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Proinflammatory and Antimicrobial Nitric Oxide in Gingival Fluid of Diabetic Patients with Periodontal Disease▽

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Abnormal nitric oxide (NO) synthesis has been implicated in the pathogenesis of both periodontal disease and diabetes mellitus. In diabetic patients, increased inducible NO synthase in inflamed gingiva correlated with NO in gingival crevicular fluid. Although increased NO reflected more-severe inflammation, it was associated with reductions in CFU of *Prevotella intermedia*, a major periodontopathogen, highlighting dual roles for NO.

Nitric oxide (NO), a toxic free radical (25) with multiple biological functions, including inhibition of neutrophil chemotaxis (14), adhesion to endothelium (17), and upregulation of tumor necrosis factor alpha (29), is generated by oxidative deamination of L-arginine by nitric oxide synthase (NOS). The inducible form of NOS (iNOS) is rapidly and durably expressed by inflammatory cells in response to bacteria or their products, such as lipopolysaccharide (LPS) (32). Small amounts of NO induced by constitutive NOS are considered beneficial, whereas excess iNOS-induced NO can mediate cell and tissue injury. Periodontal diseases are chronic inflammatory infections associated with gram-negative bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* (26), which stimulate macrophages to generate NO (1, 7, 16). Moreover, NO is increased in inflamed gingival tissue (11, 15), and mercaptoethylguanidine, a selective iNOS inhibitor, prevents bone destruction in ligature-induced rodent periodontitis (21).

Periodontal disease is often a chronic complication of diabetes mellitus (20), with evidence of increased gingival inflammation, deeper periodontal pockets, and greater clinical attachment and bone loss (22). Hyperglycemia stimulates the production of advanced glycolysated end products, enhances the polyol pathway, and activates protein kinase C, which may lead to increased oxidative stress (12). Increased NO concentrations were demonstrated in sera of patients with type I diabetes and persistent microalbuminuria (2).

The aim of our study was to evaluate expression of NO in gingivae of type I diabetic patients presenting with periodontal disease and to correlate the level of NO with *P. intermedia* infection. Gingival tissues were obtained during modified Widman flap surgery from diabetic patients (three males and two females; mean age [±standard deviation], 48.2 ± 6.9 years) diagnosed with moderate (probing depth of ≤5 mm) or advanced (probing depth of >5 mm) periodontitis. Noninflamed gingival tissue was obtained during the crown-lengthening procedure of diabetic patients (two females, aged 44 and 51 years) (protocol approved by National Medical Ethics Committee of Slovenia; patients signed informed consent). Fixed and embedded tissue sections were stained with hematoxylin-eosin (H&E) or antibodies against iNOS (monoclonal antibody 9502, 1:100 in 1% bovine serum albumin; R&D Systems, Minneapolis, MN), CD29 (monoclonal antibody 1778, 1:100; R&D Systems, Minneapolis, MN), and CD68 (M0876, 1:50; DAKO Corporation, Carpinteria, CA) by use of an indirect biotin streptavidin system for detection (basic 3,3′-diaminobenzidine tetrahydrochloride detection kit 760-001; Ventana Medical Systems, Tucson, AZ) (18). An intense inflammatory infiltrate composed predominantly of mononuclear cells, including lymphocytes and macrophages, was observed in H&E-stained gingival tissues from periodontally involved type 1 diabetic patients (Fig. 1a). Immunostaining confirmed the presence of CD68-positive macrophages (Fig. 1c) within the inflammatory site as well as CD29-positive fibroblasts (Fig. 1b) along the margins of the infiltrate. Importantly, iNOS-positive cells were identified within the lesion (Fig. 1d). Similarly to the results of Hirose et al. (11), who did not find iNOS expression in noninflamed gingival tissue of nondiabetic patients, we did not demonstrate iNOS expression in noninflamed gingival tissue of our diabetic patients (data not shown).

