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SIMVASTATIN AND RECOMBINANT ANTAGONIST OF RECEPTORS OF INTERLEUKIN-1 MODULATE ARYL HYDROCARBON RECEPTORS IN EXPERIMENTAL COLITIS IN RATS

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The pathogenesis of inflammatory bowel disease is complex and multifactorial. Studies have led to the current concept that aryl hydrocarbon receptors have recently emerged as a critical physiological regulator of immune responses affecting both innate and adaptive systems. We studied the possibility of simvastatin and antagonist of receptors of interleukin-1 for pharmacological correction of colitis in rats with a focus on the expression intensity studies of AhR with lymphocytes of colon.

Eight-month-old male Wistar rats (body mass 260–285 g) were purchased from Institution of Molecular Biology and Genetics (National Academy of Science of Ukraine, Kyiv) and kept in a 12-h light/dark cycle with controlled humidity (60–80%) and temperature (22°±1°C). Food and water were freely available. All animal experiments were performed according to international principles "of the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 18.03.1986) and "General ethical principles of animal research" (Ukraine, 2001). Rats were divided into four experimental groups: group 1 – control; group 2 – rats with oxazolone-induced colitis; group 3 – rats given simvastatin (20 mg/kg, for 5 days, intraperitoneally) ; group 4 – rats given antagonist of receptors of interleukin-1 (3 mg/kg, for 5 days, subcutaneously). Formalin-fixed, paraffin embedded colon sections (5-7 μm) placed on coated slides were sequentially deparaffinized and rehydrated using xylene and ethanol, washed in PBS (twice, 5 min each). The aryl hydrocarbon receptor immunopositive lymphocytes were determined using an indirect immunofluorescence technique with using a monoclonal rat antibody. After rinsing in 0.1 M PBS, the sections were incubated overnight at 4°C with the respective primary antibody: Aryl hydrocarbon Receptor. On the second day, after washing, sections were incubated for 1 h with a mixture of FITC-conjugated goat anti-rabbit IgG. Fluorescent images were obtained with a fluorescence microscope PrimoStar with a computer-assisted video system AxioCam 5c.

The histological observation showed inflammatory cell infiltration, including polymorphonuclear leukocytes and multiple erosive lesions in the large intestine. Occasionally, crypt abscess and regenerated epithelium were seen in the colonic mucosa. We established that development of colitis was not accompanied with the change of amount of AhR^+ lymphocytes. Drug administration during the development of experimental pathology was accompanied by changes in the expression of AhR on lymphocytes.

Simvastatin and antagonist of receptors of interleukin-1 seemed to be beneficial in oxazolone - induced colitis rat model through modulate aryl hydrocarbon receptor expression with lymphocytes of colon.

Keywords: colitis, recombinant antagonist of receptors of interleukin-1 (ARIL-1), simvastatin, aryl hydrocarbon receptor.

Relationship of work with scientific programs, plans, themes. This scientific work is a fragment of the research work «The role of violations of the relationship between the lymphoid and epithelial compartments of the immune system of mucous membranes in the development of experimental pathology», registration number 0112U005642.

Introduction. Ulcerative colitis (UC) and Crohn's disease (CD) are the main clinical phenotypes of inflammatory bowel disease (IBD). Both forms of IBD

can increase the incidence of gastrointestinal and colon cancers, and both ones affect individuals throughout life. Although the etiology and pathogenesis of UC and CD has not been fully revealed yet, it is widely accepted that both are complex and multifactorial [7]. The aryl hydrocarbon receptor (AhR), a transcription factor activated by a large number of environmental agents, modulates the activity of immune and nonimmune cells in the gut, and may represent an important link between the environment and the immune perturbations which underlie the pathogenesis of IBD. Recent findings in diverse murine models of colitis have helped to reveal the importance mechanisms of AhR dysfunction in IBD pathogenesis [13]. Although AhR seems to be a crucial co-factor in regulation of both homeostasis and inflammation, its role in the gut autoimmune pathology is poorly described.

Statin drugs are widely used worldwide for treatment of hyperlipidemia in addition to cholesterol-lowering effect, statins reduce many of the mediators involved in IBD-specific inflammation including C-reactive protein, interferon gamma, interleukins 6 and 8, and NF-kappa B [3].

