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Role of Dipeptidyl Peptidase IV/CD26 in Inflammatory Bowel Disease

Dijana Detel[1], Lara Batičić Pučar[1], Ester Pernjak Pugel[2], Natalia Kučić[3], Sunčica Buljević[1], Brankica Mijandrušić Sinčić[4], Mladen Peršić[5] and Jadranka Varljen[1*]
School of Medicine, University of Rijeka
Croatia

1. Introduction

Inflammatory bowel disease (IBD) comprises two main chronic pathologies of the gastrointestinal tract: ulcerative colitis (UC) and Crohn's disease (CD), both characterized by alternating phases of active inflammation and clinical remission with different complications and extraintestinal manifestations (Colletti, 2004; Hanauer & Hommes, 2010). The ethiopathogenesis of IBD has still not been elucidated, but it has been suggested that inflammatory processes emerge in genetically susceptible individuals as a result of an irregular, over-expressed immunological reaction to some undefined food antigens or some other agents of microbial origin (Baumgart et al., 2011).

Given the complexity of etiological factors in human IBD, a lot of current knowledge regarding IBD pathogenesis has arisen from the study of various animal models. Although no ideal model of IBD has been accomplished so far, they resemble different important clinical, histopathological and immunological aspects of human IBD (Mizoguchi & Mizoguchi, 2010). Chemically induced murine models by oral administration of dextran sulfate sodium (DSS) and intrarectal application of 2,4,6-trinitrobenzene sulfonic acid (TNBS) are the most commonly used ones, due to their onset and duration of colonic inflammation which is immediate, reproducible and shares a lot of similarities with human IBD. TNBS-induced colitis is one of the most accepted and used Crohn-like disease while the DSS-model is clinically and histologically similar to human ulcerative colitis (Wirtz & Neurath, 2007). These models, together with other animal models of IBD, have given insight in different processes at the molecular level and have revealed the importance of different molecules involved in IBD etiology, representing therefore essential tools in investigating different mechanisms underlying acute or chronic inflammation in the IBD (Uhlig & Powrie, 2009).

* Corresponding Author
[1]*Department of Chemistry and Biochemistry,*
[2]*Department of Histology and Embryology,*
[3]*Department of Physiology and Immunology,*
[4]*Department of Internal Medicine,*
[5]*Department of Pediatrics*

Growing body of knowledge proposes proteases as key factors in the occurrence of inflammatory processes due to their ability to metabolize different biologically active molecules implicated in maintaining the integrity of mucosal barrier (Ravi et al., 2007). Dipeptidyl peptidase IV, known also as CD26 molecule (DPP IV/CD26) is one of them (Gorrell et al., 2001). DPP IV/CD26 is also T-cell differentiation antigen, expressed on various cell types, having numerous functions in a variety of biological processes, as well as immunological mechanisms (Fleischer, 1994). It is also present in a soluble form circulating in body fluids in living organisms with specific peptidase function having unique features in substrate processing: it cleaves dipeptides from the N terminus of polypeptides having proline or alanin at the penultimate position. Since Xaa-Pro peptides are not easily metabolized by other proteases, the action of DPP IV/CD26 is an essential step in the degradation of many polypeptides (Gorrell et al., 2001). Numerous biologically important cytokines, chemokines and neuropeptides with potential and/or confirmed role in IBD ethiopathogenesis are effective DPP IV/CD26 substrates (Mentlein, 2004).

Previous studies proposed a role of DPP IV/CD26 in the pathogenesis of IBD, given its involvement in immune regulations via its expression on immune cells and capability to cleave biologically active molecules (Hildebrandt et al., 2001; Varljen et al., 2005). Additionally, DPP IV/CD26 inhibitors have been pointed out as therapeutic agents in ameliorating inflammatory processes in immunologically mediated diseases such as IBD (Yazbeck et al., 2009; Yazbeck et al., 2008).

The aim of this study was to review our previously published results regarding correlation between disease severity and serum DPP IV/CD26 activity in young and adult patients affected with IBD. Furthermore, our aim was to investigate and review does it and in which manner DPP IV/CD26 affect the immune homeostasis during development, progression and resolution of inflammatory events in two animal models of IBD.

2. Dipeptidyl peptidase IV/CD26 molecule

The exoprotease dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), also known as surface antigen CD26, is a transmembrane glycoprotein with molecular mass of 220-240 kDa, expressed constitutively on a variety of cell types (Lambeir et al., 2003). It is also present in a soluble form in serum, saliva, urine and other biological fluids. So far, the role of this molecule has been investigated in different fields of biochemistry, immunology, endocrinology, oncology, pharmacology, physiology and pathophysiology.

Structural and molecular characteristics

According to the current biochemical and structural data, DPP IV/CD26 is a type II transmembrane, homodimeric glycoprotein. Each monomer consists of a large extracellular part (739 amino acids), a hydrophobic transmembrane segment of 23 amino acids and a short cytoplasmic N-terminal tail. The primary sequence of DPP IV/CD26 is composed of 766 amino acids and it was found to be conserved in different species (85% similarities between rat and human and 92% similarities between rat and mouse), mostly in the C-terminal protease segment (Lambeir et al., 2003). DPP IV/CD26 is a member of the POP (prolyl oligopeptidase) gene family with an α/β hydrolase domain and a N-terminal β-propeller domain that enclose the large cavity (30-40 Å) which contains a small pocket with the active site. The catalytic site, as a part of extracellular domain of the molecule, contains Ser-630, His-740 and Asp-708, which is not common for classical serine-type peptidases, but is characteristic for the previously mentioned α/β hydrolase fold (Gorrell et al., 2006).

Based on structural and biochemical features, DPP IV/CD26 is a member of a family of DPP IV activity and/or structure homologue (DASH) proteins, which also includes quiescent cell proline dipeptidase (QPP), DPP8, DPP9, fibroblast activation protein (FAP), attractin and DPP IV-β (Sedo & Malik, 2001). Since it is well known that most DASH proteins have protease activity, having the possibility to modify the activity of biologically active peptides, it could be suggested that they are important regulatory molecules (Gorrell, 2005). However, further research is necessary in order to clarify their biological role.

Distribution and expression

DPP IV/CD26 is widely distributed in mammalian tissues, mainly on epithelial and endothelial cell surfaces, as well as on fibroblasts and lymphocytes (Boonacker & Van Noorden, 2003). The expression of DPP IV/CD26 on hematopoietic cells is well regulated according to the activation status. In humans, it is expressed on a fraction of resting lymphocytes at low density, but is strongly up-regulated following T-cell activation (Fleischer, 1987). In resting peripheral blood mononuclear cells, a small subpopulation of T cells expresses CD26 at high density on the surface (CD26-bright cells), which belongs to the CD45RO+ population of T cells (memory cells) (De Meester et al., 1999; Ishii et al., 2001). Moreover, CD26 expression on T cells may correlate with T-helper subsets. High expression is found on Th1 and Th0 cells, whereas Th2 cells display lower CD26 expression (Willheim et al., 1997).

Soluble DPP IV/CD26

Soluble DPP IV/CD26 activity was firstly discovered in the serum in 1968 by Nagatsu et al. (Nagatsu et al., 1968). Later, DPP IV/CD26 activity has been shown in other body fluids including plasma, serum, cerebrospinal and synovial fluids, semen and urine. Although soluble DPP IV/CD26 lacks the transmembrane domain and intracellular tail, due to glycosylations processes, its molecular weight is similar to the transmembrane form. The origin of the soluble DPP IV/CD26 is still not elucidated, but it was suggested that it could be released from the surface of all CD26 expressing cells in contact with blood by proteolytic cleavage (Gorrell et al., 2001). The physiological role of soluble DPP IV/CD26 in biological fluids with respect to the transmembrane DPP IV/CD26 remains poorly understood, but according to previous findings it has been proposed that, as an enzyme, it is involved in the regulation of many processes in human body (Aytac & Dang, 2004; Mentlein, 1999).

Functions in immune regulations

Immune regulation is a complex and important process in which DPP IV/CD26 as a costimulatory molecule in T-cell activation and a regulator of the functional effect of selected biological factors through its enzyme activity, certainly has an important function (Boonacker & Van Noorden, 2003). Furthermore, biochemical and immune studies provide evidence that CD26 interacts with many biologically important molecules including CD45, adenosine deaminase protein, chemokine receptor CXCR4 on the surface of human peripheral blood lymphocytes (Herrera et al., 2001) and the mannose-6-phosphate/insulin-like growth factor II receptor (Ikushima et al., 2000). The costimulatory properties of DPP IV/CD26 have been studied extensively, although different experimental settings sometimes provide conflicting results. It is generally accepted that several distinct anti-CD26 mAbs have costimulatory activities in anti-CD3-driven activation of pure T-cell subsets (either $CD4^+$ or $CD8^+$ T cells), and that the extent and kinetics of the response differs between mAbs, recognizing different epitopes. High CD26 surface expression is correlated with the production of Th1-type cytokines such as IFN-γ (Reinhold et al., 1997b).

Furthermore, $CD26^+$ $CD4^+$ T cells support differentiation of B cells into antibody-producing plasma cells (Dang et al., 1990).

The question whether the DPP IV/CD26 enzyme activity is involved in T cell activation is still controversial (Lambeir et al., 2003; Schon et al., 1985). Upon CD26-mediated costimulation, IL-2 production is higher in cells expressing wild-type CD26, suggesting that the DPP IV enzymatic activity of CD26 might contribute to, but is not essential for signal transduction. On the other hand, studies with inhibitors of DPP IV/CD26 activity have demonstrated that DPP IV/CD26 plays a key role in T cell activation (Munoz et al., 1992). It was shown that antigen-specific T cell proliferation and IL-2 production *in vitro* could be inhibited by application of the chemical inhibitor Pro-boro-Pro (Flentke et al., 1991). In addition, Lys(Z(NO_2))-thiazolidide, Lys(Z(NO_2))-piperidide, and Lys(Z(NO_2))-pyrrolidide, all synthetic competitive DPP IV/CD26 inhibitors, significantly inhibit DNA synthesis and the production of IL-2, IL-10, IL-12 and IFN-γ in pokeweed mitogen-stimulated purified T lymphocytes (Hildebrandt et al., 2000; Thompson et al., 2007). On the other hand, the presence of these inhibitors enhance the secretion of the immune-inhibitory cytokine TGF-β1, suggesting that TGF-β1 helps regulate DPP IV/CD26 effect on T cell function (Reinhold et al., 1997a).