Based on the elevated iNOS expression in inflamed gingival tissue, gingival fluid samples were obtained from diabetic patients (13 males and 5 females; mean age, 38.8 years; range, 24 to 58 years; mean duration of diabetes, 16.1 years; range, 5 to 35 years) by use of 2-μl microcapillary tubes (Drummond Co., Pennsylvania). Fluid was diluted into 50 μl phosphate-buffered saline containing gentamicin (10 μg/ml), filtered (Ultrafree microcentrifuge filter, 10,000 molecular weight), treated with nitrate reductase to convert nitrate to nitrite, and reacted with 2,3-diaminonaphthalene (23). Fluorescence was measured at a wavelength of 365/450 (excitation/emission) by use of a fluorescence plate reader (Idexx Laboratories, Westbrook, ME) and based on a standard curve, with data reported as μM nitrite plus nitrate. Patients were assessed for degree of peri-

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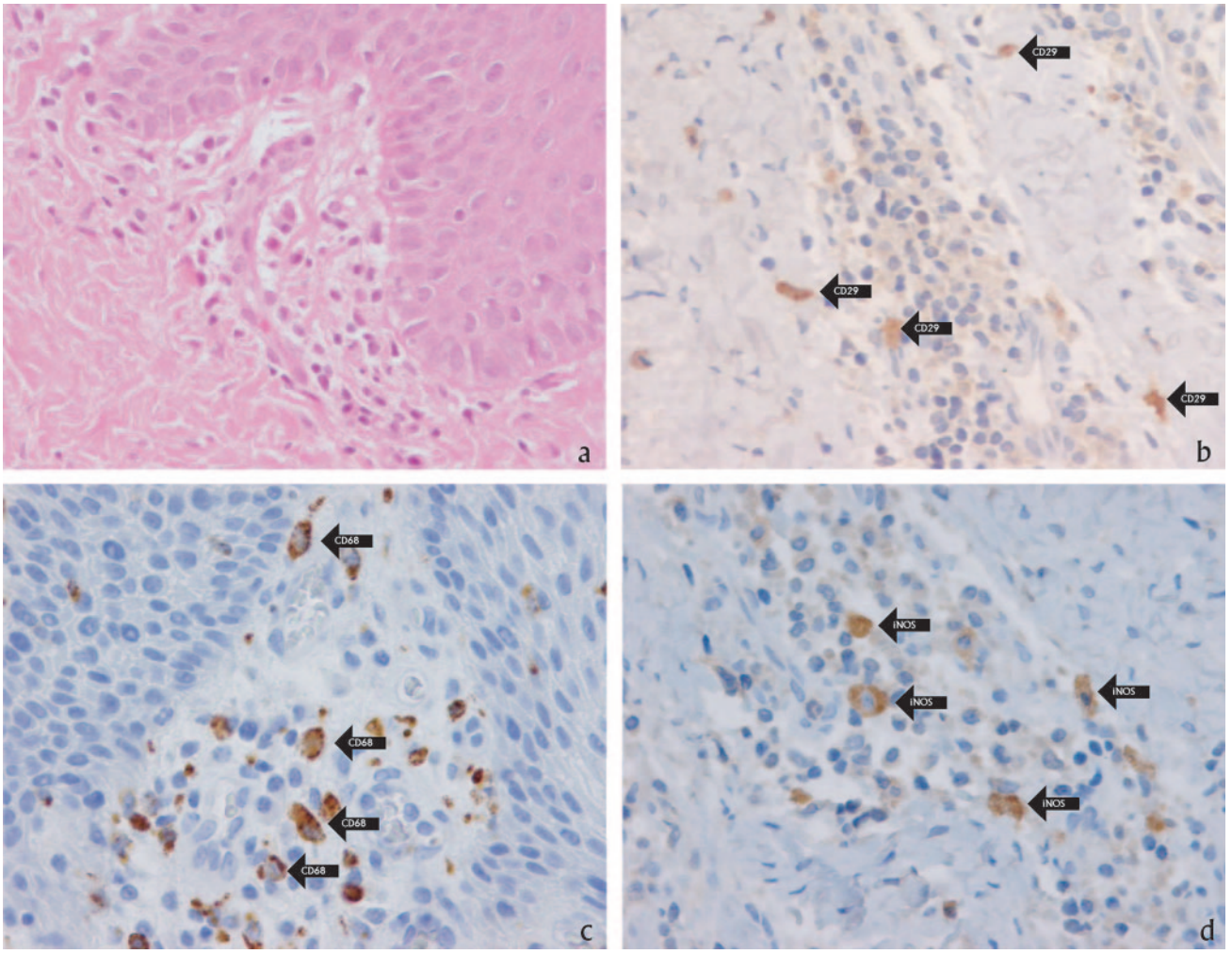

