T−786C polymorphism of the endothelial nitric oxide synthase gene and neuralgia-inducing cavitational osteonecrosis of the jaws

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Objective. We hypothesized that, similar to idiopathic hip osteonecrosis, the T−786C mutation of the endothelial nitric oxide synthase (eNOS) gene affecting nitric oxide (NO) production was associated with neuralgia-inducing cavitational osteonecrosis of the jaws (NICO).

Design: In 22 NICO patients, not having taken bisphosphonates, mutations affecting NO production (eNOS T−786C, stromelysin 5A6A) were measured by polymerase chain reaction. Two healthy normal control subjects were matched per case by race and gender.

Results. Homozygosity for the mutant eNOS allele (TT) was present in 6 out of 22 patients (27%) with NICO compared with 0 out of 44 (0%) race and gender–matched control subjects; heterozygosity (TC) was present in 8 patients (36%) versus 15 control subjects (34%); and the wild-type normal genotype (CC) was present in 9 patients (36%) versus 29 controls (66%) (P = .0008). The mutant eNOS T−786C allele was more common in cases (20 out of 44 [45%]) than in control subjects (15 out of 88 [17%]) (P = .0005). The distribution of the stromelysin 5A6A genotype in cases did not differ from control subjects (P = .13).

Conclusions. The eNOS T−786C polymorphism affecting NO production is associated with NICO, may contribute to the pathogenesis of NICO, and may open therapeutic medical approaches to treatment of NICO through provision of L-arginine, the amino-acid precursor of NO. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:548-553)

Pathophysiologic mechanisms involved in degeneration and death of cells within the jaws have been studied over the past 94 years.1-7 As early as 1915, G. V. Black, the father of modern dentistry, described a progressive “death of bone, cell by cell,” which he felt differed from osteomyelitis.1 This degenerative process, which Black called “chronic osteitis,” characteristically was able to “soften the bone, often hollowing out the cancellous portions of large areas of bony tissue.” Thirty years earlier, Noel2 had called this “bone caries” and separated it into 2 distinct categories: “bone death” and the less intense “reduced vitality.”

Over the past century, dental and orthopedic researchers have clarified that the etiology of osteonecrosis is multifactorial, the result of a wide variety of local and systemic risk factors.3-10 Whether the factors are acquired (alcoholism, glucocorticoids, estrogen replacement therapy, cancer chemotherapy, deep sea diving) or inherited (sickle cell anemia, Gaucher disease, thrombophilia, hypofibrinolysis, reduced nitric oxide [NO] production), each factor can have a compounding negative effect on the microcirculation within bone.3-16 Neuralgia-inducing cavitational osteonecrosis of the jaws (NICO)3,17,18 is commonly multifactorial, involving trauma, infection, and systemic disorders including thrombophilia and hypofibrinolysis.5,10,17-21 NICO is often multifocal, frequently lacks inflammatory cells, and is remarkably chronic, with deep bone pain and varied, persistent, and severe pain syndromes.3,17,18 NICO is associated with a relatively high failure rate with local interventions, a high prevalence of hypofibrinolysis and thrombophilia in affected patients, and primary localization at the ends of the arterial inflow (retromolar and subcrestal alveolar regions), where weak irregular blood flow favors the formation of intravascular thrombi.18

Gene polymorphisms associated with reduced NO production, eNOS T−786C, and stromelysin 5A6A, which can lead to vasoconstriction, platelet aggregation and thrombosis, may play a pathogenic role in osteonecrosis of the hip.6,7 Nitric oxide regulates bone turn-
over by osteoblasts and osteoclasts and is thought to play an important role in bone physiology.\textsuperscript{22}

Our objective in the present study was to assess whether the eNOS T\textsuperscript{−786}C mutation, associated with reduced NO production,\textsuperscript{6,7} might also be a potential cause of NICO. To this end, we assessed eNOS T\textsuperscript{−786}C and stromelysin 5A6A mutations in a cohort of individuals with NICO of the jaws.\textsuperscript{9,18} While recognizing that certain aspects of our understanding of NICO remain unclear, even contentious,\textsuperscript{23,24} we consider NICO to be an early or nascent pathologic stage of jaw osteonecrosis confined primarily to its medullary compartment and associated with ischemic injury of the marrow stroma, especially capillary endothelium and the vasa nervosa of alveolar nerves.\textsuperscript{18}