DPP IV/CD26 substrates

Neuropeptides	**Glucose regulators**
Neuropeptide Y	Glucagon
Vasoactive intestinal peptide	Glucagon-like peptide 1 (GLP-1)
Peptide YY	Glucagon-like peptide 2 (GLP-2)
Endomorfin 1 and 2	Gastrin-releasing peptide
Beta-casomorphine	
Substance P	
Mediators of inflammation	**Other bioactive peptides**
Stromal cell derived factor- 1α and 1β (SDF-1α and 1β)	Bradykinin Growth hormone-releasing factor
RANTES (regulated on activation, normal T-cell expressed and secreted)	Prolactin Enterostatin Alpha-1-microglobulin
Interleukines: IL-1, IL-2, IL-6, IL-10	Monomeric fibrin (α chain)
Tumor necrosis factor α (TNF-α)	Melanostatin
Macrophage-derived chemokine	Tripsinogen
Interferon-inducible protein 10 (IP-10)	
Eotaxin	

Table 1. Selected DPP IV/CD26 biologically important substrates (Gorrel et al, 2006)

Firstly, DPP IV/CD26 was considered to cut off distinctively after a proline or an alanine on the second position from the N-terminal end of a polypeptide chain. Meanwhile, the list of DPP IV/CD26 substrates has been enlarged as it has been shown that a DPP IV/CD26

substrate could also have a serine, glycine, valine, threonine, leucine, or hydroxyproline at the penultimate position (Lambeir et al., 2001). However, DPP IV/CD26 is unable to hydrolyze substrates with proline, hydroxyproline or N-methyl glycine on the third position from the N-terminus (Puschel et al., 1982) and therefore, these peptides are DPP IV inhibitors.

A large number of various biologically important peptides have been shown to be substrates of DPP IV/CD26. Some of the most important DPP IV/CD26 substrates are presented in Table 1.

3. Inflammatory bowel disease and DPP IV/CD26

During the past 50 years, IBD affected millions of people worldwide and therefore has become one of the major gastroenterological problems, especially in the Westernized world. It is a disorder of multiple etiologies, with generally accepted definition that occurs in genetically susceptible individuals, under influence of environmental and microbiological factors, as an overexpressed immunological response to antigens of unknown origin, characterized by chronic uncontrolled inflammation of intestinal mucosa, resulting in its destruction and lost of its function (Colletti, 2004). IBD comprises two main chronic inflammatory diseases of humans, namely ulcerative colitis and Crohn's disease, both characterized by alternating phases of active inflammation and clinical remission with diverse complications and extraintestinal manifestations (Hanauer & Hommes, 2010).

Although the scientific knowledge increases exponentially, there are still many unanswered questions in several fundamental aspects of the IBD. The most stimulating field of IBD research is the interaction among the three major factors of the pathophysiology, including genetic predisposition, environmental bacteria and immune deregulation. The early inductive phases of these diseases are particularly difficult to study in humans because patients usually come to clinic only after their symptoms have been established (Hanauer, 2006).

3.1 Clinical relevance of serum DPP IV/CD26 activity in adult patients and children with IBD

Many investigations and reviews have discussed the role of DPP IV/CD26 activity in inflammation and the potential usefulness of this protein in therapeutics and diagnostics purpose (Hildebrandt et al., 2001; Varljen et al., 2005). However, its exact role still remains unclear. In clinical practice, the differential diagnosis of CD and UC is often difficult. Different biochemical, clinical, endoscopic, pathological and histological features should be combined in order to allocate the appropriate diagnosis. However, a precise diagnosis is not possible in about 10% of patients with chronic colitis, which results in the designation »indeterminate colitis« (Geboes et al., 2008).

Given the role of DPP IV/CD26 in the modulation of the immune response, we hypothesized that DPP IV/CD26 is altered in patients with CD and UC and that changes in DPP IV/CD26 serum activity could be related to the disease activity together with other inflammatory parameters. Therefore, the aim of this study was to evaluate the clinical relevance of changes in serum DPP IV activity in adult patients IBD (CD and UC). Furthermore, given the different immune background in patients with CD and UC as well as different expression of DPP IV/CD26 on Th1 and Th2 cells, we wanted to evaluate if DPP IV/CD26 serum activity could be used as differentiating marker in the diagnosis of these diseases.

3.2 Material and methods

Adult patients

The study was performed on 62 patients, 38 with CD (mean age ± SD: 42.7±14.4; 19 males, 19 females), and 24 with UC (mean age ± SD: 45.6±17.6; 13 males, 11 females). All patients were admitted to the Department of Gastroenterology, Clinical Hospital Centre Rijeka. Diagnoses of CD or UC were established on the basis of clinical history, laboratory, endoscopic and histological data. The control group included 65 healthy donors (mean age ± SD: 41.6±12.1; 32 males, 33 females). The CD activity was evaluated using the Crohn's Disease Activity Index (CDAI), while the UC activity was evaluated according to the Truelove and Witts' (TW) classification (Truelove & Witts, 1955). The localization of the disease was determined according to the Wienna classification for CD while UC was divided into proctosigmoiditis, left-side colitis and pancolitis. Blood samples were obtained after all patients and controls signed informed consents under the protocols approved by the Ethics Committee.

Children

The study involved also young patients, 31 children with IBD. Diagnoses of CD or UC were established on the basis of clinical history, laboratory, endoscopic and histological data. CD activity was evaluated by using the Paediatric Crohn´s Disease Activity Index (PCDAI) (Hyams et al., 1991). Blood samples were obtained after all children's parents gave their signed informed consent under the protocols approved by the Ethics Committee. The study group comprised 24 patients with CD (12 with (PCDAI)≥15 and 12 with (PCDAI)<15) and 7 with UC. Their mean ± SD age at diagnosis was 13.84±1.72 years. The control group included 46 healthy children (mean age ± SD: 13.80±2.83 years; 22 males and 24 females).

3.2.1 DPP IV/CD26 assay

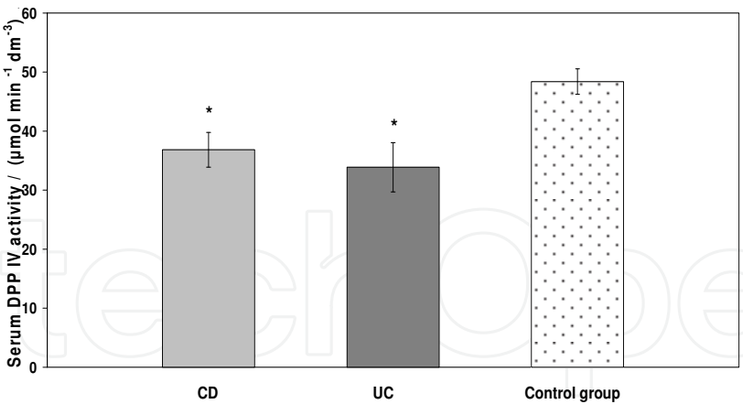
Sera were separated from fasting blood samples and stored at –80°C until thawed for enzyme activities. Determination of serum DPP IV/CD26 activities was performed as described by Kreisel et al (Kreisel et al., 1982). DPP IV/CD26 activities were determined by measuring the release of 4-nitroaniline from an assay mixture containing 0.1 mol Tris-HCl (pH 8.0), 2 mmol Gly-Pro *p*-nitroanilide (Sigma Chemical, Steinheim, Germany) as the substrate and serum in a total volume of 0.20 mL. After 30 minutes of incubation at 37°C, the reaction was stopped by the addition of 800 µL of 1 mol sodium acetate buffer (pH 4.5). The absorbance at 405 nm was measured by use of a Varian Cary UV/VIS spectrophotometer (Cary, NC). All of the reactions were performed in duplicate. Enzyme activities in serum were expressed as µmol of hydrolyzed substrate in a volume of 1 dm^3 per minute under the assay conditions.

3.3 Results and discussion

Here reviewed results for adult patients were previously published in *Croatica chemica acta,* (Varljen et al., 2005), while results of investigations that included children were previously published in *Pediatric Gastroenterology* - Reports from the 2nd World Congress of Pediatric Gastroenterology, Hepatology and Nutrition (Varljen et al., 2004).

Adult patients

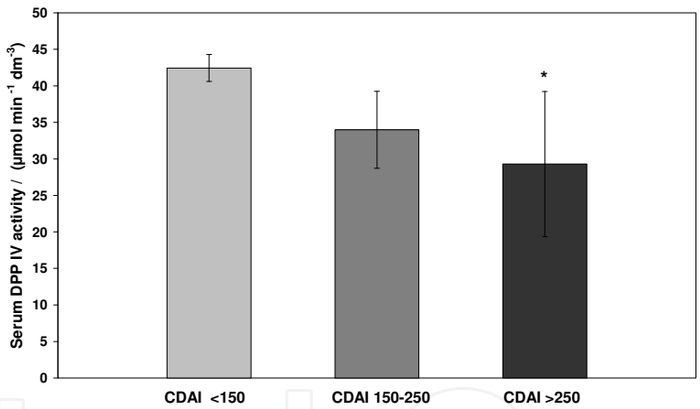
Results of serum DPP IV/CD26 activity in adult patients with CD and UC compared to the control group are presented on Fig. 1. It could be seen that both serum DPP IV/CD26 activities in CD as well as UC are statistically significantly ($P < 0.05$) reduced compared to healthy controls.



*, statistically significantly different compared to control group ($P < 0.001$)

Fig. 1. Serum DPP IV/CD26 activity in patients affected with Crohn's disease (CD) and ulcerative colitis (UC) compared to healthy controls.

When analyzing the correlation between serum DPP IV/CD26 activity in patients with CD and UC, it was noticed that patients affected with CD, having CDAI>250 had statistically significantly lower serum DPP IV/CD26 activity compared to patients having CDAI<150 (Fig. 2).



