

PhD Thesis

STUDY OF NITRIC OXIDE SYNTHASE ISOFORMS IN
INFLAMMATORY BOWEL DISEASE

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Introduction

The two major forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), are characterised by chronic inflammatory ulceration of the intestine and impaired capacity of healing. Recent experimental and clinical studies have suggested that the initiation and pathogenesis of ulcerative colitis and Crohn's disease are multifactorial and involve interactions between genetic, environmental, and immunological factors, but they are partly or entirely distinct in the initial phase of pathogenesis and in the type and the number of mediators involved in maintaining the inflammation.

Although the underlying etiology and pathogenesis of ulcerative colitis and of Crohn's disease remain unidentified, major advances have been made in every aspects, with direct impact on new treatment modalities like the biological treatment. New data on the molecular basis of intestinal epithelial function, on the impact of the enteric micrflora on the gut mucosal system, on neuroimmune interactions and on the nature of oral tolerance are now available. Epidemiological studies have further highlighted the role of the environment in the pathogenesis of IBD. New experimental models, involving genetic manipulations and in vitro epithelial cell culture models, have provided novel fundamental approaches to the investigation of IBD and the nature of intestinal inflammation. An extensive literature has developed on cytokines, growth factors, and other bioactive molecules in the maintenance of intestinal homeostasis and on the biologic features of intestinal inflammation. Immunology and genetics remain dominant and increasingly complex mechanisms in the evolution of IBD.

The pattern of reaction of the gastrointestinal tract to injury is limited. The histologic and functional characteristics of the intestinal inflammatory response are determined more by anatomic location and by the acuteness or chronicity of the response than by the etiologic agents. The reason for the similarities in the functional and histologic appearance of intestinal inflammation regardless of the initiating event is the stereotyped final common pathway that involves the same groups of inflammatory cells and the same cytokines and lipid mediators of inflammations, irrespective of the initiating event.

Nitric oxide (NO) is a ubiquitous free radical and it plays important roles either as a messenger or a destructive molecule in inflammation, and has been shown to modulate the inflammatory process. The inducible nitric oxide synthase (iNOS) is highly expressed in macrophages, neutrophils, endothelial and smooth muscle cells upon different stimuli. The increased NO synthesis is, at least partly, responsible for the cytotoxic effect of these cell types during inflammation. However, other isoforms of NOSs found in the endothelial cells (eNOS), and certain nerve terminals (nNOS) innervating the colon, regulate the blood flow and bowel motility by promoting muscle relaxation of the vessels and the bowel, respectively.

Inflammatory cytokines modify and regulate the level of NO, and they are also involved in vascular endothelial response to inflammatory injury. Apoptosis of endothelial cells induced by proinflammatory cytokines and reactive oxygen species is counteracted by the endothelial nitric oxide (NO). The suppression of apoptosis may contribute to the anti-inflammatory and pro-angiogenic effect of endothelial NO.

Despite a number of animal and clinical studies dealing with the contribution, the role of NO in IBD is still uncertain. In most studies, the

amount of NO and the expression of iNOS were found to increase in the intestinal mucosa of UC, whereas they did not show unequivocally and often presented significant differences in CD patients. Recently, a different nitrite level was detected in the serum and urine taken from patients of various types of IBD, suggesting that the level of NO production may indicate the state of, or the difference in, the process of inflammation and might also explain the changes and differences in the structure of the microvasculature and pathophysiology of the bowel motility.

Human umbilical vein endothelial cells (HUVEC) are frequently used as a specific model for studying endothelial function. HUVEC are also sensitive to pro-inflammatory stimuli induced by interleukins. NO is produced in HUVEC constitutively by eNOS and by inducible nitric oxide synthase (iNOS) in the case of different stimuli. NO also contributes to the regulation of apoptosis, cell differentiation and proliferation (angiogenesis) in this cell type.

Aim of the study

The changes in the activity of eNOS and iNOS as well as their role in the endothelium of IBD patients have not been examined in detail. The aim of the study was to characterize the expression and localization of iNOS and eNOS in colonic mucosa of IBD by histological analyses of colonoscopic biopsies taken from inflamed and uninfamed sites in the colon of UC and CD patients and patient controls. In the second phase of the study we examined the effect of sera from patients diagnosed with UC and CD on cultured HUVEC. The expression and localization of eNOS and iNOS were determined in highly confluent and in semi-confluent HUVEC cultures, and the apoptotic/necrotic characteristics and proliferation activity upon distinct treatments were also assessed.

