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Rapid communication

High nitric oxide synthase activity in endothelial cells in ulcerative colitis

ETSURO IWASHITA[1], TORU MIYAHARA[1], KUNIHIKO HINO[1], TETSUJI TOKUNAGA[1], HITOSHI WAKISAKA[2], and YOSHIO SAWAZAKI[2]

[1] Second Department of Internal Medicine and [2] First Department of Anatomy, National Defense Medical College, 3-2 Namiki Tokorozawa, Saitama, 359 Japan

Abstract: Endothelial nitric oxide (NO) synthase, a unique NO synthase (NOS) isoform that is expressed constitutively by the vascular endothelium both in vivo and in vitro, is believed to be essential to systemic and/or local vascular integrity. NOS expression by endothelial cells may indicate vascular activation. We successfully established a simple method for the culture of microvascular endothelial cells from a small amount of tissue and investigated ulcerative colitis (UC), in which condition vascular factors have not been studied extensively. We cultured endothelial cells from the mesenteries of surgical patients with UC and assayed NOS activity by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry. Strong NOS activity was demonstrated in the cells from all UC patients (5/5), whereas no activity was detected in the cells from human umbilical veins and the mesenteries of colon cancer patients (0/10 and 0/5, respectively). This strong NOS activity was not diminished by incubation with a high concentration of glucocorticoid, suggesting that it was constitutive. These results indicate a close relationship of vascular activation (high NOS activity) with the pathogenesis of UC.

Key words: nitric oxide synthase, endothelial cells, ulcerative colitis

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease of unknown etiology that is characterized by the intestinal infiltration of inflammatory and activated immunocompetent cells in the chronic stage.[1] In UC, the adhesion of circulating cells to the vascular endothelium and transendothelial migration into the tissues are very important in the process of inflammation. Thus, investigation of endothelial cells may be critical to gain complete understanding of the pathogenesis of UC. In particular, study of the microvascular endothelium obtained from the mesenteries may be significant in clarifying the earliest phase of inflammation.

Endothelial nitric oxide (NO), which was first reported as endothelium-derived relaxing factor (EDRF) in 1987,[2] is synthesized from L-arginine by several types of NO synthase (NOS). NO is a short-lived free radical and messenger molecule that mediates diverse functions, including vasodilatation,[3] neurotransmission,[4] vascular permeability,[5] and antimicrobial activity.[6] There are only a few reports referring to the pathological and clinical significance of NOS activity in UC. Some authors have suggested that NOS activity is increased in the homogenized colonic mucosa of UC.[7,8] However, endothelial NOS activity has not yet been studied in UC. To investigate the importance of the role played by endothelial NOS in UC, we cultured endothelial cells from UC patients and investigated endothelial NOS activity.

Patients and methods

Preparation of endothelial cells from UC patients

Mesenteries were obtained as fresh tissue specimens from five consecutive UC patients (M:F, 1:4; average age 38.6 years) in whom surgical resection was performed at our Department of Surgery between November 1992 and March 1994. The operatives were performed because the patients were resistant to conservative treatment. Endothelial cells were cultured by the procedure described below. The mesentery was

Offprint requests to: E. Iwashita

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treated with 10% Isodine [225 ml phosphate-buffered saline (PBS) +25 ml povidone-Iodine] for 5 min, washed five times with PBS, and minced with scissors. The specimen was then shaken in 0.2% collagenase at 37°C for 20 min and filtered through a stainless steel mesh (100 μm). The partly digested tissue retained on the mesh was incubated, with shaking, in 0.02% ethylene diamine tetraacetic acid (EDTA) + 0.04% trypsin at 37°C for 20 min and the solution was filtered again through a stainless steel mesh. The filtrate was centrifuged at 1500 rpm for 5 min and the precipitate was seeded in a 6-cm dish coated with human fibronectin. The dish contained culture medium (M199 containing 10% newborn calf serm and 10 ng/ml recombinant human basic fibroblast growth factor (FGF). Control cultures were obtained by culturing apparently normal mesenteries obtained from surgically resected specimens (patients, M : F, 3 : 2, average age 54.4 years) at a location of 6 cm or more from colon cancer (n = 5). Human umbilical vein endothelial cells (HUVEC) were also cultured, according to the method of Jaffe et al.[9] (n = 10). The collected cells were identified as endothelial cells because they were immunologically stained for von Willebrand factor and showed characteristic tube formation in collagen gel.