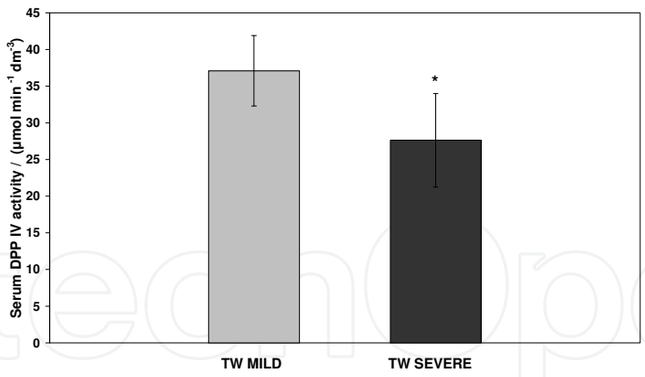
*, statistically significantly different compared to CDAI<150 ($P = 0.023$).
CDAI - Crohn's Disease Activity Index
CDAI ≤ 150 – remission
CDAI >150 – active disease

Fig. 2. Serum DPP IV/CD26 activity in three groups of patients with Crohn's disease.

Likewise, an inverse correlation between serum DPP IV/CD26 activity and disease severity was found in patients affected with UC (Fig. 3). It could be seen that patients with severe UC had statistically significantly ($P < 0.05$) lower DPP IV/CD26 activity compared to patients having mild UC.

Children

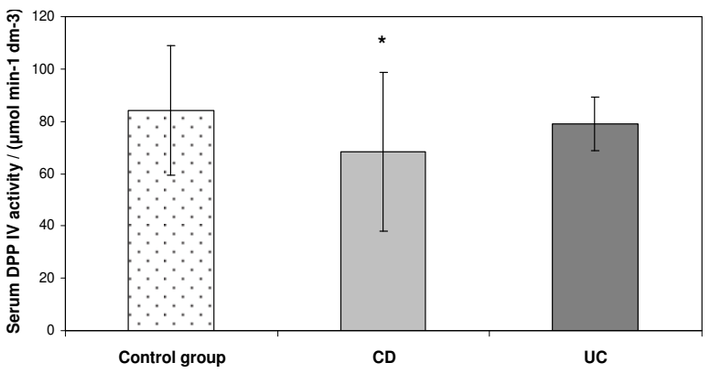
In young patients affected with IBD, DPP IV/CD26 activity in serum was also reduced compared to the levels in healthy controls, likewise in adult patients. The serum DPP



TW-mild and TW-severe - Truelove and Witts' classification (Truelove & Witts, 1955)
*, statistically significantly different compared to TW-mild (P=0.035)

Fig. 3. Serum DPP IV/CD26 activity in two groups of patients with ulcerative colitis.

IV/CD26 activity in children with CD was statistically significantly ($P < 0.05$) decreased compared to the levels in healthy controls. The DPP IV/CD26 activity in children with UC was also decreased but not statistically significantly when compared to controls (Fig. 4).

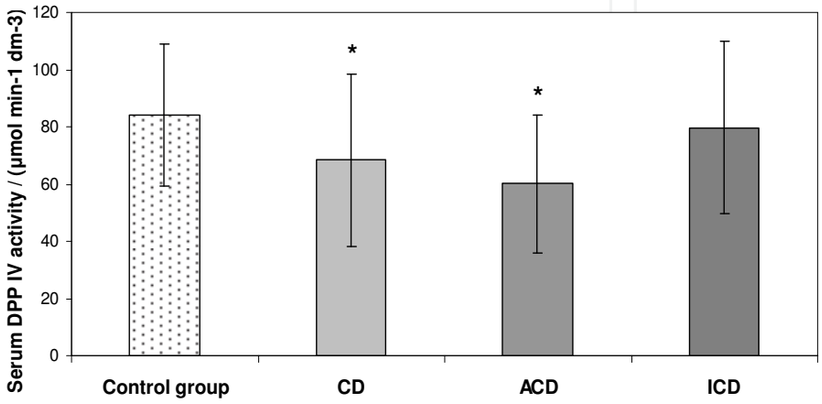


*, statistically significantly different compared to control group ($P < 0.05$)

Fig. 4. Serum DPP IV/CD26 activity in children affected with Crohn's disease (CD) and ulcerative colitis (UC)

The serum DPP IV/CD26 activity in children with active CD was statistically significantly decreased ($P < 0.05$) compared with the levels in healthy controls, while in patients with inactive CD it was also found to be decreased, but not statistically significantly (Fig. 5).
Based on obtained results, it could be concluded that soluble DPP IV/CD26 in serum seems to be involved in the pathophysiology of IBD and appears to be useful as an available non-invasive marker in the diagnosis of disease activity. Changes of DPP IV/CD26 expression and serum activity were found to occur in several clinical and experimental situations of altered immune function (Gorrell et al, 2006). Results of our study accord with previous investigation which confirmed lower serm DPP IV/CD26 activity in patients affected with IBD (Hildebrandt et al., 2001; Rose et al., 2003). Obtained data, together with previously published results, suggest that the persisted immune dysbalance could have a significant

impact on the pathogenesis of IBD. Our results can suggest a functional compartmentalization of DPP IV/CD26, which can be interpreted as an adaptive systemic immune response to a local inflammatory reaction. Meanwhile, the obtained results do not corroborate the hypothesis that the serum DPP IV/CD26 enzymatic activity differs between patients with CD and patients with UC, thus reflecting the concept of different cytokine patterns in one or the other subtype of IBD. Consequently, it seems that the serum DPP IV/CD26 activity could not be used as a specific differential diagnostic marker between CD and UC, and further investigations are necessary in order to establish a new parameter for differentiation of CD from UC.



(ACD-Active Crohn's disease, ICD-Inactive Crohn's disease)
*, statistically significantly different compared to control group (P<0.05)

Fig. 5. Serum DPP IV/CD26 activity in children affected Crohn's disease (CD),

4. Animal models of IBD

Throughout the last decade, several experimental animal models of IBD have been developed in order to define different components of the pathophysiological processes that characterize these disorders (Mizoguchi & Mizoguchi, 2010; Strober et al., 1998; Wirtz & Neurath, 2007). Experimental animal models have a number of advantages which include allowing the study of specific pathophysiological events occurring before symptoms onset. Furthermore, investigators can perform genetic and immunologic manipulations of relevant mouse genes, possibly involved in disease pathogenesis (Bhan et al., 1999).

Although no ideal model of IBD has been accomplished so far, they resemble different important clinical, histopathological and immunological aspects of human IBD. The value of the animal models is the insight they allow into the complex, multifaceted processes and mechanisms that can result in acute or chronic intestinal inflammation. Animal models of IBD have given insight in different processes at the molecular level and have revealed the importance of different molecules involved in IBD etiology, representing therefore essential tools in investigating different mechanisms underlying acute or chronic inflammation in IBD. In recent years quite a number of new experimental models of intestinal inflammation have been described (Table 2).

Animal model	Disease type
Spontaneous	
C3H-HeJBir	Colitis, superficial, acute-resolving, Th1
SAMP1/Yit	Ileitis, chronic, transmural, granulomatous, Th1
SAMP1/YitFc	Perianal disease, early onset of disease
Genetically engineered	
IL-2 knockout	Spontaneous colitis, Th1
IL-10 knockout	Colitis, acute, chronic, transmural, Th1 (early)/Th2 (late)
T-cell receptor α mutant mice	Colitis, chronic, Th2
TNF-3′ UTR knockout mice	Colitis
STAT-4 transgenic mice	Colitis, acute, chronic, transmural, Th1
IL-7 transgenic mice	Colitis, acute, chronic, Th1
HLA B27 transgenic	Spontaneous, entire colon, Th1
Chemically induced	
Trinitrobenzene sulfonic acid-induced colitis	Colitis, acute, chronic, transmural, Th1
Oxazolone colitis	Colitis, Th2
Dextran sulfate sodium colitis	Colitis, superficial, Th1 (acute), Th1/Th2 (chronic)
Peptidoglycan-polysaccharide colitis	Enterocolitis, transmural
Adoptive transfer	
CD4/CD45RB^{high} T-cell transfer colitis	Colitis, chronic transmural, Th1
Transfer of hsp60-specific CD8 T cells	Colitis, Th1

TNF, Tumor necrosis factor; UTR, untranslated region; STAT, signal transducer and activating transcription; hsp, heat shock protein

Table 2. Selected animal models of IBD (Mizoguchi & Mizoguchi, 2010)

4.1 DSS-induced colitis (ulcerative-like model of colitis)

Ulcerative colitis (UC) is a chronic inflammatory condition of the colon that may affect individuals of any age. It generally begins in the anus and extends at a variable length from the rectum in a continuous fashion. Patients usually present with a constellation of symptoms including diarrhea, lower abdominal cramping and tenesmus (Shah & Feller, 2009). The dextran sulfate sodium (DSS) model of induced colitis is an excellent preclinical animal model that exhibits numerous phenotypic features with human ulcerative colitis. It was originally described by Ohkusa et al. (Ohkusa, 1985) as a hamster model and was adapted to mice subsequently by Okayasu and its coworkers (Okayasu et al., 1990).

DSS is a polyanionic derivate of dextrane produced by esterification with chlorosulphonic acid. The exact mechanism through which DSS initiates colitis is unknown but according to previously published data, it is supposed that DSS alternates the gut permeability. It was shown that administration of DSS reduces the expression of tight junction proteins like zona occludens-1, leading to increased gut permeability. Another suggested mechanism involves direct cytotoxic action of DSS on the colonic mucosa, which leads to the alteration of integrin-α4 and M290 subunit levels on epithelial cells. Through these effects, DSS induces mucosal injury with consequent activation of immune response, leading to the development

of acute or chronic colitis (Dieleman et al., 1998). Inflammation induced by DSS is most frequent and severe in the distal part of the colon (Okayasu et al., 1990) and its severity depends on the concentration and molecular weight of DSS (Kitajima et al., 2000). Concentrations described in literature range between 1% and 7%, while the most commonly used molecular weight ranges between 30 kDa and 50 kDa.

4.1.1 Induction of DSS-colitis in mice

This study was performed using pathogen-free, male, 8-10-week-old (weighting 20±2 g) wild type (C57BL/6) mice and mice with inactivated gene for DPP IV/CD26 molecule ($CD26^{-/-}$) generated on a C57BL/6 genetic background, as described previously (Marguet et al., 2000). $CD26^{-/-}$ mice were kindly provided by Dr. Didier Marguet, Centre d'Immunologie Marseille-Luminy, France. Animals were housed and bred under standard conditions at the Central Animal Facility of the School of Medicine, University of Rijeka.