Material and methods

Collection of samples

Colonic biopsy specimens were obtained from inflamed and uninfamed sites of sigma and right colon from patients of IBD as well as from patients called in for colonoscopy to exclude colorectal cancer. The latter biopsies were used in the study only if the mucosa was found uninfamed in histopathological examination. Patients with relapsing UC and CD received no systemic or locally applied corticosteroids, and their medication was stable for the last four weeks before colonoscopy. Patients diagnosed as UC had proctosigmoiditis or left side colitis. Crohn's patients had colonic localization and non-stricturing, non-penetrating form of the disease according to the Vienna classification (1998) and an elevated C-reactive protein level (CRP>10 mg/L), without abscess or sign of infection.

Collection of blood sera

Five mL of blood was taken from 18 UC, 21 CD and 16 healthy (normal) patients, placed into collecting tubes, and centrifuged at 2000 rpm for 10 min. The sera were removed and mixed from UC, CD and healthy patient, respectively, and kept frozen at -80°C until use.

Morphological analyses

Preparation of the biopsies

Ten μm thick frozen sections were cut from 4% paraformaldehyde or acetone-fixed fresh-frozen samples. Some of the PFA-fixed biopsies were embedded into paraffin.

iNOS immunohistochemistry

Anti-iNOS polyclonal rabbit antibodies were used in 1:1000 dilution. Incubation with anti-iNOS was carried out on PFA-fixed frozen, or paraffin-embedded sections. For the development of the immunoreaction, a Universal avidin-biotin-peroxidase kit was applied, and 3,3'-diaminobenzidine (DAB) was used as a chromogen. In control experiments, the primary antibody was omitted. No immunostaining could be observed following any of these control experiments.

eNOS immunohistochemistry

Acetone-dried frozen sections were incubated in the presence of anti-eNOS antibody (rabbit, 1:100) overnight at 4 °C in a buffer similar to that used in iNOS immunolabeling. Immunoreaction was developed by a biotin-labeled secondary antibody (1:200), and avidin-conjugated FITC (1:200). The specificity of the antibody was tested by preabsorbing the primary antibody with the eNOS blocking peptide (synthetic peptide of the eNOS sequence 599-613; 250 µg/ml). No immunostaining could be observed following any of these control experiments.

NADPH-diaphorase histochemistry

NADPH-diaphorase reaction (NADPH-d), a histochemical marker of NOS was used as a quick detection for visualizing this enzyme. PFA-fixed frozen sections were incubated in a solution containing 1 mM β -NADPH, 0.2 mM nitroblue-tetrazolium, 0.3% Triton-X-100 in Tris-HCl buffer (0.1 M, pH 8.1) for about 1 hour in the dark at room temperature. In control experiments, β -NADPH was omitted from the incubation solution or substituted by α -NADPH and β -NADH. No staining could be observed when β -NADPH was omitted or substituted with the synthetic isomer α -NADPH. Using 1mM diphenylene iodonium, the inhibitor of NADPH/NADH-oxidase in the incubation media, no changes were

observed in the aldehyde-resistant NADPH-d reactivity of the sections, suggesting the reaction was related to NOS.

Double labeling immunohistochemistry for identification of iNOS-containing cells

In order to determine the type of iNOS immunoreactive (iNOS-IR) cells in the tissue, paraffin-embedded sections immunostained by anti-iNOS were incubated with mouse anti-CD68 (KP1, for macrophage identification) and anti-CD15 (C3D-1, for neutrophil identification) antibodies. In the second step, immunolabeling of the inflammatory cells was performed using the DAKO LSAB2 kit, where streptavidin was conjugated with alkaline-phosphatase. For reliable distinction of the products of the two immunoreactions, anti-iNOS was visualized by $\text{Ni}(\text{NH}_4)_2\text{SO}_4$ -enhanced DAB reaction (purple-black color), whereas cell specifying antibodies were developed by the substrate-chromogene, New Fuchsin (pinkish-red color). A number of serial sections derives from PFA-fixed frozen biopsies were stained either for NADPH-d histochemistry or iNOS immunohistochemistry to show the capability of the NADPH-d technique in identifying iNOS-IR cells.