Cytokines, such as and interleukin (IL-1), have a crucial role in the development of IBD, where they control multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD impedes the resolution of inflammation and instead leads to disease perpetuation and tissue destruction. The naturally occurring inhibitor IL-1 receptor antagonist (IL-1Ra) in part, regulates activities of IL-1. IL-1Ra specifically inhibits IL-1 activities by binding to IL-1 receptors neutralization of endogenous IL-1Ra increases the severity of intestinal inflammation, indicating that endogenous IL-1Ra plays an anti-inflammatory role. These observations suggest that IL-1 is one of the critical mediators of intestinal inflammation in IBD [10].

Objective. Investigate the possibility of Simvastatin and antagonist of receptors of interleukin-1 (ARIL-1) for correction of experimental oxazolone-induced colitis in rats with a focus on the expression studies of AhR with lymphocytes of colon.

Materials and methods

Animals and Tissue isolation

Eight-month-old male Wistar rats were purchased from Institution of Molecular Biology and Genetics (National Academy of Science of Ukraine, Kyiv) and kept in a 12-h light/dark cycle with controlled humidity (60–80%) and temperature (22°±1°C). Food and water were freely available. All animal experiments were performed according to international principles "of the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 18.03.1986) and "General ethical principles of animal research" (Ukraine, 2001). Single animals were fasted overnight and sacrificed by cervical dislocation after receiving an overdose of ether for the isolation of gut tissue. Rats were euthanized 6 days after induction of colitis. For macroscopic observation, the colon was dissected from rats. Macroscopic appearance of inflammation was scored as described specifically for oxazolone-induced colitis [18]. After removal of the colon, the tissue was flushed with cold phosphate buffered saline. For histochemical studies, they were removed and segments were fixed in formalin. After paraffin embedding 5 μm sections were cut and stained with a monoclonal antibody.

Drugs

Simvastatin was obtained from Sigma-Aldrich (St. Louis, MO) and prepared as a 4 mg/ml stock. Briefly, 4 mg was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH, incubated at 50°C for 2 h, and then pH adjusted to 7 and volume corrected to 1 ml. It was chemically activated by alkaline hydrolysis before subcutaneous injection. ARIL-1 was kindly provided by Resbio LLC (St. Petersburg, Russia). Substance ARIL-1 consists of 153 amino acids obtained by genetic engineering technology. The substance is lyophilized protein IL-1ra, which produced by a recombinant strain E. coli BL21.

Oxazolone -induced colitis

Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one) was obtained from Sigma-Aldrich (St. Louis, MO). In order to presensitize rats, a 2 × 2 cm field of the abdominal skin was shaved, and 200 μl of a 3% (w/v) solution of oxazolone in 100% ethanol was applied. 7 days after presensitization rats were rechallenged intrarectally dose of 1.5 mg/kg of body weight 0.1% oxazolone in 50% ethanol under general anesthesia with ketamine. Intrarectal injection was administered with a polyurethane umbilical catheter (Sherwood, St. Louis, MO) [18]. Mice were kept in a head-down position for 30 s and then returned to their cages. Ethanol (40%) is used to help haptens go through the intestinal epithelial barrier [8].

Animal groups

Rats were divided into four experimental groups: group 1 – control; group 2 – rats with oxazolone-induced colitis; group 3 – rats given simvastatin (20 mg/kg, 2 ml/kg in the mixture of ethanol, H_2O, NaOH and HCl for 5 days, intraperitoneally); group 4 – rats given ARIL-1 (3 mg/kg, 2 ml/kg in the phosphate buffer solution (PBS) for 5 days, subcutaneously).

Immunohistochemical staining

Formalin-fixed, paraffin embedded colon sections (5-7 μm) placed on coated slides were sequentially deparaffinized and rehydrated using xylene and ethanol, washed in PBS (twice, 5 min each). After rinsing

in 0.1 M PBS, the sections were incubated overnight at 4°C with the respective primary antibody (dilution 1:50): Aryl hydrocarbon Receptor (H-211: sc-5579) – a rabbit anti-mouse polyclonal antibody, (Santa Cruz Biotechnology, INC, CA). On the second day, after washing, sections were incubated for 1 h with a mixture of FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, INC, CA, catalog numbers sc-2012). While protected from direct light exposure, samples were washed three times in PBS and mounted. Fluorescent images were obtained with a fluorescence microscope PrimoStar (ZEISS, Germany) with a computer-assisted video system AxioCam 5c (ZEISS, Germany). Fluorescent signal intensity was quantified using ImageJ software (NIH Image version 1.46). The lamina propria of mucous layer (LAM PR) and tela submucosa (TELA SUBM) colon were studied.