Colitis was induced in both mice strains using 3% (w/v) sodium dextran sulfate sodium (DSS; MW 50 kDa; MP Biomedicals, USA) during seven days in drinking water *ad libitum* (Wirtz & Neurath, 2007). Control mice received regular drinking water throughout the experiment (days 1-15).

Handling with animals, experimental procedures and anesthesia were performed in accordance with the general principles contained in the Guide for the Care and Use of Laboratory Animals (National Academic Press). The Ethical Committee at the School of Medicine, University of Rijeka approved all of the experiments.

Experimental design

Animals included in the study were randomly divided into four groups as follows: C57BL/6 and $CD26^{-/-}$ mice treated with the 3% DSS solution for 7 days and control C57BL/6 and $CD26^{-/-}$ group treated with tap water. At day 7, in order to compare the colitis severity, treated and control animal of each genotype were anesthetized by intraperitoneal administration of ketamine (2.5 mg/mice) and sacrificed by cervical dislocation. The remaining animals were given normal drinking water until day 15 when they were sacrificed in order to compare the strain difference during colitis resolution. At each time point, 6-8 animals of each group were sacrificed. During the entire experiment, body mass was measured daily and clinical symptoms were assessed using the disease activity score. The colon segments from the ileocecal valve to the anus were excised *post mortem,* washed with ice-cold phosphate-buffered saline (PBS) and their length and weight were measured, as indirect markers of inflammation. After colon length and weight measurements, tissue samples were opened longitudinally, washed in PBS and proceeded for histology, morphometry and biochemical analysis. *Morphometrical measurements* included evaluation of crypt number, crypt depth and crypt width on hematoxilin - eosin stained tissue samples. Analyses were performed using software Issa (VAMS, Zagreb, Croatia), Pulmix camera (TMC 76S, Japan) and Olympus BX 40 microscope.

The *clinical score* was assessed as described previously (Howarth et al. 2000; Murthy et al. 1993). Briefly, weight loss of >5% was scored as 0 points, weight loss of 5 to 10% as 1 point, 10 to 15% as 2 points, 15 to 20% as 3 points, and more than 20% as 4 points. For stool consistency, 0 points were given for well formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points for liquid stools that remained adhesive to the anus. Bleeding was scored 0 points for no presence of rectal bleeding and 4 points for gross bleeding from the rectum. These scores were added and divided by three, resulting in a total clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis).

Mucosa fractions isolation from duodenum, jejunum, ileum and colon segments were prepared from mucosal scrapings according to Ahnen et al. (Ahnen et al., 1982).

4.1.2 Establishment and validation of the DSS-induced colitis at systemic and local level

Oral administration of DSS in rodents induces a colonic inflammation with many similarities to human IBD. Consistent with previous studies, as disease progressed, clinical symptoms, including loss of body mass, changes of stool consistency and appearance of rectal bleeding, were aggravated. Until day 3, no clinical symptoms of colitis were seen. From day 3 and later, both mice strain showed blood in their feces and diarrhea. From the results presented in Table 3, it could be concluded that body mass of healthy animals, control group, CD26$^{-/-}$ mice, in comparison to the control C57BL/6 mice is lower which is in agreement with previously published data (Marguet et al., 2000). Administration of the DSS solution caused a statistically significant decrease ($P < 0.05$) of body mass on day 3 in C57BL/6 mice, while in CD26$^{-/-}$ mice, extensive body mass loss began one day after, with a maximum fall on the ninth day. As the inflammation progressed, the disease activity index (DAI) in each group, increased gradually and reached its maximum on day 7 in both mice strains. Body weight increased gradually in both control groups. Variations in clinical symptoms and body mass during colitis development, established in C57BL/6 and CD26$^{-/-}$ mice, are shown in Table 3.

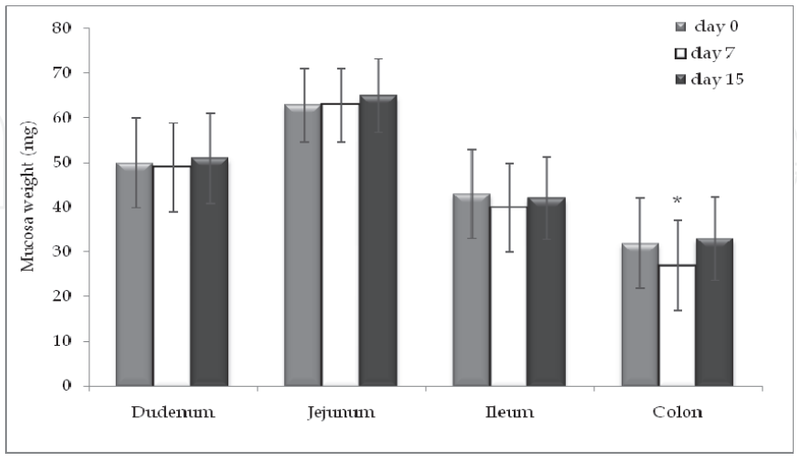
Mice strain	Day of experiment	Body mass (g)	DAI[a]	Diarrhea[b]	Gross bleeding[b]	Colon length (cm)[a]
C57BL/6	0	24.17 ± 1.97	0	0/6	0/6	8.5 ± 0.1
	7	19.19 ± 3.62	4.00 ± 0.20	6/6	6/6	7.3 ± 0.4
	15	23.35 ± 2.79	0	0/6	0/6	8.0 ± 0.2
CD26$^{-/-}$	0	23.63 ± 1.73	0	0/6	0/6	8.5 ± 0.1
	7	19.35 ± 2.16	3.66 ± 0.25	6/6	5/6	7.7 ± 0.2
	15	21.57 ± 1.55	0.33 ± 0.05	0/6	0/6	8.0 ± 0.3

[a]Data are presented as mean ± SD
[b]Number of mice with diarrhea or gross bleeding/total number of mice in each group
DSS: dextran sulfat sodium; DAI: Disease activity index

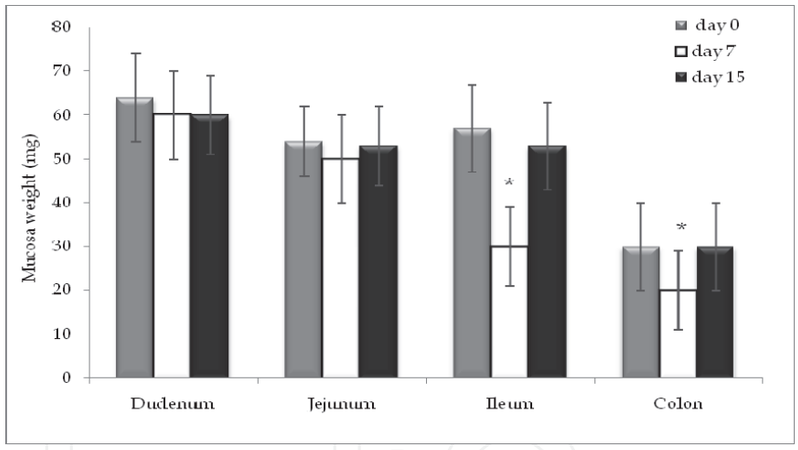
Table 3. Changes of clinical variables during DSS-induced colitis development and resolution in C57BL/6 and CD26$^{-/-}$ mice.

In order to assess the degree of inflammation at the local level, length and weight of each colon sample was measured. Statistically significant shortening of the colon was observed on day 7 of the experiment in both CD26$^{-/-}$ and C57BL/6 mice. Together with colon shortening, statistically significant increase of colon weight was observed on day 7 and 15 in CD26$^{-/-}$ and C57BL/6 mice. It is known that during colitis development, DSS induces colon tissue obliteration, but recent studies in rats showed that changes through the small intestine are also present (Geier et al., 2009; Ohtsuka & Sanderson, 2003). Therefore, in order to provide further evidence, we isolated small intestinal and colonic mucosa and measured the *changes of mucosa weight* during colitis development. A significant decrease of colonic mucosa weight was observed in C57BL/6 mice, while in CD26$^{-/-}$ mice the statistically significant decrease of ileum and colon mucosa weight was observed (Fig. 6). Our results

and results of previously published studies showed that DSS-induced damage could extend to the small intestine and therefore, further studies are necessary to validate physiological impact of this damage.



A



B

Data are presented as mean ± SD
*$P < 0.05$, statistically significantly different compared to day 0

Fig. 6. Influence of DSS-induce damage on small intestine and colon mucosa weight during colitis development and resolution in C57BL/6 (A) and CD26$^{-/-}$ (B) animals.

In compliance with our results, it could be concluded that administration of DSS in drinking water for seven days resulted in a prominent colon inflammation and gastrointestinal dysfunction, followed by regeneration of the colonic epithelium in C57BL/6 and CD26$^{-/-}$ mouse strains. Shortening of the colon and increase of colon weight, as macroscopic measures for the degree of inflammation, correlates with changes of mucosa weight and pathological changes (Okayasu et al., 1990). Given the fact that the symptoms of inflammation were the most prominent between the seventh and tenth day following DSS administration, this period was classified as acute phase, which is in accordance with

previously reported findings. Furthermore, our findings suggest and confirm that the DSS model of colitis, because of similarities to human IBD, represents a good model to study the molecular and immune mechanisms activated during colitis development and resolution.
In accordance with previously published histological data regarding colonic inflammation present in DSS model of colitis, inflammatory changes are superficial, mainly affecting the mucosa, but may extend to the submucosa and the muscularis mucosa as well. The inflammation is characterized by superficial ulcers, mucosal oedema, crypt distortion and mucosal inflammatory cell infiltration with large numbers of neutrophils, macrophages and lymphocytes (Cooper et al., 1993). *Pathohistological* and *morphometrical analyses* of colon tissue sections confirmed the presence of inflammatory changes in both mice strains. During colitis development, a statistically significant decrease in number of crypts of Lieberkühn per milimetar of mucosa followed by its shortening was recorded in both mice strains along with the infiltration of inflammatory cells in lamina propria (Fig. 7). In the acute phase (day 7), crypt architectural distortion reached its maximum and during this phase, typical sign of disease, patches of totally destroyed epithelial sheet with deep ulcerations, can be seen. The resolution of inflammation and regeneration of crypts started during the second week and finished on the day 15 (Fig. 7D). In this period, mononuclear types of inflammatory cells were predominant.