FIG. 1. Mononuclear cell infiltration in gingival tissue of diabetic patient with severe periodontitis. Gingival tissue sections were stained with H&E (a), anti-CD29 (fibroblasts) (b), anti-CD68 (macrophages) (c), or anti-human iNOS (d). Magnification, ×576.

odontitis by plaque index (PI), gingival index (GI) (19), probing depth, and clinical attachment loss (mm) by electronic periodontal probe (Peri-probe; Vivadent, Liechtenstein) and Williams periodontal probe. Consistent with iNOS detection in tissues, NO was quantified in all fluid samples, ranging from 10.7 to 86.0 μM (mean, 22.98 ± 4.32 μM). Samples from tooth sites with small or moderate plaque (PI of 1 or 2) contained increased NO, although the difference was not significant compared to sites with no plaque (PI of 0) ($P > 0.05$, Student's *t* test). Based on gingival inflammation, sites with a GI of 1 or 2 contained significantly higher NO than those with no inflammation (GI of 0) ($P = 0.012$). Likewise, significantly increased NO in gingival exudates was associated with sites of increased probing depth ($r^2 = 0.34$, $P < 0.05$, correlation and polynomial regression analysis) (Fig. 2), all consistent with higher NO in the inflamed sites.

Because *Prevotella intermedia* is one of the causative pathogens of periodontal disease and *P. intermedia* LPS induces iNOS and release of NO in murine macrophages (12), we analyzed DNA from subgingival plaque samples (isolated from the same sites where the gingival crevicular fluid was collected)

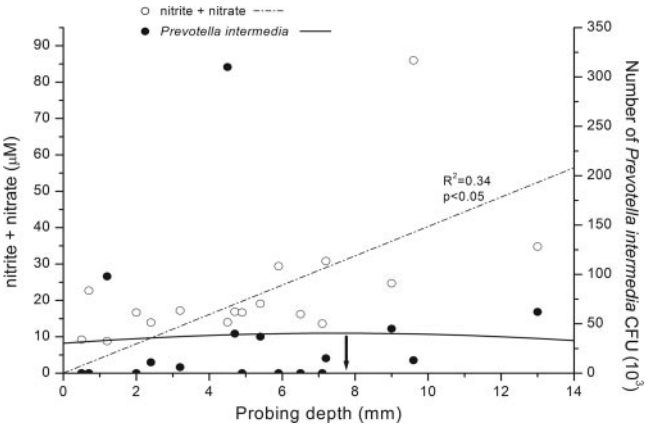

FIG. 2. Correlation between periodontal pocket depth and NO production and number of *P. intermedia* CFU. Gingival fluid and subgingival plaque samples from periodontal pockets of increasing depth were analyzed for NOx and number of bacteria, respectively. Increased NO production was associated with increased periodontal pocket depth. In contrast, the numbers of *P. intermedia* CFU in deeper (>6 mm) periodontal pockets decreased considerably from the numbers found in moderate pockets.

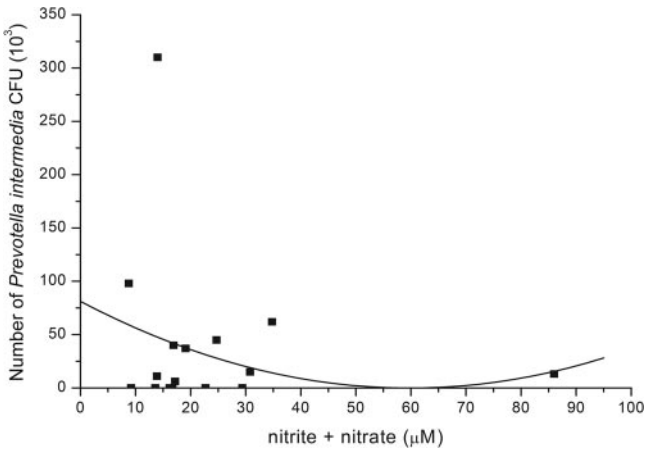

FIG. 3. Correlation between NO levels and numbers of *P. intermedia* CFU in periodontal pockets from diabetic patients. The concentrations of NOx in gingival crevicular fluid and the numbers of *P. intermedia* CFU in subgingival plaque samples were compared in diabetic patients with various degrees of periodontal disease. Increased concentrations of NOx in gingival crevicular fluid were associated with reduced numbers of *P. intermedia* CFU.