Identification of the vascular endothelium

Monoclonal antibodies against PECAM-1 (CD31, JC70A) were applied. Antigen retrieved paraffin sections were incubated by the primary antibody (used in 1:100 dilution), then labeled with an anti-mouse secondary antibody conjugated with alkaline phosphatase (1:100). Visualization was performed by the New-Fuchsin substrate, and the sections were counterstained afterwards with methyl green.

Quantification of the immunoreaction

Slides were examined with an *Olympus Provis AX/70A* microscope and images were digitalized with an *Olympus Camedia C4040* digital camera.

Quantitative evaluation was performed with a computer image analysis software *analysis 2.11*, developed and validated by Olympus. The number of iNOS-IR cells presented in the figures, was given as a mean of the surface mean (mm²).

iNOS immunoblotting

Groups of five to six biopsies obtained from inflamed and endoscopically intact bowels of UC and CD, as well as control patients were immediately frozen in liquid nitrogen, then ground down to dust, and stored at – 70 °C until processing. The samples were homogenized in RIPA lysis buffer and protein concentration was determined. The concentration of each sample was adjusted to 1 mg protein/ml. Twenty µl of samples were loaded onto a 10% SDS-polyacrylamide-gel, and electrophoresed at 120V for 1 h. Separated proteins were blotted onto a nitrocellulose membrane at 100 V for 90 min during the continuous mixing and cooling of the transfer buffer. For quantitative analysis, the intensity of three bands taken from different homogenates was determined using standard densitometric procedures. The densitometric analysis was optimized to a stained BSA standard and to the immunoblot made on different concentrations of the myosine phosphatase protein.

Preparation and culturing HUVEC

Freshly isolated umbilical cords were obtained from the Department of Obstetrics and Gynecology (University of Debrecen). Umbilical cords were immersed into a sterile flask containing culturing medium and carried to the cell culture room. Both ends of the cords were bound and the ends of the umbilical vein were canulated. The vein vessel was washed through the canules by sterile PBS, followed by Hank's Balanced Salt Solution (HBSS), and M199 media. Then, the vein vessel was filled with 1 mg/mL collagenase in M199, and placed in a CO₂ incubator for 12

min at 37 C°. Endothelial cells were isolated in a sterile centrifuge tube from the vessel by washing twice with 20 ml M199 medium. Cell suspension was centrifuged at 1000 rpm for 15 min, the supernatant was discarded and the cells were resuspended in the culturing medium.

The cells (2×10^6 cells/ml) were inoculated in a plastic culturing dish. After 4-5 days of culturing, during which time cells grew extensively over the entire space of the dish, cells were washed with HBSS, then digested with 1 ml trypsin-EDTA (trypsin:/EDTA = 1:25). The cells were suspended in one ml culturing medium and collected into a sterile flask, then mixed well. Then were passed onto culturing plates at about 2×10^6 cell/ml concentration. Cell monolayers that reached 20-30% or 70-80% confluency were used for the experiments. The cells were kept in 50% normal, 50% UC or 50% CD sera dissolved in culturing medium for 2, 4, 8, 12, 20, 24, 36, and 48 h, then processed for visualization of eNOS or iNOS.

Immunoblot (Western blot)

After removing the culturing media, cells were washed with ice-cold PBS, then harvested by a cell scraper into 50 μ L RIPA lysis buffer.

The protein concentration of the lysates was determined by the BCA method using BSA as standard in an ELISA reader at 540 nm. From each solution, aliquots (30 μ g protein) were applied to SDS-PAGE and electrophoresed at 120 V for 1 h. Separated proteins were blotted onto a nitrocellulose membrane. The membranes were incubated with the anti-eNOS or anti-iNOS polyclonal antibodies raised in rabbits. After washing with PBS-Tween for 3x5 min, membranes were incubated with horseradish peroxidase conjugate of anti-rabbit IgG. Densitometry of the blots was performed and the scans were analyzed by the Volume Analyse feature of the Molecular Analyst Software. The densities for the NOS

bands was normalized in each blot to the NOS-IR band obtained from the sample of 2 h treatment with normal serum and expressed as percentage of this control.. The significance of the data between groups (normal, UC, CD) was tested by analysis of variance (ANOVA). Differences were considered significant for values of $P < 0.01$.

Immunofluorescence

Cells were cultured on coverglasses coated with sterile filtered 10% gelatine in PBS. Cultures showing 20-30% confluence were used for studying proliferation, cell distribution and apoptosis/necrosis characteristics, whereas those with 70-80% confluency were used for visualization of NOS isoforms..