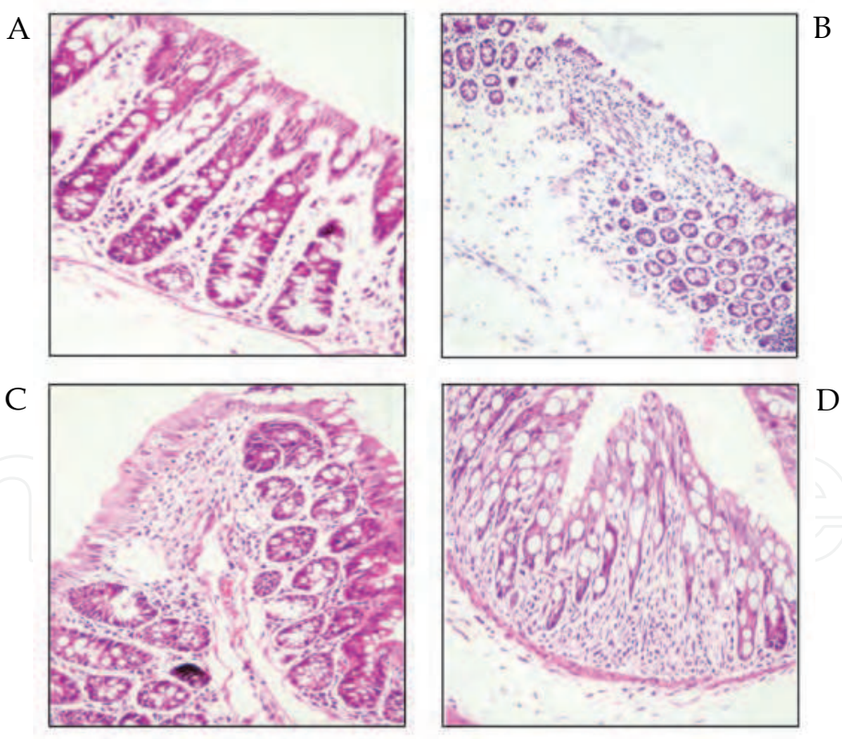


Fig. 7. Histological changes in colon tissues during dextran sulfate sodium-induced (DSS) colitis development and resolution in CD26$^{-/-}$ animals. Normal colon (A), acute phase of colitis (B) and process of tissue damage resolution (C, D). Colon sections (2 μm) were stained with hematoxylin and eosin and examined for histological properties. Magnification: 20x (A, C, D); 10x (B).

There is evidence that susceptibility to DSS varies with the animal species and mice strain. Guinea pig is the most susceptible, with inflammation usually fully established in less than 72 h (Iwanaga et al., 1994). In mice, some strains such as C3H/HeJ and C3H/HeJ Bir were found to be highly susceptible, while others such as NON/LtJ were quite resistant to DSS colitis (Mahler et al., 1998). Our results are in agreement with previously published study regarding the intensity of clinical symptoms between C57BL/6 and CD26$^{-/-}$ mice. Given the fact that there is no statistically significant difference in the intensity of clinical symptoms between mice strains it could be suggested that CD26 deficiency does not increase resistance to the development of DSS-induced experimental colitis (Geier et al., 2005).

4.1.3 DPP IV/CD26 and DPP IV/CD26-like activity in DSS-colitis

Functional studies have demonstrated that inhibition of DPP IV/CD26 enzyme activity may lead to changes in chemokine regulation and a subsequent immunological effect, while *in vitro* studies using activated T lymphocytes have shown that inhibition of DPP IV/CD26 activity can result in a decreased secretion of proinflammatory cytokines, including TNF-α and IFN-γ as well as an increase in the anti-inflammatory cytokine TGF-β. This evidence suggests that DPP IV/CD26 enzyme activity plays an essential role in the immune response and therefore, its enzymatic role is being extensively investigated. In our study, a statistically significant decrease in serum DPP IV/CD26 activity was observed in the acute phase in serum of C57BL/6 mice. The results regarding serum DPP IV/CD26 activity established in a DSS mouse model of colitis are consistent with our previous work in patients with IBD (Varljen et al., 2005). Furthermore, a decrease in DSS colitis disease activity was observed in wild type mice treated with inhibitors but on the other hand the inhibitors were not effective in CD26$^{-/-}$ animals (Yazbeck et al., 2010). Concurrently, during colitis development, an increased expression of DPP8 in wild type and CD26$^{-/-}$ animals and DPP2 mRNA expression in wild type animals was observed.

Considering that DPP IV/CD26 is a member of a large S9b family of structurally homologous serine proteases that possess a unique catalytic activity, and since two recent studies have demonstrated a broad tissue distribution of DPP IV-like enzyme activity in both wild type and CD26$^{-/-}$, a possible explanation of results obtained in our study could be that other DPP IV-like protease are involved in the activation of the inflammatory response in animal model of colitis (Ansorge et al., 2009; Yu et al., 2009). Furthermore, it was recently demonstrated by Yazbeck and its coworkers (Yazbeck et al., 2008) that inhibition of DPP-like activity ameliorates the severity of inflammation in experimental colitis in mice. However, further studies are required to characterize the role of DPP IV-like proteins in the initiation and activation of immune mechanisms leading to intestinal inflammation and development of IBD.

4.2 TNBS-induced colitis (Crohn-like model of colitis)

One of the most widely used and accepted Crohn-like colitis in scientific research is the TNBS-induced colitis. The TNBS-colitis resembles human Crohn's disease in different aspects, from the clinical manifestation, histological appearance and immunological features. TNBS-colitis is induced in experimental animals by rectal application of TNBS in an adequate, experimentally determined dilution of ethanol, usually 30 to 50%. Ethanol serves as a barrier-breaker which allows TNBS molecules, a contact-sensitizing agent, to enter in deeper layers of the colonic mucosa. The mechanism of TNBS-induced

inflammation involves reaction of TNBS, which is a hapten, with tissue host proteins. TNBS is a covalently reactive compound that attachs to autologous proteins and stimulates a delayed-type hypersensitivity response (Camoglio et al., 2000). This generates a variety of new antigens in situ, as well as stimulates the production of proinflammatory molecules and free radicals which initiate a whole cascade of complex immunological interactions (Grisham et al., 1991). The colonic administration of a single dose of TNBS/ethanol solution induces in mice and rats a granulomatous, transmural inflammation with tissue destruction, mainly localized in the distal part of the colon (Scheiffele & Fuss, 2002).

4.2.1 Induction of TNBS-colitis in mice

Two mice strains were used in our study: wild type mice strain C57BL/6 and mice with inactivated gene for molecule CD26 (C57BL/6 Jbom-ob, CD26$^{-/-}$), generated on a C57BL/6 genetic background. CD26$^{-/-}$ mice were kindly provided by Dr. Didier Marguet, Centre d'Immunologie Marseille-Luminy, France. Generation of CD26$^{-/-}$ mice has been described previously (Marguet et al., 2000). Male, 8-10-week-old mice were used in the study. Animals were housed and bred under standard conditions at the Central Animal Facility of the School of Medicine, University of Rijeka. Laboratory animals were housed in plastic cages, fed with standard pellet food (MK, Complete Diet for Laboratory Rats and Mice, Slovenia), given tap water *ad libitum* and maintained under a 12/12 hours dark/light cycle at constant temperature (20±1)°C and humidity (50±5)%. Each study group comprised 8-10 experimental animals. Handling with animals, experimental procedure and anesthesia were performed in accordance with the general principles contained in the Guide for the Care and Use of Laboratory Animals (National Academic Press). The Ethical Committee of the School of Medicine, University of Rijeka, approved all experimental procedures.

TNBS-colitis was induced by rectal administration of 5% (w/v) TNBS (Sigma-Aldrich, Germany) dissolved in 50% ethanol (Kemika, Croatia). Each animal received 0.1 mL of TNBS-ethanol solution, using a vinyl catheter that was positioned 4 cm from the anus, according to the protocol of (Scheiffele & Fuss, 2002). Two control groups of mice were used for each mice strain. Control mice underwent identical procedures, but were instilled equal volumes of saline (NaCl 0.9%) or ethanol solution. Mice were anesthetized with ketamine/xylazine while receiving TNBS, saline or ethanol solution.

4.2.2 Analytical methods

Experimental animals were sacrificed by cervical dislocation after 2, 7, 15 and 30 days upon administration of TNBS, saline or ethanol solution. Peripheral blood samples were taken and serum samples were collected by centrifugation at 3000 rpm for 10 minutes. Livers and spleens were isolated and their weights were noted. Colons were freed from adhering tissue and macroscopic changes were noted. The colon lumen was carefully washed with ice-cold saline, its weight and length was measured after which underwent homogenization procedure. Brains were separated immediately after sacrifice, washed in ice-cold saline and then homogenized on ice. Colon and brain homogenates were centrifuged at 14000 rpm for 20 minutes at +4°C. Resulting supernatants were measured for total protein concentrations according to the method of Bradford (Bradford, 1976).

Colon tissues for *histological and histomorphometrical analyses* were collected and fixed in 4% formalin for 24 h. Samples were processed and embedded in paraffin wax. Two-micrometer sections were stained with hematoxylin and eosin. An experienced pathologist blinded to

treatment allocation scored microscopical changes, which included overall severity of mucosal damage, number of crypts of Lieberkühn and their depth and width.
The DPP IV/CD26 (in C57BL/6) and DPP IV/CD26-like *enzymatic activities* (in CD26$^{-/-}$ mice) in mice serum, brain and colon homogenates were measured according to the protocol of (Kreisel et al., 1982), as described in section 3.2.1.
Brain and colon samples for *Western blot analyses* of CD26 molecule expression were homogenized on ice using RIPA lysis buffer including inhibitors of proteases and phosphatases. After that, homogenates were centrifuged at 14000 rpm for 20 minutes at +4°C and resulting supernatants were measured for total protein concentrations according to the method of Bradford (Bradford, 1976). Equal amounts of total proteins were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Proteins were transferred from the gels to polyvinylidenedifluoride membranes by semi-dry electroblotting. Membranes were incubated overnight with primary anti-CD26 (Santa Cruz Biotechnology Inc., CA), followed by 45 min incubation with secondary antibody, horseradish peroxidase-conjugated mouse-anti-rabbit IgG (Santa Cruz Biotechnology Inc., CA). Membranes were incubated with chemiluminescent Amersham ECL-plus Western blotting detection reagents (Amersham, Little Chalfont, UK) and bands revealing protein expression of the CD26 molecule were visualized after exposure to photosensitive films (AGFA Ortho CP-G plus). Equal total protein loading was ensured with use of the primary mouse ß-actin antibody (Chemicon International, USA), and secondary horseradish peroxidase-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnology Inc., CA).

