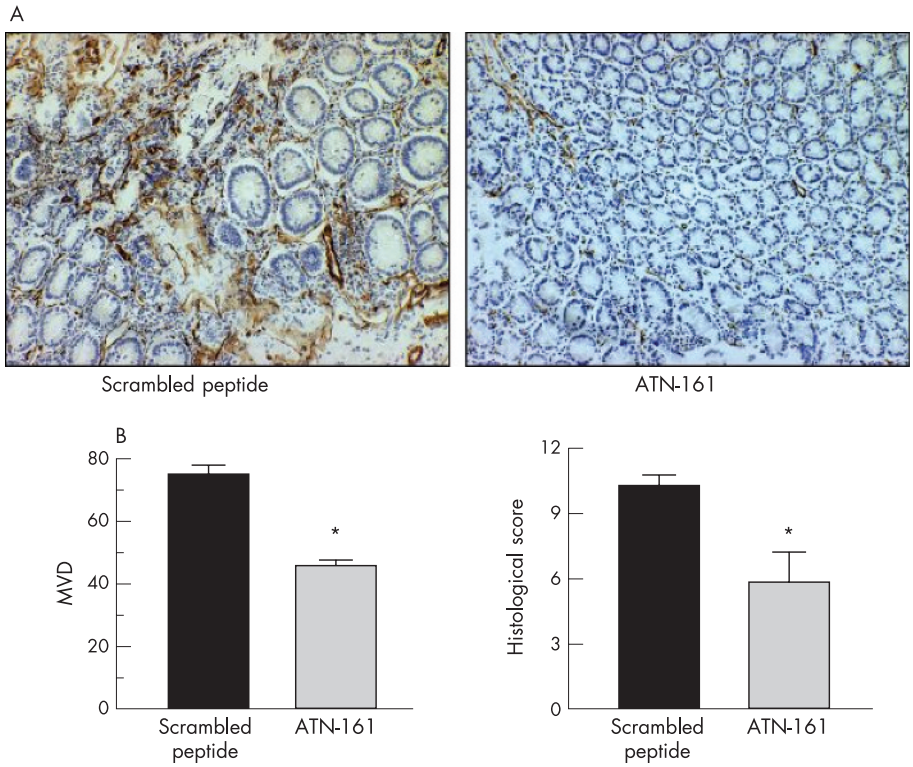

Figure 5 Reduction in vascular density in the inflamed gut mucosa by angiogenesis blockade. Fifteen-week-old mice with fully established colitis were given intraperitoneal injections of ATN-161 (n = 12) or its scrambled peptide ATN-163 (n = 10) on alternate days. After 6 weeks, animals were killed, their colons were removed and colonic tissue sections immunostained for CD31. (A) Decreased vascularisation in the colon of an interleukin 10-deficient mouse treated with ATN-161 compared with a mouse treated with ATN-163. (B) Decrease in vascular density and histological score after ATN-161 treatment. * $p<0.05$. MVD, mean vascular density.

dropped to 50% of the initial DAI by the end of week 6. By contrast, the ATN-163-treated group continued to deteriorate clinically (fig 4). The decrease in DAI was significantly different ($p<0.002$) between the ATN-161-treated and ATN-163-treated groups. No significant differences in DAI were observed between untreated mice and ATN-163-treated mice (data not shown). ATN-161 seemed to work only in animals with established disease, as prophylactic treatment with ATN-161 or ATN-163 failed to prevent disease progression.

The anti-angiogenic activity of ATN-161 was also tested in the DSS model of colitis. No improvement of colitis and differences in DAI were observed between colitic mice that received ATN-161 and those that received ATN-163 over 10-days of treatment (data not shown). The clinical scores in ATN-163-treated mice were comparable to those of mice that developed DSS-induced colitis but were left untreated (data not shown).

Effect of angiogenesis blockade on colonic inflammation and vascular density

To demonstrate that the clinical benefits observed in colitic $IL10^{-/-}$ mice were due to anti-angiogenic effects of ATN-161, animals were killed at week 22 and the colons examined. After zinc-based fixation, tissue was stained for CD31 and examined for the presence of inflammation and number of vessels in the mucosa. Animals that received the scrambled peptide showed the same degree of inflammation as those that were untreated (data not shown), with numerous vessels scattered among inflammatory infiltrates, whereas the animals treated with ATN-161 displayed an essentially normal mucosa (fig 5A). When vascular density was quantified, ATN-161-treated mice contained 30% fewer vessels in the mucosa than those treated with control peptide (fig 5B).