Statitical analysis

Results were statistically treated with Student's t-test using STATISTICA 6.0 (StatSoft Inc. 2001, USA) and presented as mean±SEM. Statistical differences were considered significant if the P value was <0.05.

Results. The histological observation showed inflammatory cell infiltration, including polymorphonuclear leukocytes and multiple erosive lesions in the large intestine. Occasionally, crypt abscess and regenerated epithelium were seen in the colonic mucosa. The study of serial sections of ileum showed that the development of colitis is not accompanied by changes of total number of AhR immunopositive lymphocytes (AhR^+) in lymphoid structures of colon. But, the administrations of Simvastatin in experimental animals during the development of experimental pathology was accompanied by decrease of AhR^+ cells by 38% (in LAM PR, p<0.05) in proximal colon (**Fig. 1B**); by 24% (in TELA SUBM, p<0.05) in distal colon in comparison with colitis (**Fig. 1E**). The administrations of ARIL-1 to experimental animals during the development of experimental pathology was accompanied by the decrease of AhR^+ cells by 34% (in TELA SUBM, p<0.05) in proximal colon (**Fig. 1C**); by twice (in TELA SUBM, p<0.05) in distal colon in comparison with colitis (**Fig. 1F**).

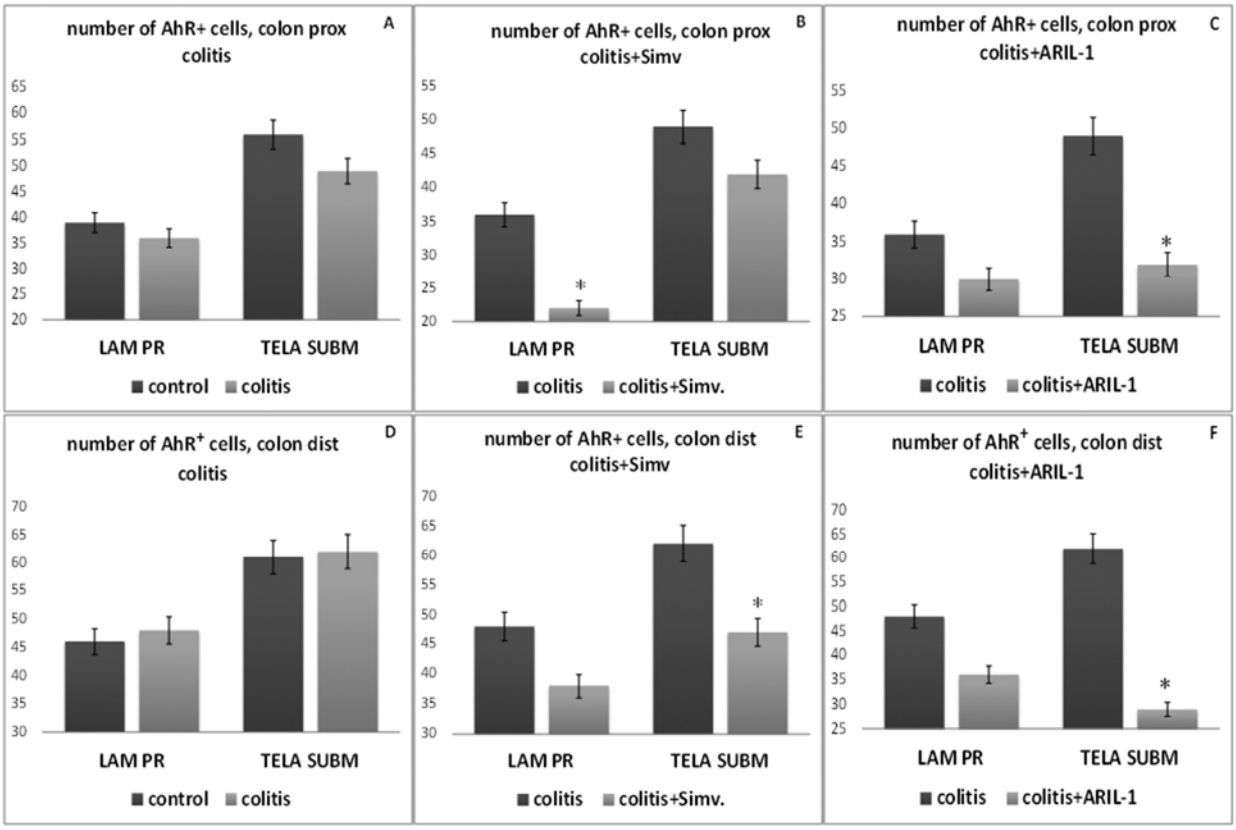

Fig. 1. The number (on 1 mm^2) of AhR^+ cells in lamina propria of mucous layer (COLON PROX, LAM PR) and in tela submucosa (COLON PROX, TELA SUBM) proximal colon during the development of colitis (A) and after administration of Simvastatin (B) or ARIL-1 (C) to experimental animals during the development of colitis; in lamina propria of mucous layer (COLON DIST, LAM PR) and in tela submucosa (COLON DIST, TELA SUBM) distal colon during the development of colitis (D) and after administration of Simvastatin (E) or ARIL-1 (F) to experimental animals during the development of colitis

Note: * — P < 0.05.

Discussion. We found that the development of colitis is not accompanied by changes of total number of AhR^+ in lymphoid structures of colon, but these results do not corroborate recent studies Furumatsu K. et al., because they have described the development of colitis during DSS administration is associated with increased the expression levels of AhR and CYP1A1 mRNA in the colon