Therapeutic DNA vaccination and RNA interference in inflammatory bowel disease

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Abstract. Angiogenesis plays a significant role in numerous diseases. Recently, dysbalanced angiogenesis was found to be a component of pathogenesis of inflammatory bowel disease (IBD). Therefore, inhibition of angiogenesis is a novel therapeutic strategy that alleviates the inflammation and clinical outcomes of IBD. Bacteria act as vectors for the delivery of therapeutic sequences, a process that is particularly suitable in IBD, due to the natural occurrence of bacteria in gut. The main focus of the present study was the application of bacterial gene therapy in the modulation of angiogenesis in IBD. As a target molecule we used the main proangiogenic factor, vascular endothelial growth factor (VEGF). Bacterial strain *Salmonella typhimurium* SL7207 was used as a gene delivery vector for oral application. DNA vaccination and RNA interference were examined and their efficiency in improving the course of the disease in dextran sulfate sodium-induced colitis in mice was compared. The two approaches yielded similar beneficial results in evaluation of the disease activity parameters (stool consistency, weight loss and colon length) as well as VEGF expression and tumor necrosis factor-α (TNF-α). Improvement in all of the parameters compared with the control groups not treated by therapeutic bacterial strains was also observed. All the bacterial groups showed similar improvement in histopathological scoring. Results of this study are consistent with the literature and provide a basis for additional studies on the modulation of angiogenesis in IBD.

Introduction

The pathogenesis of inflammatory bowel disease (IBD) is of a complex nature and remains to be fully elucidated. One of the key factors involves dysfunctional immunoregulation manifested by the inappropriate production of mucosal cytokines. However, an abnormal microcirculatory system also plays a role in the pathogenesis of IBD (1). A connection between angiogenesis and the pathology of IBD was proposed over 10 years ago, at which time, it was identified that serum vascular endothelial growth factor (VEGF) correlated significantly with disease activity, as well as with elevated levels in patients with moderate/severe Crohn's disease and ulcerative colitis (2). In both diseases, vascular density in colon was found to be significantly higher than that observed in normal tissue, and local microvasculature likely undergoes an intense process of inflammation-dependent angiogenesis (3). VEGF levels found in IBD exert a proinflammatory effect that is similar to that of other inflammatory agents and suggest that this cytokine may serve as an intermediary between angiogenic stimulation and cell-mediated immune responses (4). Thus, angiogenesis appears to be an integral component of IBD pathogenesis, providing a practical and conceptual framework for anti-angiogenic therapies in IBD.

Angiogenesis has emerged as an additional vascular mechanism contributing to chronic inflammation in IBD (3). However, the increased blood supply does not automatically equate with adequate perfusion of the intestine in IBD. One of the potential confounding areas in our understanding of the role of the microvasculature in IBD has been a series of observations from both humans and animal models of IBD that suggest microvascular dysfunction and impaired perfusion of the mucosal surfaces affected by chronic inflammation. This relative ischemia has also been observed in animal studies, where diminished perfusion at the level of microcirculation has been demonstrated (5).

Dextran sodium sulfate (DSS) model of colitis stimulates angiogenesis resulting in increased blood vessel density concomitant with increased histopathology, suggesting that neovasculature contributes to tissue damage during colitis (6). Of note, treatment with the anti-angiogenic agents significantly reduced angiogenic activity and associated tissue histopathology during experimental colitis. Direct evidence of the usefulness of anti-angiogenic therapy was provided recently in mice using gene transfer techniques. Overexpression of VEGF-A in mice with DSS-induced colitis exacerbated their condition, whereas the overexpression of soluble VEGF

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receptor-1 (VEGFR-1) had the opposite effect (7). On the other hand, it has been found that pre-emptive VEGF inhibition does not significantly attenuate angiogenesis but worsens inflammation in a model of acute colitis (8). Preventive VEGF blockade may thus disrupt healing and exacerbate injury via alternative angiogenic or inflammatory pathways. Therefore, recent data indicate that the role of angiogenesis in the pathology of IBD is of more than only an accessory nature. Although slight discrepancies exist, the inhibition of blood vessel formation seems to be promising in the therapy of IBD.