For specific detection of eNOS and iNOS proteins, non-specific binding sites of primary antibodies were blocked by incubation of the fixed cells. NOS primary antibodies used for immunocytochemistry were the same as those used for immunoblotting. Both eNOS and iNOS antibodies were applied in 1:200 dilution in solution-A overnight at 4C°. Cells were incubated with FITC conjugated anti-rabbit IgG diluted to 1:40 in solution-A for 30 min at room temperature

For evaluation of the proliferation activity, HUVEC were incubated in a medium containing 50% normal, 50 %UC or 50 % CD sera, or in a medium containing 50% normal sera plus 10^{-4} M nitro-L-arginine methyl-ester, a NOS inhibitor. Cells were exposed to a monoclonal antibody (in 1:100 dilution) specific for the proliferation marker Ki-67 antigen for 2 h at room temperature. In this case, FITC-conjugated anti-mouse IgG was used as a secondary antibody.

For testing the effect of normal and IBD sera on the viability of HUVEC, apoptotic/necrotic cells were assessed by the annexin-V-biotin kit. Briefly, after removing the incubation media and washing with PBS,

cells were labeled with the annexin-V-biotin/propidium iodide solution. After subsequent washing with HEPES, cells were fixed with methanol-ethanol (1:1) solution for 1 min, and labeled with 5 $\mu\text{g}/\text{mL}$ avidin-fluorescein for 15 min. Preparations used for the assay of proliferation activity and to assess apoptosis/necrosis were stained prior to covering with 1% toluidin blue in PBS. The integrity of the cytoskeleton was examined by visualization of actin filaments by incubation with 0.2 $\mu\text{g}/\text{mL}$ phalloidin-FITC for 1 h.

Examination was carried out and digital images were captured with an Olympus AX-70 fluorescence microscope. Images were taken from Ki-67 immunoreactive (IR), annexin-labeled, and propidium iodide reactive cell nuclei, respectively. The same sites of the preparations were also captured in bright visual illumination to count the toluidin blue stained nuclei (total cell number). The images were quantitatively analyzed by the computer image analysis software Olympus analysis 2.11 (SiSoft), developed and validated by Olympus. At least 10^3 cells were counted for each data point and the ratio of proliferative, apoptotic and necrotic cells were given as the percentage of total cell numbers. Statistical analyses were calculated from the data obtained for the different treatments comparing the IBD and normal group using the ANOVA test.

Statistics

Data were presented as the mean \pm SEM. The significance of the results was determined by an unpaired Student's *t*-test, and the group (inflamed versus uninfamed, and UCi versus CDi, respectively) means were tested by analysis of variance (ANOVA). *P* values are provided in the figure legends. Differences were considered significant for values of $p < 0.01$.

Results

Distribution and characterization of iNOS-IR cells in IBD mucosa

Inflamed mucosa of patients with active UC exhibited numerous inflammatory cells detected by the histochemical NADPH-diaphorase reaction. NADPH-diaphorase reactive cells accumulated mainly around the base and along the wall of the Lieberkühn crypts. Parallel sections with anti-iNOS revealed that the distribution and morphological characteristics of the iNOS-IR cells were similar to those that showed NADPH-diaphorase reactivity. In contrast, sections of CD biopsies exhibited only a limited number of NADPH-diaphorase reactive or iNOS-IR cells in the mucosa.

Applying double labeling immunohistochemistry for the identification of inflammatory cells in IBD demonstrated that iNOS-IR cells displayed CD68 or CD15 membrane antigens, suggesting that these are macrophages (CD68-IR) or neutrophils (CD15-IR). In UC, many of the CD15-IR cells found in the mucosa displayed iNOS immunoreactivity. In contrast, in CD the dominant inflammatory cell type was macrophage. However, in sections developed for iNOS immunohistochemistry, only some of the CD68-IR cells displayed iNOS immunoreactivity.

Quantification of iNOS immunoreactivity confirmed that the number of iNOS-IR cells were significantly higher in biopsies of inflamed sites of UC (UC_i , 64 ± 4 cells/mm²) compared to the sections of inflamed CD mucosa (CD_i , 4 ± 2 cells/mm²).

Significant differences were observed between the inflamed and uninfamed (UC_u) groups within UC patients (UC_u , 19 ± 8 cells/mm²).