epithelium. In addition, oral administration of β-naphthoflavone (βNF), a non-toxic agonist of AhR, suppressed the pathogenesis of DSS-induced colitis. βNF also attenuated DSS-induced colitis. In cell culture experiments, downregulation of AhR in human colon carcinoma SW480 cells enhanced the inflammatory responses evoked by lipopolysaccharide (LPS), and furthermore, AhR activation attenuated LPS-induced inflammatory responses, suggesting that AhR expressing intestinal epithelial cells are involved in the prevention of colitis [2].

Activation of AhR by dietary ligands is necessary for the maintenance or expansion of innate immune cells in the gut, such as intraepithelial lymphocytes (IELs) and interleukin (IL)-22-producing lymphoid cells (ILC22). AhR-deficient mice lack IELs, have reduced number of ILC22 cells, and are more susceptible to bacterial infections and experimental colitis. In animal models, AhR activators inhibit proinflammatory cytokine synthesis and attenuate colitis by a pathway that involves IL-22. Analysis of AhR in the human gut reveals that intestinal T cells and natural killer cells isolated from Crohn's disease patients express low levels of AhR and respond to AhR ligands by downregulating inflammatory cytokines and upregulating IL-22 [13].

Furthermore, the AhR pathway has been shown to play a significant role in the development of both Th17, and Treg cells. The effect of AhR activation on T cells is ligand dependent. TCDD induces persistent activation of AhR in Treg cells [4]. On the other hand dietary derived, short acting ligands, such as FICZ [6-formylindolo, 3, 2-b-carbazole] induce Th17 differentiation [17]. The relative abundance of the different ligands, along with the AhR system polymorphisms may further modulate the response [15]. The increased expression of colonic macrophages in wild type mice compared to $AhR^{-/+}$ further supports the role of this receptor in colitis. Recent in-vitro studies in macrophages described an ARNT independent, non-genomic pathway downstream of AhR that induces an inflammatory response [14].

Lee J.Y. et al. demonstrated that simvastatin inhibits proinflammatory gene expression by blocking NF-kappaB signaling in IEC, and attenuates DSS-induced acute murine colitis, and could be a potential agent for the treatment of IBD [9].