The rationale for bacterial therapy of IBD has previously been shown and proven legitimate (9,10). Bactofection of colonic mucosa using the *Salmonella typhimurium* SL7207-carrying gene-encoding superoxide dismutase has been effective in the therapy of DSS-induced colitis in both rats and mice (9,10). Moreover, a previous study focused on bacteria-mediated anti-angiogenic therapy using various molecular approaches, including bactofection, protein delivery, DNA vaccination and transkingdon RNA interference (11).

The aim of this study was to examine the effect of the bacteria-mediated inhibition of angiogenesis in a model of inflammatory bowel disease in mice. DNA vaccination strategy was employed to induce systemic immune response against VEGF and the course of colitis was monitored. Bacterial strain *Salmonella typhimurium* SL7207 was used as a vector for the delivery of plasmid-encoding human VEGF. The strain SL7207 with plasmid for RNA interference against VEGF was used as an alternate therapeutic group and SL7207 with no therapeutic plasmid was used as the blank control.

Materials and methods

Animals and colitis model. Male C57BL/6 mice (n=20, age 12 weeks) were obtained from Charles River Laboratories (Prague, Czech Republic). Mice were kept in a controlled environment with 12:12 light-dark cycle with *ad libitum* access to water and feed. Animals were divided into four groups (n=5): DSS PBS, DSS SL7207, DSS siVEGF and DSS CA-VEGF. On days 0, 15 and 30 of the experiment mice in all four groups were given 0.25 ml of the respective bacterial strain [109 colony forming units (CFU); groups DSS SL7207, DSS siVEGF and DSS CA-VEGF] and phosphate-buffered saline (group DSS PBS), respectively, using a gastric gavage. The mice received 2% DSS [molecular weight (MW) 36,000-50,000; no. 160110; MP Biomedicals, Solon, OH, USA] for 7 days *ad libitum* in drinking water starting from day 30. Starting from day 37 DSS was changed back to water. Body weight and stool consistency (0, normal; 1, soft-formed; 2, watery; 3, watery with blood) were monitored on a daily basis starting from day 30 until the end of the experiment. Mice were sacrificed on day 40. The schedule of the experiment is shown in Fig. 1. The animal experiment was approved by the institutional review board and Ethics Committee of Comenius University Faculty of Medicine.

Bacteria and plasmids. Bacterial strain *Salmonella typhimurium* SL7207, engineered for the transfection of eukaryotic cells (12), was transformed with plasmids pUC-CAGGS/hVEGF165 (CA-VEGF) containing the cDNA-encoding human VEGF isoform 165 under eukaryotic chicken β-actin promoter and plasmid pSilencer 2.0/

Gavage	Bacteria/ PBS	Bacteria/ PBS	Bacteria/ PBS										
Drinking	H2O	H20	DSS	DSS	DSS	DSS	DSS	DSS	DSS	H2O	H2O	H2O	sacrifice
Day	0	15	30	31	32	33	34	35	36	37	38	39	40

Figure 1. Schedule of the experiment. Mice were gavaged three times in a 15-day interval, following 7 days of 2% DSS treatment and 3 days of drinking water.

siVEGF (siVEGF) containing the cassette for the expression of shRNA against human/mouse VEGF under eukaryotic U6 promoter. The pUC-CAGGS/hVEGF165 plasmid was a kind gift from Professor Yoshikazu Yonemitsu (Department of Gene Therapy, Chiba University Graduate School of Medicine, Chiba, Japan) and the pSilencer 2.0/siVEGF plasmid was purchased from Addgene (Cambridge, MA, USA). Bacteria SL7207 with appropriate plasmid were grown in standard LB medium in the presence of ampicillin (100 μg/ml) and streptomycin (50 μg/ml). Bacterial culture was incubated without shaking until an OD_{600} of 0.4 was reached. The culture was then centrifuged (10 min, 5,000 x g, 4˚C) and the pellet washed three times with 15% glycerol in PBS. Bacteria were then resuspended in 15% glycerol in PBS to achieve CFU $4x10^9$. The final solution was divided in 1 ml aliquots and stored at -80˚C until use for oral gavage. The bacterial count was confirmed by plating serial dilutions of bacterial stock onto LB plates containing appropriate antibiotic.

Collection of colon samples. Mice were anesthetized and the entire colon was removed from cecum to anus. The length of the colon was measured. Blood samples were obtained and plasma was extracted. Samples from colon were taken, snap-frozen in liquid nitrogen and stored at -80˚C until use. Samples for histological examination were stored in 4% formaldehyde.