**MATERIALS AND METHODS**

**Case and control subjects**

This prospective case-control protocol was approved by the Institutional Review Board of the Jewish Hospital of Cincinnati and was carried out with signed informed consent. Case subjects included 22 patients with NICO of alveolar bone of the jaws,\textsuperscript{18} as documented by presenting symptoms, imaging, diagnostic anesthesia test results, and microscopic evaluation.\textsuperscript{25} Nineteen patients were referred from a single dental surgery practice, 1 in October 1966 (restudied on January 5, 2007) and 21 from January 5, 2007, to March 31, 2009. To minimize the risk of bias in the control group, 2 asymptomatic healthy controls were matched to each subject by gender and race, for a total of 44 control subjects.\textsuperscript{26} These 44 control subjects came from a previously described cohort of 72 healthy adults, including 40 hospital personnel and 32 subjects evaluated during family studies of hyperlipidemic patients.\textsuperscript{7,27,28}

Patients and control subjects provided detailed medical histories, were given physical examinations, and had blood drawn (fasting, seated) for assessment of the T\textsuperscript{−786}C eNOS and stromelysin 5A6A mutations. In the NICO patients, information on the duration and severity of jawbone pain was systematically obtained. No subjects or controls had taken oral or intravenous bisphosphonates, and none had active cancer and/or hypercalcemia. None of the 22 NICO patients took corticosteroids, none were cigarette smokers, and 1 was a recovered alcoholic.

**Laboratory methods**

As previously described,\textsuperscript{28} after an overnight fast, at 8:30-9:00 a.m. blood was collected in 3.2% buffered sodium citrate (1 part citrate:9 parts blood). The samples were immediately transported and centrifuged at 2,600g for 15 minutes to obtain platelet-poor plasma. The samples were run in batches. The plasma was frozen in aliquots and stored at minus seventy degrees centigrade. Blood for polymerase chain reaction (PCR) analysis was drawn in tubes containing the appropriate anticoagulant (ethylene diamine tetraacetic acid), and the DNA was extracted for subsequent analysis.

The DNA was isolated with the Capture Column (Gentra Systems, Minneapolis, MN). PCR measures of the 5A/6A stromelysin\textsuperscript{29,30} and the T\textsuperscript{−786}C eNOS\textsuperscript{31-34} mutations were performed using previously published techniques. The forward primer for the stromelysin polymorphism\textsuperscript{30} was 5-ggt tct tca ttc tga tgg ggg gaa aga-3, the reverse primer was 5-ctt cct gga att cac atc act gcc acc aga-3. One hundred nanograms of patient DNA was denatured at 95°C for 5 minutes, then 31 cycles of 95°C for 1 minute and 60°C for 1 minute, and then 72°C for 1 minute. The product was digested with Tth111 I (New England Biolabs, Beverly, MA) per the supplier’s instructions. The forward primer for the eNOS polymorphism was 5-tgg aga gtg ctg gtg tacc cca-3. The reverse primer was 5-gcc tcc acc ccc acc ctg tc-3.\textsuperscript{34} One hundred nanograms of patient DNA was denatured at 95°C for 5 minutes, then 32 cycles of 94°C for 0.5 minute and 63°C for 0.5 minute, and then 72°C for 0.5 minute. The product was digested with Msp I per the supplier’s instructions (New England Biolabs). The products of the PCR reactions were then electrophoresed on a 10% polyacrylamide gel and the bands visualized with ethidium bromide.

As reported by Nakayama et al.,\textsuperscript{34} the PCR analysis that identifies the eNOS T\textsuperscript{−786}C polymorphism was confirmed by complete sequencing of the eNOS gene from nucleotide -1533 to + 44.

**Statistical methods**

Based on our previous studies of osteonecrosis of the hip\textsuperscript{7} (95 cases: 14 [15%] eNOS T\textsuperscript{−786}C homozygous mutant, 42 [44%] heterozygous, and 39 [41%] wild-type normal; and 72 normal control subjects: 1 [1%] homozygous mutant, 27 [38%] heterozygous, and 44 [61%] wild-type normal), with alpha = 0.05 and beta = 0.20, we estimated that we would need to study 58 cases to have optimal power to distinguish them from controls.