4.2.3 Evaluation of TNBS-colitis assessment

All groups of experimental mice were monitored daily for their body weights, stool consistence and presence of blood, eventual occurrence of rectal bleeding and general clinical state. Characteristic findings associated with intrarectal administration of TNBS solution in mice included their poor clinical state, body weight loss up to 15% and a mortality rate of approximately 11% in both CD26$^{-/-}$ and C57BL/6 mice. Disease symptoms were mostly pronounced in the first five days of experiment.
Body mass of individual experimental animals of all groups of both investigated mice strains were determined each day at about the same time, starting from the day of application of TNBS-ethanol solution, ethanol solution or saline, until the day of sacrifice (the second, seventh, fifteenth and thirtieth day). It has been noticed that body weights of CD26$^{-/-}$ animals, as compared to C57BL/6 strain of animals of the same age and gender, are statistically significantly different ($P < 0.05$) and amount (25.29±2.23)g for C57BL/6 and (23.41±2.51)g for CD26$^{-/-}$ animals. Lower body mass of CD26$^{-/-}$ animals in comparison with their genetic background (C57BL/6) mice as well as enhanced insulin secretion and improved glucose tolerance in mice lacking CD26 was reported before (Marguet et al., 2000).
The weights of the brain, liver and spleen were measured analytically for each experimental animal and presented in Table 4.
It was noticed that weights of livers and spleens were slightly higher in CD26$^{-/-}$ animals as compared to the corresponding groups of C57BL/6 animals, nevertheless their lower body mass. The trend of reduction in weight of liver and spleen on the second day of induction of colitis was observed in both strains of experimental animals. The hepatosomatic index (relative ratio of liver weight and body mass) and the relative ratio of spleen weight and body mass of animals were calculated for all animals. It was found that CD26$^{-/-}$ mice have statistically significantly higher ($P < 0.05$) values of the hepatosomatic index compared to

Mice strain	Experimental group	Day of experiment	Brain mass (g)	Liver mass (g)	Spleen mass (g)
C57BL/6	physiological	0	0.44776 ± 0.02273	1.32092 ± 0.11335	0.07822 ± 0.00951
	colitis	2	0.44733 ± 0.02117	0.89232 ± 0.14574	0.07596 ± 0.01806
	colitis	7	0.44019 ± 0.01498	1.12842 ± 0.19671	0.08650 ± 0.02623
	colitis	15	0.44037 ± 0.03687	1.24861 ± 0.12537	0.08424 ± 0.02037
	colitis	30	0.44113 ± 0.02342	1.36642 ± 0.14692	0.07707 ± 0.01311
C57BL/6	physiological	0	0.44776 ± 0.02273	1.32092 ± 0.11335	0.07822 ± 0.00951
	control	2	0.44716 ± 0.01527	1.27890 ± 0.15756	0.07680 ± 0.01630
	control	7	0.44488 ± 0.00435	1.23160 ± 0.05487	0.07462 ± 0.00938
	control	15	0.44838 ± 0.25067	1.26048 ± 0.05868	0.07630 ± 0.01327
	control	30	0.43701 ± 0.03043	1.35711 ± 0.17414	0.07728 ± 0.00697
CD26$^{-/-}$	physiological	0	0.41532 ± 0.02409	1.43343 ± 0.12470	0.08170 ± 0.01447
	colitis	2	0.41836 ± 0.02203	1.08765 ± 0.12792	0.06012 ± 0.01982
	colitis	7	0.42448 ± 0.02631	1.53770 ± 0.07895	0.11080 ± 0.04752
	colitis	15	0.42556 ± 0.01740	1.47498 ± 0.18033	0.11300 ± 0.01758
	colitis	30	0.41040 ± 0.01315	1.36693 ± 0.10567	0.09158 ± 0.02720
CD26$^{-/-}$	physiological	0	0.41532 ± 0.02409	1.43343 ± 0.12470	0.08170 ± 0.01447
	control	2	0.40117 ± 0.02964	1.33858 ± 0.22979	0.08222 ± 0.03727
	control	7	0.41232 ± 0.02331	1.38604 ± 0.07852	0.08058 ± 0.00259
	control	15	0.41924 ± 0.03450	1.24388 ± 0.08975	0.08260 ± 0.00995
	control	30	0.42334 ± 0.02402	1.34925 ± 0.24189	0.08338 ± 0.01257

Table 4. Average brain, liver and spleen weights (g) for different groups of experimental animals at scheduled days of experiment.

C57BL/6 animals. Hepatosomatic index decreased the second day of colitis induction in both strains of experimental animals, as a result of reduction in liver weight, despite of body weight loss. On the other hand, analyzed mice strains did not differ statistically significantly in the relative values of the ratio of spleen weight and body weight in physiological conditions, as well as in the control group treated with ethanol solution. Regardless of the

reduction of the weight of the spleen on the second day of induction of colitis in both strains of experimental animals, due to a reduction in body weights no statistically significant reduction in their relative ratios was observed. Statistically significant increase in the relative ratio of spleen weight and body mass was recorded in the group of CD26$^{-/-}$ animals on the fifteenth day of the induction of colitis, compared to the corresponding control group and compared to C57BL/6 animals sacrificed on the same day.

Macroscopic examination of the distal part of the colon discovered localized inflammation with several ulcerations, mucosal erosions and bowel obstruction with enhanced edematous changes. Colon shortening and thickening with marked colonic edema, accompanied by increased colon weight and presence of hemorrhagic changes was most prominent two days following TNBS administration (Fig. 8). Therefore, day 2 of experiment was classified as acute phase of colitis, which is in accordance with previously reported findings (Scheiffele & Fuss, 2002).

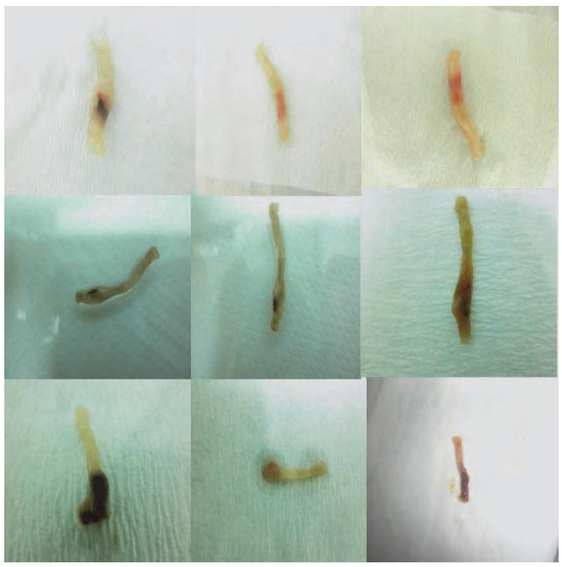


Fig. 8. Macroscopic appearance of the distal part of mice colons in the acute phase of colitis, two days after administration of TNBS-ethanol solution.

Results of our histopathological analyses of colon tissue sections in wild type and CD26$^{-/-}$ mice that received TNBS-ethanol solution confirmed the presence of inflammatory processes and accomplishment of colitis induction. *Microscopic changes*, as well as macroscopic, were most conspicuous in the acute phase of colitis. *Pathohistological analyses* confirmed the presence of inflammatory changes very similar to those seen in human Crohn's disease and revealed that under physiological conditions no differences in histological architecture was observed between analyzed mice strains (Fig. 9).

Pathohistological analyses of a wider number of colonic section samples discovered some differences in the manifestation of inflammatory processes between CD26$^{-/-}$ and wild type mice: in CD26$^{-/-}$ mice, ulcerations were mainly localized in one part of the mucosal surface, and inflammatory changes did not overtake the entire mucosa. In most analyzed colon samples from CD26$^{-/-}$ mice, a part of the colonic mucosa was preserved with physiological appearance of crypts, but a transmural inflammation was observed in a number of mice (Fig. 10A). On the other hand, no transmural inflammatory changes were observed in wild

type animals, but in a number of experimental animals, inflammatory processes affected the entire colonic circumference with very little or no areas of preserved mucosa (Fig. 10B).

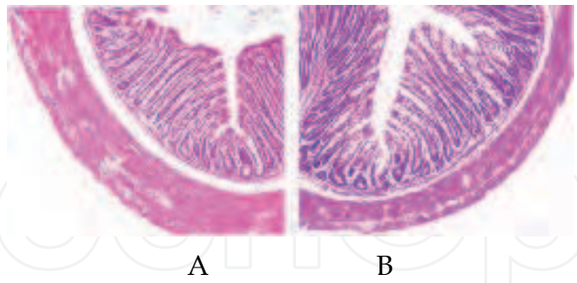


Fig. 9. Histological appearance of colonic tissue sections of CD26$^{-/-}$ (A) and wild type mice (B) two days after application of saline solution. Colon sections (2 μm) were stained with hematoxylin and eosin and examined for histological properties. Magnification: 10x.