of 18 diabetic patients for *P. intermedia*. Loosely adherent supragingival plaque in the intercanine sector was gently removed by cotton gauze. A sterile paper point was inserted into the apical extent of periodontal pocket sulcus for 10 s, and after elution and denaturation, samples were analyzed by slot blot analysis for bacterial species, including *P. intermedia* (Omnigene Laboratory, Cambridge, MA), as described by French et al. (6). Questions regarding the sensitivity and specificity of the Omnigene probe for *P. intermedia* have been raised by van Steenbergen et al. (30), who claimed that the Omnigene probe did not distinguish between *P. intermedia* and *Prevotella nigrescens*. Quantitative differentiation between *P. intermedia* and *P. nigrescens* by oligonucleotide probes to specific rRNA sequences, as described by Gmür and Thurnheer (8), was not performed in our study. However, Moore et al. (24) detected five times as many *P. intermedia* CFU as *P. nigrescens* CFU in periodontal pockets but found seven times more *P. nigrescens* CFU than *P. intermedia* CFU at healthy sites. In addition, Dahlen et al. (4) identified two-thirds of their *P. intermedia*-like isolates from "destructive periodontal disease" as *P. intermedia* isolates, whereas 75% of the isolates from healthy control subjects were *P. nigrescens* isolates. According to these results and the fact that our subgingival plaque samples were collected predominantly from moderate and deep periodontal pockets, we assume that *P. intermedia* and not *P. nigrescens* was the main detected species in our samples. *P. intermedia* isolates were detected in our plaque samples, ranging from 0 to 310 $\times$ 10^3 bacteria. When *P. intermedia* levels were compared by polynomial regression analysis, based on the depth of the periodontal pocket source, we found a modest increase in *P. intermedia* DNA in moderate depth pockets (<6 mm) but decreased numbers in pockets deeper than 8 mm (Fig. 2). Importantly, polynomial regression analysis of NO and *P. intermedia* in periodontal pockets confirmed the reduction in *P. intermedia* DNA in pockets where NO levels were increased (Fig. 3) ($r^2 = 0.34$, $P < 0.05$).

The correlation between increased NO in gingival fluid and decreased CFU of the periodontal pathogen *P. intermedia* in deep periodontal pockets (>6 mm) suggested a possible microbicidal effect. Nitric oxide induced by iNOS has been shown to possess immunomodulatory, cytotoxic, and antibacterial effects (33), consistent with a role for reactive oxygen and nitrogen species in periodontal tissue damage as well as in microbial killing. Recent data indicate that modulation of superoxide levels by NO influences phagocytic functions of neutrophils and macrophages and that NO is an important element of host defense against *P. gingivalis* (9). Since *P. gingivalis* LPS can also induce macrophages to produce NO in an L-arginine- and gamma interferon-dependent mechanism (27), this NO may potentially be an important mediator of bone resorption. In this regard, iNOS null mice demonstrate a significantly reduced osteoclast response (13). In addition, iNOS influences both osteoblast and osteocyte function in bone remodeling (31).

The assessment of NO stable end products, nitrite and nitrate (NOx), is commonly used as a measure of NO production in biological fluids. Both NOx and vascular endothelial growth factor concentrations are increased, although not related, in the vitreous fluid of diabetic patients with proliferative diabetic retinopathy (10), and NO has been implicated in angiogenesis (3). Rapid serum diffusion of NO could contribute to increased aqueous NOx (10), implicating NO in the pathophysiology and progression of diabetic retinopathy (28) as well as in periodontal disease. These molecules may serve as therapeutic targets for the treatment and/or prevention of systemic and ocular microvascular complications in diabetes (5). Similarly, subgingival local delivery of NO inhibitors might be useful in the treatment of periodontal inflammation, particularly as systemic delivery of an NO inhibitor was shown to reduce bone resorption in an animal model of experimental periodontitis (21).

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