The number of CD15-IR cells far exceeded the number of CD68-IR ones in UC_i , revealing a percentage distribution for CD15-IR and CD68-IR of 92.0% and 8.0%, respectively. Among CD15-IR cells, 92.4% were iNOS-IR ($85 \pm 10\%$ of the total (CD15-IR + CD68-IR) cell number),

whereas 87.5% of CD68-IR cells displayed iNOS immunoreactivity (7±5% of the total cell number). In UC_u, the percentage of the iNOS-IR cells within CD15-IR (9.6%) and CD68-IR (11.8%) cells was significantly lower. Regarding to the total (CD15-IR + CD68-IR) cell number, only the ratio of iNOS positive CD15-IR cells represented a significant change (in UC_u 8±3%, in UC_i 85±10%).

In CD_i, the occurrence of CD68-IR cells was more prominent (90.0%) than CD15-IR cells (10.0%) Only 14.3% of the CD15-IR cells were iNOS-IR, and similarly only a small proportion (4.4%) of CD68-IR cells exhibited iNOS immunoreactivity. In CD_u, the percentage of the iNOS-IR cells within CD15-IR cells did not change significantly (16.7%), whereas within the CD68-IR cells, the percentage of iNOS-IR cells (33.8%) was higher than in CD_i. Regarding to the total (CD15-IR + CD68-IR) cell number, the ratio of iNOS positive CD68-IR cells also represented a significant change (in CD_u 23±8%, in CD_i 4±3%).

In the biopsies, where the number of iNOS-IR cells were low, the apical surface of the epithelium displayed strong staining for NADPH-diaphorase, or for iNOS in inflamed compartments of the colon in UC and CD. In control mucosa the NADPH-diaphorase reactive or iNOS-IR epithelium was never seen.

Immunoblotting of tissue homogenates revealed a 130 kDa iNOS-IR protein band matched to the molecular mass of human iNOS. The intensity of the band was significantly stronger in the inflamed versus the intact mucosa in UC, whereas in CD, no significant changes were observed according to the control.

Localization of eNOS and comparison of the vascular status in IBD

NADPH-diaphorase staining of the blood vessels, exhibited distinct patterns in UC and CD. The enlarged vessels observed in UC displayed intense NADPH-diaphorase reactivity in the endothelia. In contrast, the

number of small diameter blood vessels was high in the mucosa of CD, and no NADPH-diaphorase reactivity of the endothelial cells could be detected. In the lamina propria, the blood vessels displayed thickening of the wall and narrowing of the lumen as well. In control, NADPH-diaphorase reactivity in the wall of capillaries was moderate. eNOS immunoreactivity of capillaries and other small diameter blood vessels were prominent in the lamina propria of UC, whereas the eNOS-IR endothelium could rarely be observed in CD. Myoepithelial cells around the crypts displayed eNOS immunoreactivity irrespectively of the section origin.

The endothelial marker PECAM-1 (CD31) was visualized by immunoreaction in order to demonstrate the vascular state of the IBD colon. Qualitative differences observed in CD31 and eNOS immunoreactivity were confirmed by quantitative image analyses of the sections: in control, the number of CD31-IR capillaries was 24 ± 8 blood vessel/ mm^2 , in UC 17 ± 6 blood vessel/ mm^2 , in CD 48 ± 12 blood vessel/ mm^2 . Whereas in control and UC samples the ratio of eNOS/CD31-IR capillaries was 92.0%, and 82.3%, respectively, in CD this ratio was significantly low (8.3%).

Detection and densitometric analyzes of eNOS by immunoblotting and immunofluorescence

Western blots from HUVEC lysates detected eNOS immunoreactive bands at approximately 160 kDa, corresponding to the molecular mass of human eNOS. Culturing HUVEC in a medium containing 50% normal human serum, the amount of eNOS did not change significantly during the experimental period (0-48 h). However, in the presence of 50% UC serum, the eNOS level increased after 8h, and peaked at 12 h (relative optical density value was $188 \pm 23\%$ and

229±26%, $P<0.01$, respectively). At 12 h, the eNOS level appeared to have doubled compared to the normal level at 2h (100±15%). Over the 12 h period in the presence of 50% UC serum, the eNOS level began to decline (36 h, 153±23%, $P<0.01$), and reached control level at 48 h (100±8%). In the presence of 50% CD serum the level of eNOS was close to normal until 8h, decreased after 12 h (41±12%, $P<0.01$) reaching the lowest level at 20-24h (18±16% and 23±15%, $P<0.01$, respectively), then slowly increased rising close to normal at 48h (65±8%).