Our results about the ability of simvastatin to affect proinflammatory signaling in the gut are indirectly confirmed by other authors. Simvastatin has been shown to inhibit acute as well as chronic inflammatory responses in a cholesterol-independent manner by interfering with endothelial adhesion and leukocyte migration to sites of inflammation [12]. In rats with normal blood cholesterol levels, simvastatin was found to ameliorate immunopathology in an acute TNBS colitis model by blocking neutrophil accumulation in the small intestine and lowering serum TNF-α level [6]. Lee J et al. show Simvastatin blocked TNF-alpha-induced NF-kappaB transcriptional activity, IkappaB phosphorylation/degradation and DNA binding activity of NF-kappaB. Administration of simvastatin significantly reduced the severity of DSS-induced murine colitis as assessed by body weight, colon length and histology in a dose-dependent manner [9]. Bereswill S et al. show that after peroral administration of Simvastatin, mice were protected from acute ileitis development. Simvastatin treated animals displayed significantly increased numbers of regulatory T cells and augmented intestinal epithelial cell proliferation/regeneration in the ileum mucosa compared to placebo control animals. In contrast, mucosal T lymphocyte and neutrophil granulocyte numbers in treated mice were reduced. In addition, levels of the anti-inflammatory cytokine IL-10 in ileum, mesenteric lymph nodes and spleen were increased whereas pro-inflammatory cytokine expression (IL-23p19, IFN-γ, TNF-α, IL-6, MCP-1) was found to be significantly lower in the ileum of treated animals as compared to Placebo controls [1].

One of the factors participating in the initiation and perpetuation of inflammation in IBD may be an inappropriate production of anti-inflammatory cytokines, resulting in a disturbed balance of proinflammatory versus anti-inflammatory cytokines. Although increased levels of IL-1 and an imbalance between IL-1 and IL-1Ra have been documented, the role of other IL-1 modulators in IBD has not yet been elucidated. An imbalance between the production of IL-1 and IL-1Ra has been described in freshly isolated intestinal mucosal cells and in colonic mucosal biopsies obtained from inflamed intestinal tissue of IBD patients [5]. Administration of recombinant IL-1Ra prevents mucosal inflammation and necrosis in a rabbit model of dextran-induced colitis [19]. Conversely, neutralization of endogenous IL-1Ra increases the severity of intestinal inflammation, indicating that endogenous IL-1Ra plays an anti-inflammatory role. The importance of IL-1 and IL-1Ra in the pathogenesis of IBD has been corroborated by the association between carriage of IL-1RN allele 2, low production of IL-1Ra and severity of disease in UC patients [16]. Maeda S et al. show mucosal imbalance of interleukin-1β and interleukin-1 receptor antagonist in

canine inflammatory bowel disease. A significant decrease in the intestinal IL-1Ra: IL-1β ratio of mRNA and protein was observed in IBD cases when compared with healthy control dogs [11]. Our data demonstrate ability of ARIL-1 to influence the level of expression of pattern recognition receptors and show therefore potential in the correction of immune disorders in IBD. Characteristically, ARIL-1 operates as pure antagonist by blocking communication between the molecules of IL-1 (IL-1-alpha, IL-1-beta) and IL-1 receptor that allows providing effective control for the whole IL-1 system in the body.

Conclusions. Simvastatin and antagonist of receptors of interleukin-1 seemed to be beneficial in oxazolone -induced colitis rat model through modulate AhR expression with lymphocytes of colon.

Prospects of further researches. Investigate the possibility of Simvastatin and antagonist of receptors of interleukin-1 (ARIL-1) for correction of experimental ileitis in rats with a focus on the expression studies of AhR with lymphocytes of ileum.

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СИМВАСТАТИН І РЕКОМБІНАНТНИЙ АНТАГОНІСТ РЕЦЕПТОРІВ ІНТЕРЛЕЙКІНУ-1 МОДУЛЮЮТЬ АРИЛ-ГІДРОКАРБОНОВІ РЕЦЕПТОРИ ПРИ ЕКСПЕРИМЕНТАЛЬНОМУ КОЛІТІ У ЩУРІВ

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Резюме. Патогенез запальних захворювань кишечника складний і багатофакторний. Дослідження показують, що аріл-гідрокарбонові рецептори є критичним фізіологічним регулятором імунних відповідей, і що вони впливають як на вроджений, так і на адаптивний імунітет. Ми вивчали можливість застосування симвастатину та антагоніста рецепторів інтерлейкіну-1 для фармакологічної корекції коліту у щурів з акцентом на вивчення інтенсивності експресії AhR лімфоцитами товстої кишки.