Histological scoring. Each colon sample was graded by a pathologist blinded to the treatment group using a scoring system to evaluate the inflammation (0-3), crypt damage (0-4), regeneration (4-0), extent (0-3) and percentage of involvement (0-4) (13). The highest injury score was 18 and the lowest was 0.

Biochemical analyses. Samples taken from the terminal colon tissue were homogenized using TissueLyser II (Qiagen, Hilden, Germany). Following the centrifugation of colon homogenates, tumor necrosis factor-α (TNF-α) and VEGF levels were measured in supernatants using mouse ELISA kits (Bender MedSystems, Vienna, Austria). Plasmatic VEGF was measured using the mouse VEGF ELISA kit (Bender MedSystems). The concentration of proteins in the colon homogenates was analyzed using the Lowry assay (14). Plasma cytokine concentrations were expressed in pg/ml of plasma and colon concentrations in pg/mg of proteins.

Statistical analysis. Data were analyzed using the one-way analysis of variance. The Student's t-test was used to evaluate the differences between groups. $P<0.05$ was considered to indicate statistically significant differences. The calculations were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± SEM.

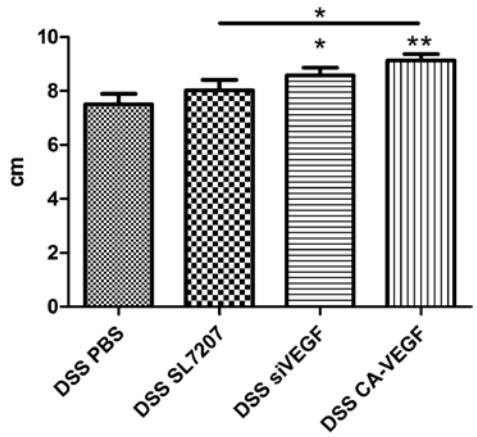

Figure 2. Colon length. The two groups treated with anti-angiogenic bacteria showed higher colon length compared to the PBS group. A significant difference was also found between CA-VEGF and control SL7207. Data are presented as mean ± SEM. *P<0.05; **P<0.01.

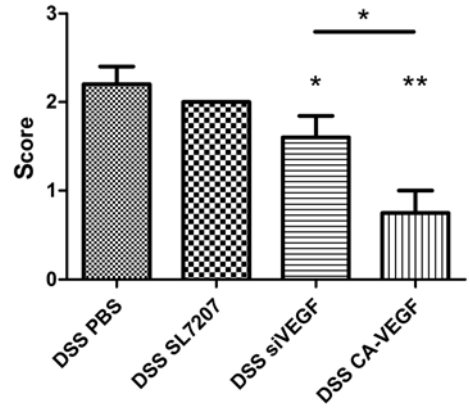

Figure 3. Stool consistency at day 39. The two therapeutic groups showed improved stool consistency compared to the control group. A significant difference was also found between CA-VEGF and siVEGF. Data are presented as mean ± SEM. *P<0.05; **P<0.01.

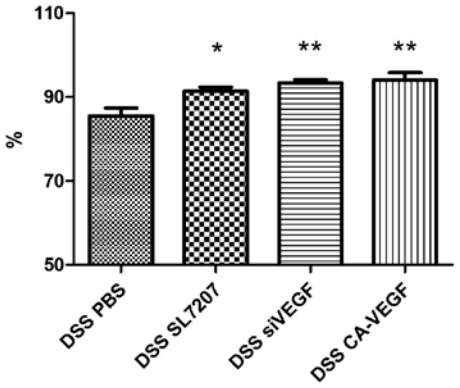

Figure 4. Percentage of initial weight at day 40. The two therapeutic groups had a significantly higher percentage of initial weight compared to that of the PBS-treated control group. Data are presented as mean ± SEM. *P<0.05; **P<0.01.