Detection of NOS activity (NADPH-diaphorase histochemical assay)

NOS detection in cultured endothelial cells was performed according to the method of Janssens et al.[10] Briefly, endothelial cells cultured through at least four passages (population doubling; 20) were seeded in culture chamber slides (Nunc, Roskilde, Denmark) and incubated overnight. The cultures were then washed with PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 3 h, and washed three times with PBS for 10 min each time. The cultures were then incubated at room temperature for 1 h with a staining solution prepared by adding 0.1 M phosphate buffer (pH 7.4) to 150 μl of Triton X-100, 50 mg of NADPH (reduced form), and 10 mg of nitroblue tetrazolium to make a volume of 50 ml. Finally, the cultures were washed with distilled water and NOS was demonstrated as NADPH-diaphorase activity by blue cytoplasmic staining.

Results

Endothelial cells from the mesenteries of all five UC patients (E-UC) showed abundant blue cytoplasmic staining in the NADPH-diaphorase histochemical assay. Endothelial cells from control patients (E-Ca) and HUVEC, in contrast, were not stained at all in the simultaneous assays (0/5 and 0/10). However, E-Ca and HUVEC were both stained intensely when stimulated with lipopolysaccharide (LPS) from *Escherichia coli* (serotype O55 : B5; Sigma St. Louis, USA). To compare NOS activity in E-UC and HUVEC, cultures were treated with 10 ng/ml or 10 μg/ml of LPS and incubated for 24 h. HUVEC with 10 μg/ml of LPS stained more intensely than HUVEC with 10 ng/ml of LPS. E-UC exhibited even higher NADPH-diaphorase activity than HUVEC incubated with 10 μg/ml of LPS (Fig. 1). In addition, when 10 μg/ml of LPS was incubated with E-UC for 24 h, there was no change in NADPH-diaphorase activity. E-UC were also cultured with prednisolone sodium succinate (PSL) for 36 h. E-UC cultured with 100 ng/ml or 10 μg/ml of PSL were stained as strongly as those without PSL in the NADPH-diaphorase assay (Fig. 2).

Discussion

It has been reported that the NOS activity of homogenized colonic mucosa is significantly elevated in UC.[7,8] In these studies, NOS activity was determined by measuring ^{14}C-citrulline, a co-product of the conversion of L-^{14}C-arginine to NO. With this method, however, it cannot be clarified whether high NOS activity in the colonic mucosa is derived from the endothelium. The possibility cannot be ruled out that numerous macrophages and neutrophils infiltrating the colonic mucosa in the process of UC inflammation have secondarily elevated the NOS activity of the tissue as a whole. It thus remains unclear whether the colonic epithelial cell in UC patients have high NOS activity, making it difficult to attach a pathogenic significance to elevated NOS activity. As to the vascular endothelium, the authors cited above considered it only in the context of secondary involvement, and no specific vascular role was proposed.

In the present study, E-UC, which had divided at least 20 times, were examined in vitro for NOS activity, using the NADPH-diaphorase method. We performed the experiment in vitro primarily because such an approach facilitates the sensitive detection of injury to endothelial cells. Histochemical staining with NADPH-diaphorase involves the detection of activity in the C-terminal of the isoform of paraformaldehyde-resistant NOS.[10] Our results showed that only E-UC had high NOS activity, even after repeated subculture. In addition, the strong activation of NOS was resistant to the effect of a high concentration of PSL, suggesting that the source of NOS is a constitutive isoform[11] and that cell division has little to do with the expression of NOS activity. It is possible that a minimal amount of LPS was present in the culture medium, but its effect