The histological colitis score was significantly lower in ATN-161-treated mice than in ATN-163-treated mice (5 (1) vs 10 (1) points, respectively; $p = 0.04$; fig 5B). No differences were observed between ATN-161-treated and ATN-163-treated animals in the DSS model of colitis in terms of histological score of colitis or inhibition of angiogenesis (data not shown).

Effect of angiogenesis blockade on colonic cytokine levels

Intestinal inflammation is associated with a locally increased production of cytokines and its attenuation generally parallels a decrease in mucosal cytokine levels. To test whether the improvement of clinical and histological colitis observed in ATN-161-treated mice was associated with a decrease in cytokine production in the intestinal mucosa, the production of IL6 and IL12 in the colonic mucosa was measured in an organ culture system. In colitic $IL10^{-/-}$ mice, levels of IL12 and IL6 decreased dramatically ($p<0.05$) in colons cultured from ATN-161-treated animals compared with ATN-163-treated animals (fig 6). By contrast, no change in cytokine levels was observed in the DSS model of colitis (data not shown). To elucidate whether the decrease in cytokine levels was the consequence of a decreased leucocyte influx caused by a reduced microvascular bed or was an unanticipated inhibitory effect of ATN-161 directly on local inflammatory cells, LPMCs from healthy mice were isolated and cultured with anti-CD3 or LPS in the presence or absence of ATN-161, and IL12 was measured in the culture supernatants. Unstimulated LPMCs spontaneously produced IL12, but its production was

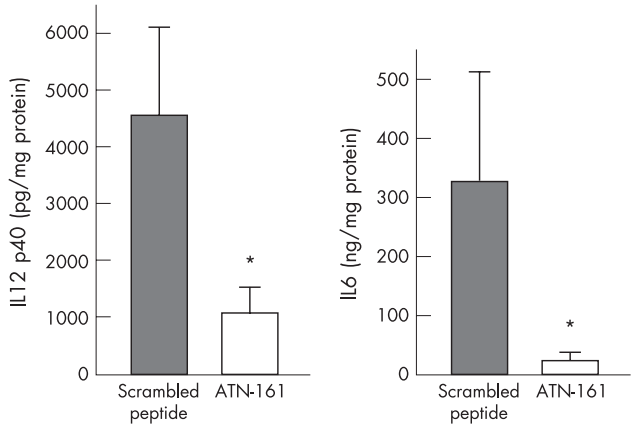

Figure 6 Reduction of cytokine production in the inflamed gut mucosa by angiogenesis blockade. Intraperitoneal injections of ATN-161 (n = 12) or its scrambled peptide ATN-163 (n = 10) were given on alternate days to 15-week-old mice with fully established colitis. After 6 weeks, animals were killed, their colons removed, maintained in organ cultures and supernatants collected for the analysis of interleukin (IL) 12 and IL6 content by ELISA. *$p<0.05$.

significantly upregulated when LPMCs were stimulated with LPS (fig 7A). Moreover, incubation of LPS-stimulated LPMCs with 10 μg/ml ATN-161 had no effect on the production of IL12 by these cells (fig 7A). We also investigated whether ATN-161 could inhibit LPMC proliferation. Anti-CD3 stimulation induced the proliferation of LPMC, but ATN-161 failed to inhibit this effect (fig 7B).

DISCUSSION

The results of this study show that the pathogenic tissue reaction accompanying experimental colitis includes, in addition to well-known immune abnormalities, a major angiogenic component. Inhibiting newly expanded mucosal neovasculature has a clear therapeutic effect on the progression or maintenance of established colitis in $IL10^{-/-}$ mice.