Тварини, використані в експериментах (самці щурів лінії Вістар у віці 8 місяців (маса тіла 260–285 г)), перебували в умовах природного освітлення при температурі повітря 18-21 °С, при світловому дні 7-00 – 19-00, з вільним доступом до їжі та води. Всі експерименти на тваринах проводилися відповідно до міжнародних принципів "Європейської конвенції про захист хребетних тварин, які використовуються для експериментальних та інших наукових задач" (Страсбург, 18.03.1986) і "Загальні етичні принципи досліджень на тваринах" (Україна, 2001). Щурів поділяли на чотири експериментальні групи: група 1 – контрольна; група 2 – щури з оксазолон-індукованим колітом; група 3 – щури, яким вводили симвастатин (20 мг/кг, протягом 5 днів, внутрішньочеревно); група 4 – щури, яким вводили антагоніст рецепторів інтерлейкіну-1 (3 мг/кг, протягом 5 днів, підшкірно). Імунопозитивні AhR^+-лімфоцити визначали за допомогою методу непрямої імунофлюоресценції з використанням моноклональних антитіл щура.

Гістологічне дослідження показало інфільтрацію запальних клітин, включаючи поліморфноядерні лейкоцити і множинні ерозивні ураження в товстому кишечнику. Іноді в слизовій оболонці кишечника спостерігали абсцеси крипт і регенерований епітелій. Встановлено, що розвиток коліту не супроводжувався зміною кількості AhR^+-лімфоцитів. Прийом препарату під час розвитку експериментальної патології супроводжувався змінами експресії AhR на лімфоцитах.

Симвастатин та антагоніст рецепторів інтерлейкіну-1 мають позитивний вплив на перебіг оксазолон-індукованого коліту, через модуляцію експресії AhR лімфоцитами товстої кишки.

Ключові слова: коліт, рекомбінантний антагоніст рецепторів інтерлейкіну-1 (ARIL-1), симвастатин, аріл-гідрокарбоновий рецептор.

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СИМВАСТАТИН И РЕКОМБИНАНТНЫЙ АНТАГОНИСТ РЕЦЕПТОРОВ ИНТЕРЛЕЙКИНА-1 МОДУЛИРУЮТ АРИЛ-ГИДРОКАРБОНОВЫЕ РЕЦЕПТОРЫ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ КОЛИТЕ У КРЫС

Жеребятьев А. С., Войтович А. В.

Резюме. Патогенез воспалительных заболеваний кишечника сложный и многофакторный. Исследования показывают, что арил-гидрокарбоновые рецепторы является критическим физиологическим регулятором иммунных ответов, и они влияют как на врожденный, так и на адаптивный иммунитет. Мы изучали возможность применения симвастатина и антагониста рецепторов интерлейкина-1 для фармакологической коррекции колита у крыс с акцентом на изучение интенсивности экспрессии AhR лимфоцитами толстой кишки.

Животные, использованные в экспериментах (самцы крыс линии Вистар в возрасте 8 месяцев (масса тела 260–285 г)), находились в условиях естественного освещения при температуре воздуха 18–21 °С, при световом дне 7-00 - 19-00, со свободным доступ к пище и воде. Все эксперименты на животных проводились в соответствии с международными принципами "Европейской конвенции о защите позвоночных животных, используемых для экспериментальных и других научных задач" (Страсбург, 18.03.1986) и

"Общие этические принципы исследований на животных" (Украина, 2001). Крыс разделяли на четыре экспериментальные группы: группа 1 – контрольная; группа 2 – крысы с оксазолон-индуцированным колитом; группа 3 – крысы, которым вводили симвастатин (20 мг/кг в течение 5 дней, внутрибрюшинно) группа 4 – крысы, которым вводили антагонист рецепторов интерлейкина-1 (3 мг/кг в течение 5 дней, подкожно). Имунопозитивные AhR^+-лимфоциты определяли с помощью метода непрямой иммунофлюоресценции с использованием моноклональных антител крысы.

Гистологическое исследование показало воспалительную клеточную инфильтрацию, включая полиморфноядерные лейкоциты и множественные эрозивные поражения в толстой кишке. Изредка в слизистой оболочке толстой кишки наблюдались абсцессы крипт и регенерированный эпителий. Установлено, что развитие колита не сопровождалось изменением количества AhR^+-лимфоцитов. Прием препарата во время развития экспериментальной патологии сопровождался изменениями экспрессии AhR на лимфоцитах.

Симвастатин и антагонист рецепторов интерлейкина-1 имеют положительное влияние на протекание оксазолон-индуцированного колита, через модуляцию экспрессии AhR лимфоцитами толстой кишки.

Ключевые слова: колит, рекомбинантный антагонист рецепторов интерлейкина-1 (ARIL-1), симвастатин, арил-гидрокарбоновый рецептор.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of coauthors of the article.

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