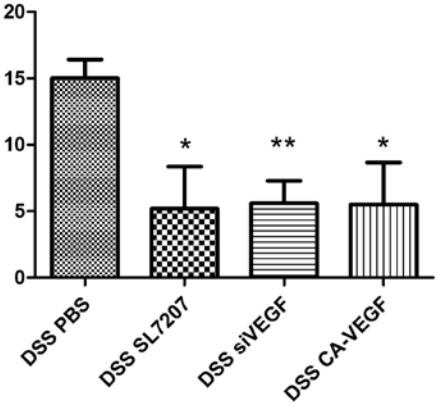

Figure 5. Colon histopathology score. The groups treated with bacteria had a significantly lower score compared to the PBS-treated control group. No significant differences were found among the three groups receiving bacteria. Data are presented as mean ± SEM. *P<0.05; **P<0.01.

Results

Macroscopic examinations. Colon length as a marker of intensity and stage of colitis was measured on the day of sacrifice. The two groups treated with anti-angiogenic bacteria (siVEGF and CA-VEGF) had higher colon length compared to the PBS-treated control group. Moreover, in the vaccination group (CA-VEGF), colon length was higher compared to the sham vaccination group (unmodified SL7207) (Fig. 2). Stool consistency as a marker of ongoing inflammation was evaluated at day 39. The two therapeutic groups showed improved stool consistency compared to the control group (Fig. 3). Moreover, the vaccination group CA-VEGF showed improvement compared to the siVEGF group. The weight of the mice was measured and the percentage of initial weight at day 0 was calculated. The two therapeutic groups showed a significantly higher percentage of initial weight compared to the PBS-treated control group at day 40 (Fig. 4).

Histological scoring. The four groups showed a histopathological score of >0. Groups treated with bacteria (SL7207, siVEGF and CA-VEGF) had a significantly lower score compared to the PBS-treated control group (Fig. 5). No significant differences were found among the three groups receiving bacteria.

Cytokine expression. The VEGF protein level in colon tissue and plasma was measured. In colon, the two therapeutic groups had significantly lower VEGF levels compared to the PBS-treated control group. In addition, CA-VEGF had a lower VEGF level compared to unmodified SL7207 (Fig. 6). In plasma, only the siVEGF group showed a lower VEGF level compared to the PBS-treated control group. However, VEGF was lower in the two therapeutic groups compared to SL7207 (Fig. 7). Expression of the pro-inflammatory cytokine TNF-α protein level in colon was also measured. Results showed that the two therapeutic groups had significantly lower TNF-α levels compared to the PBS-treated control group. There was no significant difference between the therapeutic groups (Fig. 8).

Discussion

In the present study, we aimed to analyze the effect of angiogenesis by means of modern genetic modifications including

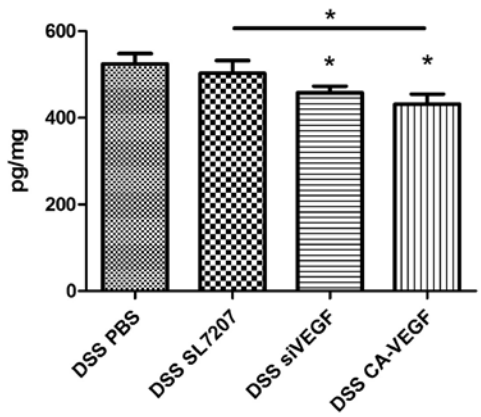

Figure 6. Expression of VEGF in colon. VEGF levels in the two therapeutic groups were significantly lower compared to the PBS-treated control group. CA-VEGF had a lower VEGF level compared to SL7207. Data are presented as mean ± SEM. *P<0.05.

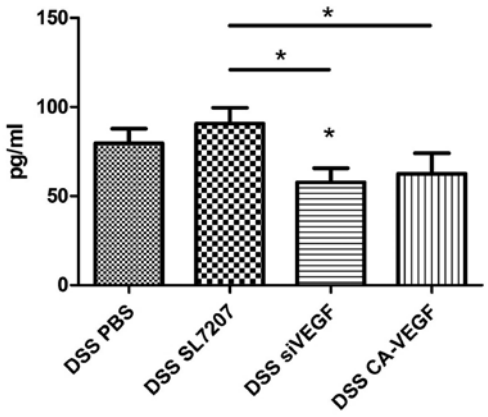

Figure 7. Expression of VEGF in plasma. siVEGF group had lower VEGF levels compared to the PBS-treated control group. VEGF was lower in the two therapeutic groups compared to SL7207. Data are presented as mean ± SEM. *P<0.05.