Localization of eNOS in HUVEC by immunofluorescence revealed that the enzyme mostly accumulated in the nuclear area of the cytoplasm and was also detected at the periphery of the cells. Normal human serum had no effect on the distribution and intensity of eNOS immunoreactivity in HUVEC. Prolonged treatment (12 h) with UC serum extended the eNOS immunoreactive area and simultaneously enhanced slightly the intensity of eNOS immunofluorescence in HUVEC. In contrast, in the presence of CD serum (after more than 4 h), the eNOS immunoreactivity seemed to contract toward the centre of the cells and its immunofluorescence intensity decreased. At the end of the experimental period (48h treatment), no visible difference could be detected in the eNOS immunofluorescence of HUVEC in the presence of either normal, UC or CD sera.

Detection and densitometric analyzes of iNOS by immunoblotting and immunofluorescence

Western blots in HUVEC lysates detected faint iNOS immunoreactive bands at around 130 kDa, corresponding to the molecular mass of human iNOS. Culturing HUVEC in the presence of 50% normal human serum, the relatively low level of iNOS expression did not change significantly during the 48h incubation period. In the

presence of 50% UC serum, the iNOS level increased at 4h (relative optical density value was $225\pm 20\%$, $P<0.01$), peaked at 8h ($300\pm 21\%$, $P<0.01$), then declined to about normal level at 48h ($117\pm 15\%$). In the presence of 50% CD serum the iNOS level in HUVEC increased after 2 h ($201\pm 19\%$, $P<0.01$) and peaked at 8 h ($275\pm 27\%$, $P<0.01$). From 12 h the iNOS level declined and was not distinguishable from the control during the 24-48h period (at 48 h: $92\pm 18\%$). In the presence of 50 % normal serum HUVEC exhibited faint iNOS immunofluorescence. Expression of iNOS in HUVEC by immunofluorescence could obviously be detected in the presence of IBD sera at the 4-12 h period. iNOS was dispersed in the cytoplasm, accumulated in certain areas showing a high intensity of fluorescent signal. Both UC and CD sera significantly increased iNOS immunofluorescence at the 4-12 h period. Before and after this time interval, iNOS could not be detected unequivocally in HUVEC by immunofluorescence.

Effect of IBD sera on proliferation activity of HUVEC

Ten to fifteen percent of HUVEC was observed to be in the state of proliferation in the cultures with 20-30% confluency at the beginning of the experiments as revealed by Ki-67 immunostaining . From the 2 h to 48 h period no difference was detected in the ratio of Ki-67-IR/toluidin blue stained cell nuclei in cultures in the presence of 50% normal (at 48 h: $19\pm 10\%$) or 50% UC sera (at 48 h: $10\pm 3\%$). In the presence of 50 % CD serum a significant increase in the number of proliferating cells is apparent at 20 h ($41\pm 12\%$, $P<0.01$). About 50-65% of HUVEC nuclei exhibited Ki-67 immunofluorescence in the presence of CD serum for 24-48 h. The presence of 10^{-4} M L-NAME in the medium containing 50%

normal serum, increased the proliferation activity significantly from 24 h ($42\pm 5\%$, $P<0.01$) to 48 h ($48\pm 7\%$, $P<0.01$).

Effect of IBD sera on viability of HUVEC

Using the annexin-V-biotin method, early apoptotic cells revealed fluorescent signals only on the cell membrane, whereas necrotic cells showed propidium iodide binding to DNA (nuclear fluorescent signal). Both signals could be detected in late apoptotic cells. During the experimental period (2-48h), the number of apoptotic cells was low (1-3%) in the presence of 50% normal serum, but a continuous increase of apoptotic cell numbers was observed in the presence of 50% CD serum (2-5%, with a maximum level at 36h), which proved not to be significant. In the presence of UC serum the apoptotic index did not differ from the control. Incubation of HUVEC with both normal and CD sera slightly increased the number of necrotic cells during the examination period (2-48h), but no significant changes were observed between the necrotic indices. Actin filaments visualized by phalloidin-FITC seemed to be intact in cells independent of the media used and the time of incubation .