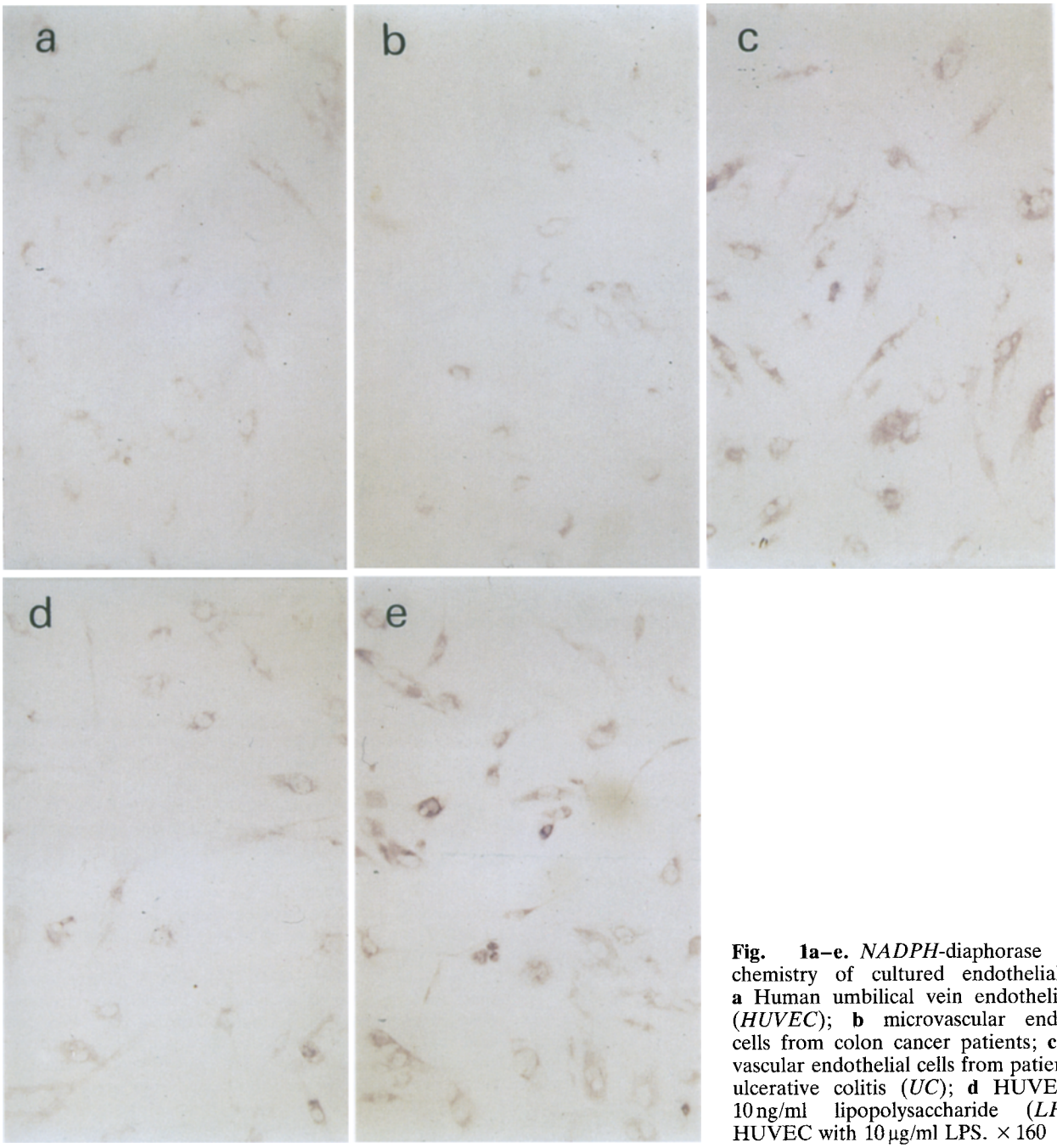

Fig. 1a–e. *NADPH*-diaphorase histochemistry of cultured endothelial cells. **a** Human umbilical vein endothelial cells (*HUVEC*); **b** microvascular endothelial cells from colon cancer patients; **c** microvascular endothelial cells from patients with ulcerative colitis (*UC*); **d** HUVEC with 10 ng/ml lipopolysaccharide (*LPS*); **e** HUVEC with 10 μg/ml LPS. × 160

was considered to be negligible, since NADPH-diaphroase activity did not appear in HUVEC unless LPS was deliberately added.