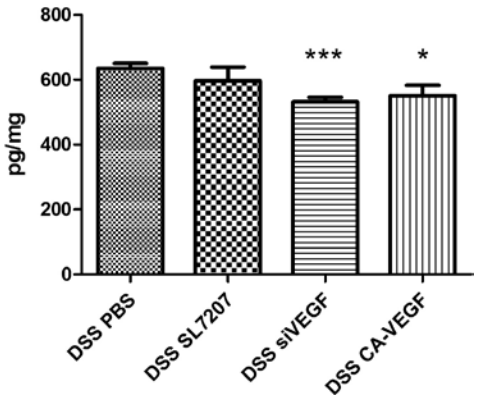

Figure 8. Expression of TNF-α in colon. The two therapeutic groups had significantly lower TNF-α levels compared to the PBS-treated control group. Data are presented as mean ± SEM. *P<0.05; ***P<0.001.

DNA vaccination and RNA interference in an animal model of IBD. Data from the literature indicate that the suppression of angiogenesis may alleviate severity of the disease and provide a therapeutic effect.

The main focus of the study was to employ the principle of bacteria-mediated DNA vaccination against the main proangiogenic factor VEGF in a DSS model of IBD by administration of SL7207 bacteria bearing the plasmid-encoding VEGF cDNA. SL7207 is a commonly used strain for gene delivery and DNA vaccination strategies. DNA vaccination is based on the delivery of target antigen-encoding DNA, expression of the antigen by the host cells and the subsequent immunological response to it. The time needed to induce a sustained immune response against the delivered antigen following oral delivery is known to be 30-45 days. Paglia *et al* (15) reported the finding that after three courses of oral administration of SL7207 at 15-day intervals, mice developed both cell-mediated and systemic humoral responses against the antigen. In this study, we used a similar protocol of triple oral application of the involved bacterial carrier at 15-day intervals. Control groups used sham immunization by non-modified carrier strain SL7207 as well as SL7207 carrying the plasmid designed to induce RNA interference against VEGF (siVEGF). As the effect of RNA interference is usually only of a short-term nature, we did not expect the siVEGF strain to have a pronounced effect in terms of inhibiting the expression of VEGF at the end of the experiment.

The two therapeutic groups, CA-VEGF and siVEGF, showed similar results in the evaluation of disease activity. Weight loss, colon length and stool consistency were significantly improved in the two groups compared to the control. In addition, stool consistency was improved in the CA-VEGF group compared to the siVEGF and CA-VEGF groups, and a higher colon length was noted compared to the sham-vaccinated group (SL7207), suggesting a slightly improved therapeutic outcome of vaccination as compared to RNA interference.

VEGF expression in colon and plasma was also analyzed. In colon, the two therapeutic groups showed significantly lower VEGF levels compared to the control PBS group, while CA-VEGF had lower VEGF levels compared to the sham-vaccinated group with SL7207. By contrast, in plasma only the siVEGF group expressed lower VEGF levels compared to the PBS-treated control group, whereas VEGF was lower in both therapeutic groups compared to SL7207. We also measured the expression of the pro-inflammatory cytokine TNF-α. The level of TNF-α corresponded to ongoing acute inflammation in colon tissue. The two therapeutic groups expressed significantly lower TNF-α levels compared to the PBS-treated control group, however, no significant difference was observed. In terms of the histopathological score, the three bacterial groups exhibited similar and substantial improvement. No difference was observed between the therapeutic and control SL7207 groups, indicating that the effect may be, at least in part, mediated by the carrier bacterial strain itself.

Although several positive effects of VEGF vaccination were observed in this study, there is only a small difference in benefit between DNA vaccination and the RNA interference strategy. It seems that RNA interference against VEGF had a positive therapeutic effect even if used in a DNA vaccination protocol. This was a noteworthy finding as RNA interference is not expected to have such a long-term effect. However, the immune response that may help elucidate the effects was not monitored in this experiment.

Increased vascular density has been associated with progression of human IBD and animal models of colitis. Direct evidence of the usefulness of anti-angiogenic therapy was provided recently in mice using gene transfer techniques. Overexpression of VEGF-A in mice with DSS-induced colitis exacerbated their condition, whereas overexpression of soluble VEGFR-1 had the opposite effect (7). Moreover, treatment of IBD using natural angiogenesis inhibitor endostatin also showed a beneficial effect in experimental IBD (16). Our results are consistent with the above-mentioned findings and support the hypothesis of the therapeutic inhibition of angiogenesis in IBD. However, a study on anti-angiogenic DNA vaccination in IBD has yet to be published.