Effect of IBD sera on the shape and structure of HUVEC culture

HUVEC cultures of 20-30% confluency were used also to investigate the effect of IBD sera on the distribution and shape of the cells. Cells visualized by eNOS immunofluorescence were partly found to be accumulated in islands exhibiting polygonal shape and attached to each other. In addition, single cells exhibiting long processes were also present. In the presence of 50% normal or 50% UC sera cells did not change distribution and shape over the 48 h experimental period. However, in the presence of 50% CD serum, after 20 h cells were

distributed in a two dimensional reticular network, changing the shape of the culture reminiscent of the appearance of early vascular vessels in tissues.

5. Conclusions

1. In UC, intensive iNOS immunoreactivity was present in the epithelial cells and in mucosal inflammatory cells, according to the data obtained by histochemical, immunohistochemical or immunoblot methods. The eNOS level was found to be unchanged compared to the control, in the endothelium. iNOS-IR cells were mainly neutrophils in UC.

2. A lower level of iNOS and eNOS was observed in mucosal leukocyte type cells and endothelium of CD patients, respectively. CD68-IR (macrophage) cells are high in CD mucosa, but as in the case of relapsed inflammation, they exhibited low iNOS immunoreactivity.

3. The diminished NADPH-diaphorase reactivity and eNOS immunoreactivity in CD endothelial cells together with the presence of a high number of capillaries provoke speculation supporting the role of microvascular system in the pathogenesis of CD. Part of the remodeling is neovascularisation as was also revealed in our study by the increased number of CD31-IR capillaries within low eNOS-IR material. Microvascular endothelial dysfunction found only in areas of chronic inflammation is probably an acquired alteration, a consequence of tissue remodeling in chronically inflamed bowel segments.

4. Histological data do not show correlation with the actual clinical and endoscopic activity index of IBD, therefore this does not reflect strictly the state of inflammation.

5. iNOS expression and presumably the constitutive eNOS activity are regulated in a different manner in UC and CD, therefore NO may play different roles in eliciting and maintaining the inflammation in the inflammatory bowel diseases.

6. Differences observed in expression and distribution of NOS isoforms could be of importance in the histological differentiation of UC and CD.

7. The NADPH-diaphorase histochemical reaction was successfully applied for the assessment of iNOS and eNOS in the cells. Since a significant difference was observed in the number of cells showing NADPH-diaphorase reactivity and iNOS immunoreactivity between UC and CD, this simple, cheap and easy to carry out procedure might be useful in the differential-diagnosis of these two types of IBD

8. Induction of both iNOS and eNOS was observed in the presence of UC serum. In contrast, upon addition of CD serum to HUVEC media a transient increase in the expression of iNOS was observed parallel with a marked decrease in the expression of eNOS. The number of apoptotic and necrotic cells, was not altered significantly, and the structural integrity of the cytoskeleton was not changed either.

9. IBD sera used in our study may include a mixture of numerous cytokines and inflammatory adhesion molecules with a non-specific

inflammatory effect. Up-regulation of iNOS by IBD sera may be an adaptive response of HUVEC to this culture environment. The constitutive expression of eNOS in the microvascular endothelium is believed to be essential for systemic and/or local vascular integrity. The effect of UC and CD sera on eNOS expression could be of special interest and may be implicated in the distinct pathophysiological and morphological features of the two diseases. The reciprocal reaction of cytokines and angiogenic molecules results in changes in the expression of eNOS and iNOS and causes an imbalance between the two isoforms which may play a role in the dysfunction of angiogenesis in IBD.

10. HUVEC, in the presence of CD serum, but not in the presence of UC serum, showed morphological changes reminiscent of vascular like structures. This special loop formation of the cells may suggest an angiogenic activity of CD sera.

11. Results correlate well with immunohistochemical findings on biopsies, and suggest the presence of a cytokine composition in the blood of IBD patients which is able to evoke changes in the eNOS/iNOS ratio in endothelial cells. Simultaneous down-regulation of eNOS and up-regulation of iNOS, together with the increase of the cell proliferation activity may reduce the anti-inflammatory capacity of endothelial cells. The latter may contribute to the initiation of pro-inflammatory cascade reactions leading to endothelial barrier dysfunction and causing structural changes in the microvessels in Crohn's mucosa. Our present findings imply that some components of the Crohn's serum decrease eNOS protein level in the endothelium which may influence the pathomechanism and cause abnormal alterations in the vascular wall of the inflamed tissue.

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