The microvascular changes associated with angiogenesis are key contributors to the tissue injury and remodelling process that inevitably accompanies chronic inflammation.[5 7 9 10] We have recently shown that intense angiogenesis occurs in human IBD (both Crohn's disease and ulcerative colitis), but only few preliminary observations are available on the possible role of angiogenesis in experimental colitis. Information on angiogenesis in animal models of IBD is limited to two reports using the DSS-induced model of murine colitis.[27 28] In the first study, in vivo confocal microscopy showed diffuse hypervascularity and vessel tortuosity and dilation of the mucosal capillaries as early as 5–7 days after DSS administration.[27] In the second study, the authors evaluated the effect of smoking on inflammation-driven colon tumorigenesis, and detected an increase in vascular endothelial growth factor (VEGF) and mucosal angiogenesis in animals with DSS-induced colitis exposed to cigarette smoke.[28]

Precise quantification of angiogenesis by imaging of vessels is essential to reveal vascular abnormalities associated with neoplastic or inflammatory diseases. Among various techniques, direct microscopic imaging is the most validated approach for quantification of vasculature in normal and pathological tissues.[23 29] To investigate the vascularisation state in experimental colitis, we used two different animal models: the $IL10^{-/-}$ and the DSS models, widely accepted as a chronic and an acute model of intestinal inflammation, respectively. In both models, quantification of vessel number revealed that angiogenesis parallels the progress of mucosal inflammation. Importantly, increased vascularisation was present only in areas containing active inflammatory infiltrates, a finding compatible with the concept of immune-driven angiogenesis,[6 7 9 30 31] and indicative of a direct codependency between angiogenesis and inflammation.[5 7 10]

The ultimate goal of any study investigating mechanisms of disease is to identify those pathogenic mechanisms whose blockade or elimination may induce clinical improvement or, ideally, a cure. Given the complexity of most chronic diseases, multiple cells and pathways become potential therapeutic objectives. Recently, the disease-feeding tissue microvasculature has emerged as a prime target in both cancerous and inflammatory conditions. In support of the importance of immune-driven angiogenesis in chronic inflammatory and autoimmune diseases, anti-angiogenic interventions aimed at integrin blockade have been shown to be effective in various animal models of inflammation, including arthritis,[8 32] psoriasis,[33] airway inflammation,[34] atherosclerosis[35] and retinal neovascularisation.[36] Thus, anti-angiogenic treatment could also be therapeutically beneficial in experimental colitis.

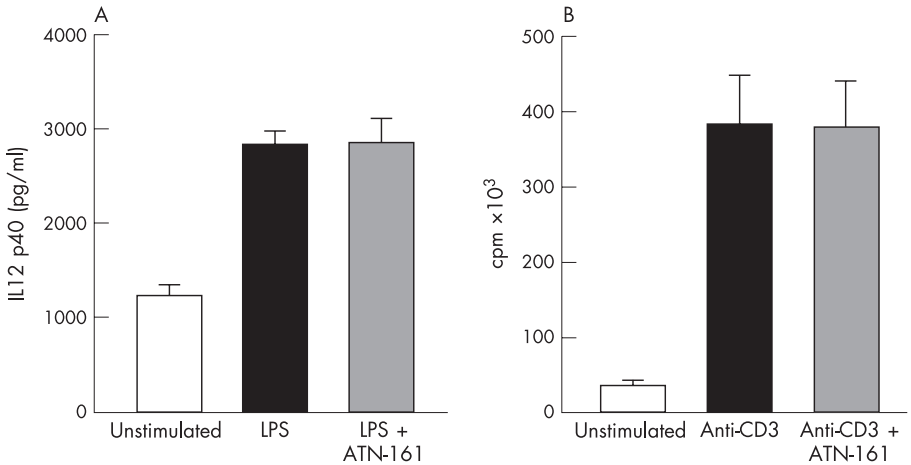

Figure 7 Lack of effect of ATN-161 on lamina propria mononuclear cell (LPMC) cytokine production and proliferation. (A) LPMCs isolated from the normal colon of interleukin (IL) 10-deficient mice kept under the ultra-barrier facility were cultured alone or stimulated with lipopolysaccharide (LPS), in the presence or absence of ATN-161. After 3 days, supernatants were collected and the content of IL12 was measured by ELISA. (B) Isolated LPMCs were cultured alone or stimulated with anti-CD3 in the presence or absence of ATN-161, and proliferation was measured by thymidine incorporation.