On the other hand, it has been found that pre-emptive VEGF inhibition does not significantly attenuate angiogenesis, but worsens inflammation in a model of acute colitis. Chernoguz *et al* (8) reported that preventive VEGF blockade may disrupt healing and exacerbate injury via alternative angiogenic or inflammatory pathways. In the present study, we used a similar strategy of pre-emptive VEGF blockade. The results showed an opposite tendency, which, however, is most likely caused by a different anti-VEGF agent used (monoclonal antibody against VEGF - bevacizumab vs. DNA vaccine).

Additional studies employing non-DSS control groups may identify potential non-specific effects of the therapeutic bacterial strains. Moreover, the cellular and humoral immune response should be measured to prove the ongoing vaccination. This is particularly important for studies on DNA vaccination. The expression of other angiogenetic and inflammation-related factors should be analyzed to provide a complete view of the ongoing processes. Nevertheless, our approaches and findings are valid but remain to be confirmed.

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References

1. Kanazawa S, Tsunoda T, Onuma E, Majima T, Kagiyama M and Kikuchi K: VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. Am J Gastroenterol 96: 822-828, 2001.
2. Bousvaros A, Leichtner A, Zurakowski D, Kwon J, Law T, Keough K and Fishman S: Elevated serum vascular endothelial growth factor in children and young adults with Crohn's disease. Dig Dis Sci 44: 424-430, 1999.
3. Danese S, Sans M, de la Motte C, *et al*: Angiogenesis as a novel component of inflammatory bowel disease pathogenesis. Gastroenterology 130: 2060-2073, 2006.
4. Goebel S, Huang M, Davis WC, Jennings M, Siahaan TJ, Alexander JS and Kevil CG: VEGF-A stimulation of leukocyte adhesion to colonic microvascular endothelium: implications for inflammatory bowel disease. Am J Physiol Gastrointest Liver Physiol 290: G648-G654, 2006.
5. Mori M, Stokes KY, Vowinkel T, *et al*: Colonic blood flow responses in experimental colitis: time course and underlying mechanisms. Am J Physiol Gastrointest Liver Physiol 289: G1024-G1029, 2005.
6. Chidlow JH Jr, Langston W, Greer JJ, *et al*: Differential angiogenic regulation of experimental colitis. Am J Pathol 169: 2014-2030, 2006.
7. Scaldaferri F, Vetrano S, Sans M, *et al*: VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. Gastroenterology 136: 585-595, 2009.
8. Chernoguz A, Crawford K, Vandersall A, Rao M, Willson T, Denson LA and Frischer JS: Pretreatment with anti-VEGF therapy may exacerbate inflammation in experimental acute colitis. J Pediatr Surg 47: 347-354, 2012.
9. Palffy R, Behuliak M, Gardlik R, Jáni P, Kadasi L, Turna J and Celec P: Oral in vivo bactofection in dextran sulfate sodium treated female Wistar rats. Folia Biol (Krakow) 58: 171-176, 2010.
10. Palffy R, Gardlik R, Behuliak M, *et al*: Salmonella-mediated gene therapy in experimental colitis in mice. Exp Biol Med (Maywood) 236: 177-183, 2011.
11. Gardlik R, Behuliak M, Palffy R, Celec P and Li CJ: Gene therapy for cancer: bacteria-mediated anti-angiogenesis therapy. Gene Ther 18: 425-431, 2011.
12. Darji A, Guzman CA, Gerstel B, *et al*: Oral somatic transgene vaccination using attenuated *S. typhimurium*. Cell 91: 765-775, 1997.
13. Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SG and Van Rees EP: Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol 114: 385-391, 1998.
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951.
15. Paglia P, Medina E, Arioli I, Guzman CA and Colombo MP: Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. Blood 92: 3172-3176, 1998.
16. Tolstanova G, Deng X, Khomenko T, *et al*: Role of anti-angiogenic factor endostatin in the pathogenesis of experimental ulcerative colitis. Life Sci 88: 74-81, 2011.