In our study, the high level of endothelial NOS activity in UC patients showed evidence of vascular activation. Even endothelial cells in the histologically uninvolved areas far from the sites of inflammation had high NOS activity. It seems possible that endothelial cells, at least those in the mesentery, play an important role in the earliest phase of inflammation in UC. Endothelial cells would utilize NO from the constitutive NOS and thus increase vascular permeability.[5] NO induces vasodilatation through vascular smooth muscle relaxation;[3] as a result, an early inflammatory reaction is likely to occur. NO also protects endothelial cells against the adhesion of circulating immunoactive cells and the aggregation of platelets.[12,13] Because of this function of NO, typical pathological vasculitis may not develop. In conventional NOS inhibitory experiments,[7,8,14,15] such an autocrine regulator process has not been studied. The findings that circulating von Willebrand factor[16] and submucosal endothelin-1[17] are

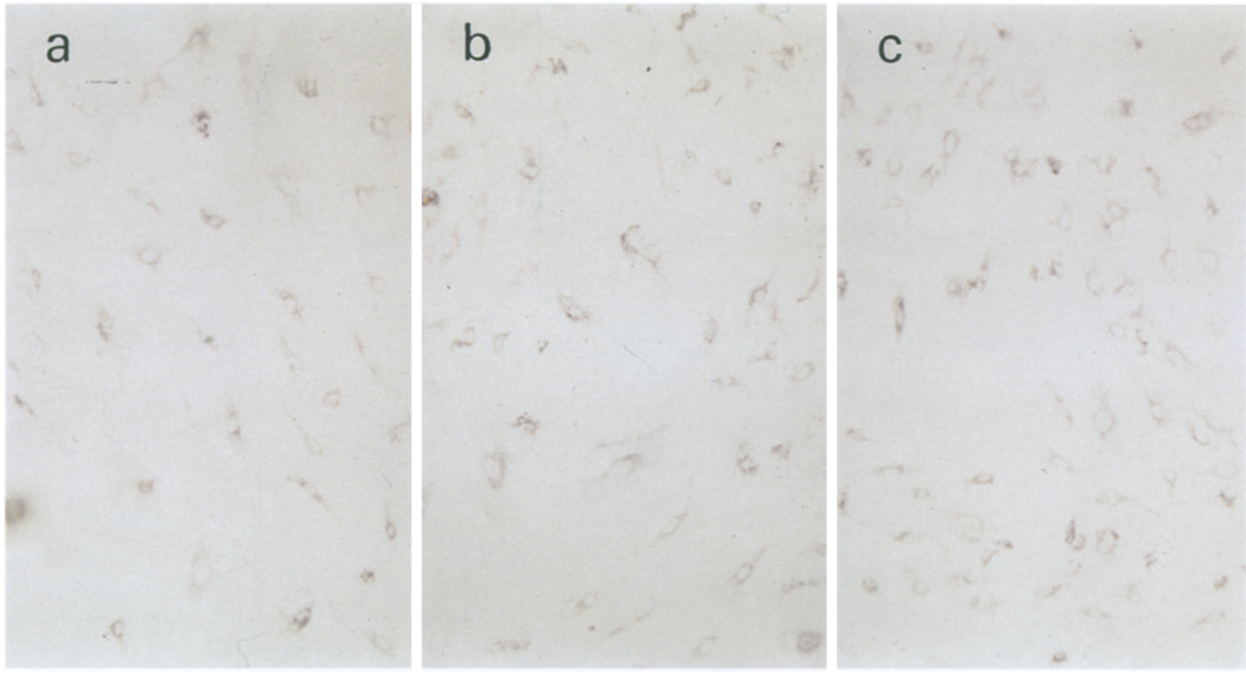

Fig. 2a–c. Effects of prednisolone sodium succinate (*PSL*) on histochemical reactivity in cultured endothelial cells from UC patients; **a** without PSL; **b** with 100 ng/ml PSL; **c** with 10 μg/ml PSL. × 160

increased in UC, although the typical pathology of vasculitis was not found, provides support for our idea.

Since it is not sufficient to study the pathogenesis of UC without taking into account vascular factors, our new approach to endothelial NOS may be useful. Although further studies are necessary to investigate the role played by NO in the pathogenesis of UC, NO production by endothelial NOS appears to play an important role in the initiation and development of this disease.

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