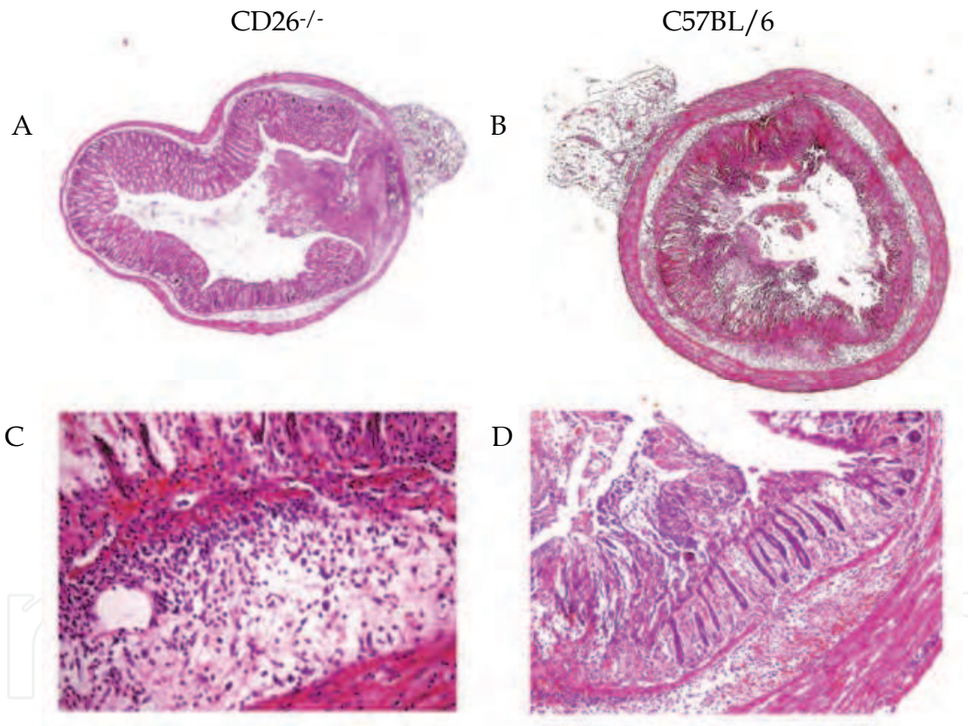


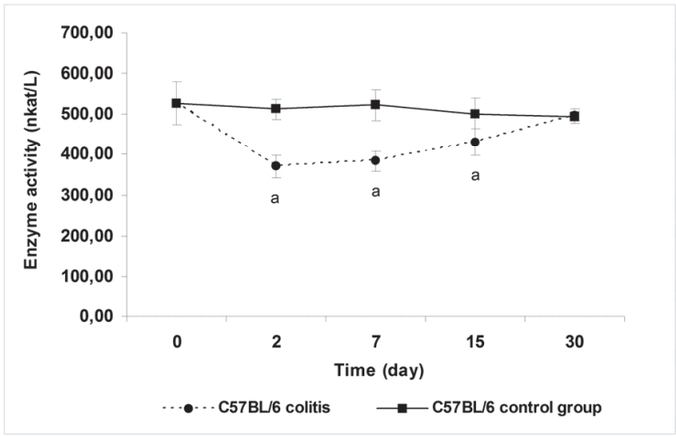
Fig. 10. Pathohistological appearance of colonic tissue sections of CD26$^{-/-}$ (A, C) and wild type mice (B, D) in the acute phase of colitis, two days after application of TNBS-ethanol solution. Colon sections (2 μm) were stained with hematoxylin and eosin and examined for histological properties. Magnification: 4x (A, B), 10x (D) and 20x (C).

Results of *histomorphometrical analyses* also confirmed the presence of inflammatory changes in both mice strains that received TNBS-ethanol solution. Number of crypts of Lieberkühn per mm of mucosa, and their depth and width for different groups of both mice strains at given days of experiment were measured (data not shown). Statistical analyses of obtained results among both control groups of animals did not reveal statistically significant changes

in observed parameters, nor at different days of sacrifice. In both mice strains with induced colitis, a statistically significant ($P < 0.05$) decrease in number of crypts of Lieberkühn per mm of mucosa was observed in the acute phase of colitis. Changes persisted even during tissue healing in CD26$^{-/-}$ mice. The width of crypts of Lieberkühn was increased in the acute phase of colitis in both mice strains, but it took longer to achieve physiological values in wild type mice. Furthermore, the depth of crypts of Lieberkühn was decreased in acute colitis in both mice strains. All those changes represent consequences of inflammatory processes in the colon which include mucosa thickening and formation of edema due to TNBS-ethanol-induced tissue damage.

4.2.4 DPP IV/CD26 and DPP IV/CD26-like activity in TNBS-colitis

Measurements of DPP IV/CD26 activity and protein expression in wild type mice were performed at systemic and local levels, in the serum and within the gut-brain axis respectively. Furthermore, in order to evaluate whether in conditions of DPP IV/CD26 deficiency other DPP IV/CD26-like enzymes could partially undertake its enzymatic function, DPP IV/CD26-like systemic and local activities were determined in CD26$^{-/-}$ mice. Results of investigations concerning DPP IV/CD26 and DPP IV/CD26-like molecules in TNBS-induced colitis in mice reviewed here are accepted for publication in *Croatica Chemica Acta* (*in press*, vol.no.4, 2011). Fig. 11 shows results of serum DPP IV/CD26 activity in wild type mice with induced colitis compared to control groups.

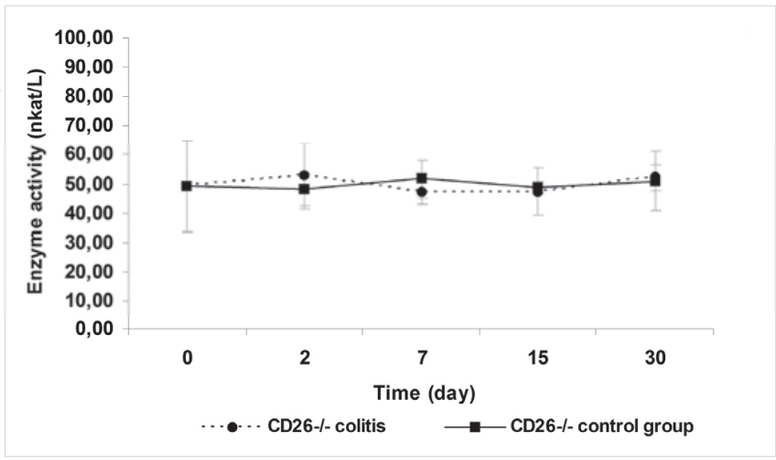


[a], statistically significantly different compared to control group ($P < 0.05$)
0 – control group, physiological condition; 2, 7, 15, 30 – days after administration of TNBS-ethanol solution (colitis group) or ethanol solution (control group).

Fig. 11. Serum DPP IV/CD26 activity in C57BL/6 mice during colitis development and resolution compared to control group.

A statistically significant decrease ($P < 0.05$) in serum DPP IV/CD26 activity, starting in the acute phase of colitis and achieving physiological values after disease healing could be seen. Our results accord with previously published results that included determination of serum DPP IV/CD26 activity in patients with IBD, as described before in this chapter. Furthermore, our results are in accordance with the observation that serum DPP IV/CD26 activity correlates inversely with disease severity in patients with IBD (Varljen et al., 2005), since the lowest DPP IV/CD26 activity in mice was found in the acute phase of disease.

Furthermore, we wanted to evaluate possible changes in serum DPP IV/CD26-like activities during colitis development and healing as well. Therefore, CD26$^{-/-}$ mice with induced colitis as well as their control groups were analyzed. Obtained results showed that CD26$^{-/-}$ mice express approximately 10% of total serum DPP IV/CD26 activity detected in wild type mice. Fig. 12 shows results of serum DPP IV/CD26-like activity in CD26$^{-/-}$ mice with induced colitis compared to their control groups.



0 – control group, physiological condition; 2, 7, 15, 30 – days after administration of TNBS-ethanol solution (colitis group) or ethanol solution (control group).

Fig. 12. Serum DPP IV/CD26-like activity in CD26$^{-/-}$ mice during colitis development and resolution compared to control group.

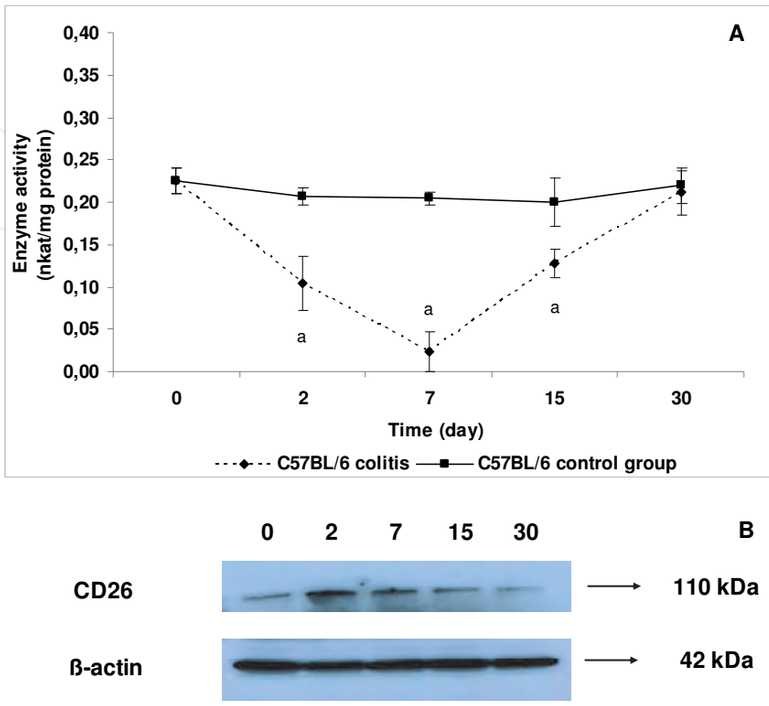
Our results indicated that there are no statistically significant differences in serum DPP IV/CD26-like activity between groups of CD26$^{-/-}$ animals with colitis and their control groups. Therefore, the significance of DPP IV/CD26 over DPP IV/CD26-like serum activity is proposed.

4.2.5 DPP IV/CD26, IBD and the gut-brain axis

Growing scientific evidence emphasizes neuroimmunomodulation as an important factor in the occurrence of inflammatory and autoimmune processes, as described in different investigations in the last few years (Ohman and Simren, 2010). The complex causal connection between central and enteric nervous system caused the introduction of the term **gut-brain axis** (Romijn et al., 2008). DPP IV/CD26 has previously been shown to play a key role in the metabolism of important bioactive neuro- and immunopeptides, as well as in the activation of the immune response (Vanderheyden et al., 2009). Due to its localization on the cell surface of the nervous and digestive system, likewise on the surface of important immune cells (Matteucci & Giampietro, 2009), we aimed to investigate possible changes in DPP IV/CD26 activity and protein expression at sight of inflammation, in the colon, and in which way those changes reflect on examined parameters in the brain.