One of the main mechanisms by which an immune-driven inflammatory response stimulates the surrounding microvasculature is induction or activation of integrins—key molecules essential to the generation of new blood vessels.[14 37–39] One of these integrins, $\alpha_{V\beta3}$, is not expressed on resting vessels but is upregulated on angiogenic vessels undergoing active proliferation.[14 37–39] In fact, $\alpha_{V\beta3}$ is essential to the survival of proliferating endothelial cells,[14 37 38] and inhibiting $\alpha_{V\beta3}$-mediated adhesion is one way of selectively targeting angiogenic endothelial cells.[12–15 40] Another integrin that has recently been demonstrated to be integral to angiogenesis is $\alpha_{5\beta1}$.[41]

Since angiogenesis blockade through targeting of $\alpha_{V\beta3}$ and $\alpha_{5\beta1}$[38 41] has been shown to be effective in several inflammatory experimental models, we tested the effect of the dual $\alpha_{V\beta}3/\alpha_{5\beta1}$ inhibitor ATN-161 in a chronic and in an acute model of experimental colitis. Blocking these integrins had a convincing therapeutic effect in the chronic IL10$^{-/-}$ colitis model. This effect was associated with a significant reduction of vascular density and an attenuation of the histological and clinical scores of inflammation, providing undisputable evidence that angiogenesis can be manipulated for therapeutic purposes in the inflamed gut. Interestingly, when ATN-161 was tested in the acute model of DSS colitis, no beneficial effect was found. This result is consistent with the concept that angiogenesis plays a pathogenic role in chronic but not in acute gut inflammation. Moreover, ATN-161 failed to show any prophylactic effect as it failed to prevent the development of experimental colitis in the IL10$^{-/-}$ model, even though it was clearly effective in the therapeutic setting—that is, once disease was fully established. This observation suggests that the targets for ATN-161 (eg, $\alpha_{v\beta3}$ and $_{\alpha5\beta1}$) may not be activated in the normal intestinal mucosa but become activated with disease evolution. In agreement with these findings, Chidlow *et al*[42] have recently shown that ATN-161 has a strong therapeutic effect in the T cell transfer model of colitis, another model of chronic experimental colitis, reinforcing the notion that pathological angiogenesis plays a key role in the chronic phases of intestinal inflammation. Indeed, angiogenesis can be viewed as a double-edged sword: on the one hand, it is required for wound healing and repair, but, on the other hand, it promotes tissue damage in neoplastic and inflammatory disorders. The dichotomy of the two responses may be because in a healing process angiogenesis is tightly regulated and self-limited, whereas in cancer and chronic inflammation angiogenesis is progressive and unregulated.[1 5 7 9] This possibility is reinforced by evidence showing that promoting angiogenesis in animal models of rheumatoid arthritis, lung inflammation, psoriasis and atherosclerosis leads to disease progression.[34 43–45] This seems to be also true in experimental IBD, based on preliminary experiments with VEGF gene transfer or the administration of recombinant VEGF, which leads to progressive inflammation and worsening of disease (S Danese, unpublished observations). On the basis of these observations, it seems reasonable to postulate that angiogenesis blockade could be beneficial for the treatment of chronic inflammation associated with IBD, as has been documented in several other conditions.[9 46–50]

In addition to improving clinical parameters of colitis, histological score and vascular density, ATN-161-mediated angiogenesis blockade was accompanied by a significant reduction in inflammatory cytokines. In fact, when IL6 and IL12p40 were measured as a read-out for intestinal inflammation, a dramatic inhibition in the colonic levels of both cytokines was observed. This effect was not a consequence of a direct inhibition of cytokine production or LPMC proliferation by ATN-161, as shown by our in vitro experiments on LPMCs. This is important, because it further supports the notion that the therapeutic effect of ATN-161 was mediated by its anti-angiogenic properties, and was not an unsuspected inhibitory effect on the immune and inflammatory response.

In conclusion, the results of this study show that active angiogenesis is present in experimental colitis and that targeting new blood vessel formation decreases inflammation and tissue damage, probably by interrupting the dependency of chronic mucosal inflammation on a pathologically expanded microvascular bed. Some of the drugs effective in the treatment of IBD in humans, such as infliximab and thalidomide, also have potent anti-angiogenic activity as part of their mechanism of action, suggesting that they may act at least in part through angiogenesis blockade.[51 52] Combined with these observations, our results provide a rationale for considering anti-angiogenic agents as new strategies for IBD treatment.

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