Results of our research showed an accentuated decrease in DPP IV/CD26 activity at site of inflammation, in the inflamed colon in wild type animals compared to their control groups

(Fig. 13A). On the other hand, an increased CD26 protein expression in the acute phase of disease was revealed by Western blotting technique (Fig. 13B).

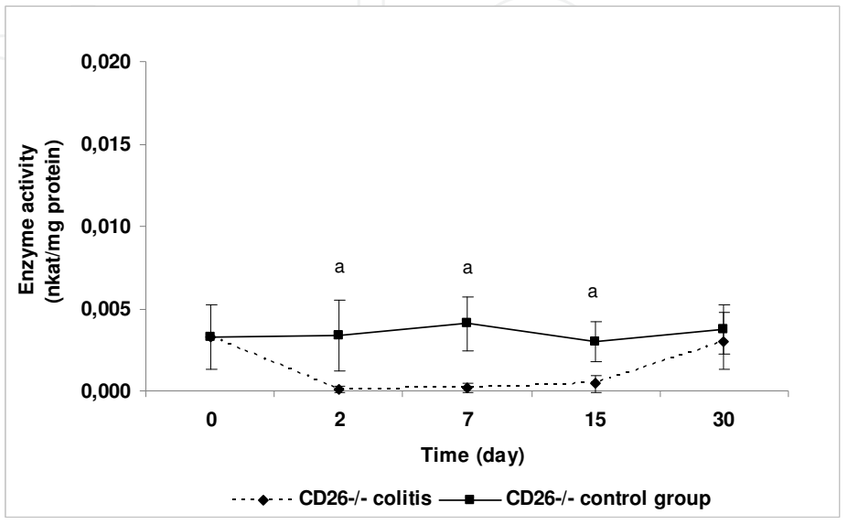


[a], statistically significantly different compared to control group ($P < 0.05$).
0 – control group, physiological condition; 2, 7, 15, 30 – days after administration of TNBS-ethanol solution (colitis group) or ethanol solution (control group).

Fig. 13. DPP IV/CD26 activity (A) and protein expression (B) in colon of C57BL/6 mice during colitis development and resolution.

Besides a regulatory system at the enzymatic level, this observed fact could also be partly explained as a compensatory mechanism, considering that a part of the decreased DPP IV/CD26 activity in the colon of wild type mice is indeed a consequence of severe mucosal damage induced by TNBS-ethanol solution. Consequently, increased CD26 protein expression in the acute phase of disease could represent an effort to realize a compensatory mechanism. Since damaged DPP IV/CD26 conformation is present in inflamed tissue, with the consequence of an improper enzyme activity, enhanced DPP IV/CD26 protein expression could represent a mechanism of feed-back. Our results are in agreement with previously reported observations regarding enhanced CD26 mRNA production in inflamed tissue (Nemoto et al., 1999).
We have determined that, in physiological conditions, CD26$^{-/-}$ mice express less than 2% of total DPP IV/CD26 activity detected in the colon of wild type mice. This enzyme activity was defined as DPP IV/CD26-like activity in the colon and was investigated during colitis development and resolution, as well. Our results showed statistically significantly ($P < 0.05$) decreased DPP IV/CD26-like activity in inflamed colon homogenates in CD26$^{-/-}$ mice, compared to their controls (Fig. 14).

Nevertheless, this observation could not entirely be explained as a consequence of an intrinsic regulatory mechanism which downregulates the activity of DPP IV/CD26-like enzymes in inflammatory processes, but could also partially be attributable to tissue damage induced by TNBS/ethanol solution.



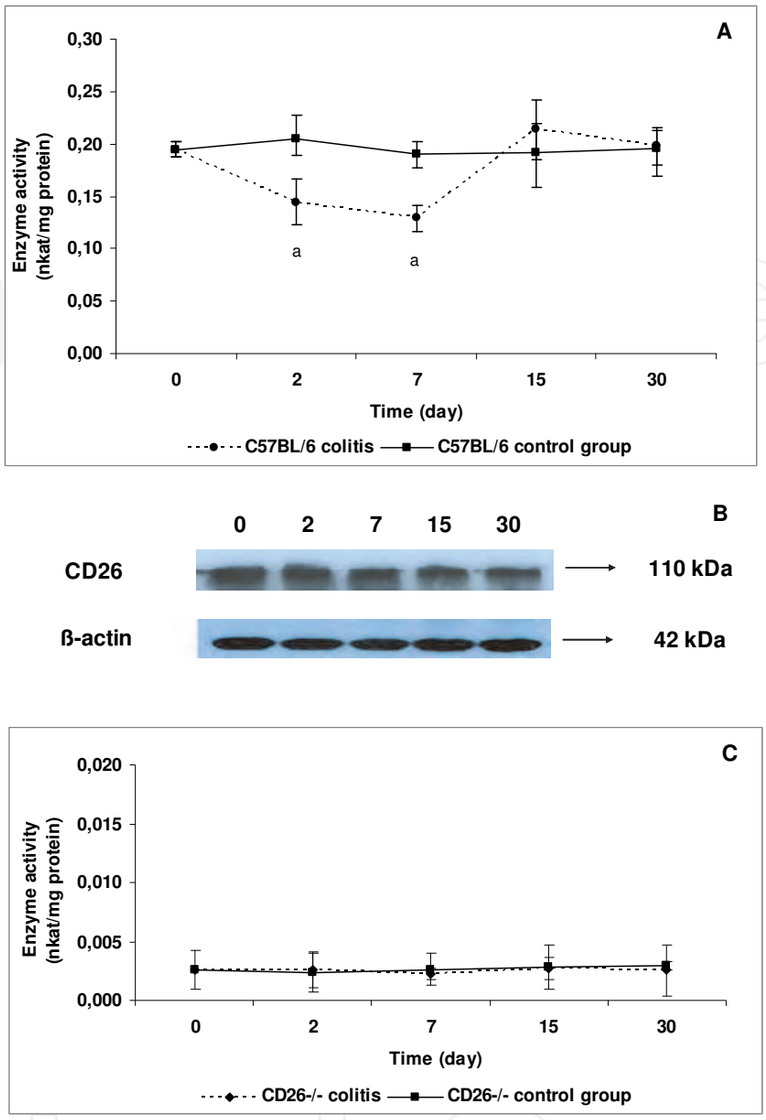
[a], statistically significantly different compared to control group ($P < 0.05$).
0 – control group, physiological condition; 2, 7, 15, 30 – days after administration of TNBS-ethanol solution (colitis group) or ethanol solution (control group).

Fig. 14. DPP IV/CD26-like activity in colon of CD26$^{-/-}$ mice during colitis development and resolution compared to control group.

DPP IV/CD26 and DPP IV/CD26-like activities were also analyzed in brain homogenates during colitis development and resolution in wild type and CD26$^{-/-}$ mice. Our results showed that DPP IV/CD26 activity in brain is statistically significantly decreased ($P < 0.05$) in the acute phase of colitis compared to control groups (Fig. 15A). On the other hand, CD26 protein expression, as confirmed by Western blotting (Fig. 15B) remains constant. Furthermore, the activity of DPP IV/CD26-like enzymes was found to remain unchanged (Fig. 15C).

It could be seen that changes in DPP IV/CD26 activity in the colon during inflammatory events, reflect on its activity in the central nervous system, which accentuates the importance of the gut-brain axis in IBD pathogenesis. Therefore, a decreased DPP IV/CD26 activity in the brain is most probably causally connected with its accentuated changes in the colon. Furthermore, a regulatory mechanism which regulates DPP IV/CD26 activity in brain, independently of its protein expression is proposed.

This study reveals new data about DPP IV/CD26 activity and protein expression in a model of Crohn-like colitis in mice. Likewise, due to very little available results of colitis investigation under conditions of CD26 deficiency, our study gives new insights in inflammatory manifestations induced by TNBS-ethanol administration in CD26 $^{-/-}$ mice.



[a], statistically significantly different compared to control group ($P < 0.05$).
0 – control group, physiological condition; 2, 7, 15, 30 – days after administration of TNBS-ethanol solution (colitis group) or ethanol solution (control group).

Fig. 15. Brain DPP IV/CD26 activity and protein expression in C57BL/6 mice (A, B), DPP IV/CD26-like activity in CD26$^{-/-}$ mice (C) during colitis development and resolution.

5. Conclusions

Results of our studies show that DPP IV/CD26 is involved in the pathogenesis of IBD. In patients, its activity seems to be a good marker of disease activity, given its inverse correlation with disease severity. Given the potential role of DPP IV/CD26 in IBD, animal models of UC and CD have been established in CD26$^{-/-}$ and wild type mice. Our results showed that CD26$^{-/-}$ mice are not protected from two chemically induced colitis (DSS and TNBS colitis), but show specificity in histological damage compared to wild type mice, as well as differences in the

time course of the disease. When analyzing targeted immunobiochemical parameters, it was noticed that changes occurring during inflammatory processes in the colon reflect on investigated parameters in the central nervous system. Therefore, our results indicate and confirm the importance of the gut-brain axis in the pathogenesis of IBD.

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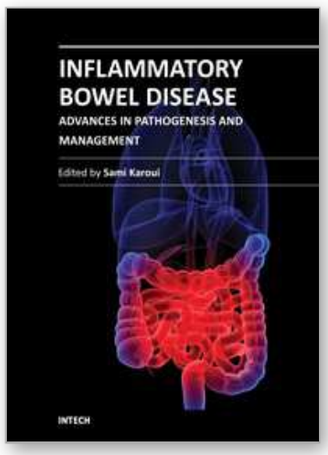
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This book is dedicated to inflammatory bowel disease, and the authors discuss the advances in the pathogenesis of inflammatory bowel disease, as well as several new parameters involved in the etiopathogeny of Crohn's disease and ulcerative colitis, such as intestinal barrier dysfunction and the roles of TH 17 cells and IL 17 in the immune response in inflammatory bowel disease. The book also focuses on several relevant clinical points, such as pregnancy during inflammatory bowel disease and the health-related quality of life as an end point of the different treatments of the diseases. Finally, advances in management of patients with inflammatory bowel disease are discussed, especially in a complete review of the recent literature.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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