Distributions of genotypes of the eNOS and stromelysin polymorphisms were compared in subjects and race and gender–matched controls by chi-squared analyses and Mantel-Haenszel \( \chi^2 \) tests (Table I; Fig. 1). Mutant homozygosity (TT) versus wild-type normal (CC) plus heterozygosity (TC) were compared by Fisher exact tests (Table I). Risk ratios with 95% confidence intervals (CIs) were reported (Table I). The CIs for sensitivity and specificity were calculated by asymptotic normal distribution for binomial variables, when cell frequency was 0 using continuity adjustment correction +0.5 (Table I).
RESULTS

The 22 caucasian subjects with NICO included 17 women and 5 men, with a mean ± SD age of 53 ± 13 years. The 44 caucasian healthy normal controls included 34 women and 10 men, aged 43 ± 13 years.

The mean ± SD duration of jawbone pain in the 22 NICO patients was 6 ± 3.3 years, median 5 years, and the interquartile range (25th-75th percentiles) was 3-7 years. In 18 patients, pain was unrelenting and chronic, 4 were totally disabled by pain, 2 of whom described their lives as “ruined” by pain. To achieve pain relief, 73% of the 22 patients required opiate and/or fentanyl analgesia. Five patients required daily opiates, 11 took opiates intermittently, 2 used nonnarcotic analgesics, 3 used over-the-counter analgesics, and 1 required no pain therapy. As shown in Fig. 2, technetium-99 MDP (methylene disphosphonate) scintigraphy scans of alveolar regions of painful ischemic bone damage revealed intense uptake. Biopsy material from alveolar bone in these subjects demonstrated degenerative marrow and bone changes consistent with NICO, with areas of thrombosis, marrow congestion, ischemic myelofibrosis, and chronic low-grade inflammation (Fig. 3).

Homozygosity for the mutant eNOS allele (TT) was present in 6 out of 22 patients (27%) versus 0 out of 44 (0%) race/gender–matched control subjects (Fig. 1; Table I). Heterozygosity for the mutant eNOS allele (TC) was present in 8 patients (36%) versus 15 control subjects (34%), and the wild-type normal genotype (CC) was present in 9 patients (36%) versus 29 controls (66%) (\(\chi^2 = 14.3; \text{df} = 2, P = .0008\); Table I). The distribution of the eNOS T-786C genotype was shifted toward heterohomozygosity in cases, compared with control subjects (Mantel-Haenszel \(\chi^2 = 10.8; P = .001\); Table I; Fig. 1). The mutant eNOS T-786C allele was more common in case subjects than in control subjects: 20 out of 44 (45%) versus 15 out of 88 (17%) (\(\chi^2 = 12.2; P = .0005\); Table I; Fig. 1). The relative risk ratio of eNOS T-786C homozygosity versus heterozygosity/wild-type normal was 3.75, with 95% CI 2.5-5.7 (Table I). The specificity of eNOS T-786C genotype (homozygosity vs. nonhomozygosity) was high, with 44 out of 44 of 44 healthy control subjects (100%) free of eNOS homozygosity.

As shown in Table I, the distribution of the stromelysin 5A6A genotype in case subjects did not differ from control subjects (\(\chi^2 = 4.2; \text{df} = 2; P = .13\); Mantel-Haenszel \(\chi^2 = 3.75; P = .053\)). Homozygosity for the stromelysin 5A6A (7 out of 22, 32%) was more common in cases than in controls (8 out of 44, 18%), but not significantly (\(P = .21\)). The mutant stromelysin 5A6A allele was more common in case subjects than in control subjects (28 out of 44 [64%] vs. 42 out of 88 [48%]), but not significantly (\(\chi^2 = 2.98; P = .084\). The

| Table I. Case-control differences in eNOS T-786C and stromelysin 5A6A mutations |
|----------------------------------|-----------|-----------|-----------|-----------|-----------------|-----------------|-----------|
|                                  | n         | TC        | CC        | Mutant allele frequency | Mutant allele | Fisher | RR (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) |
| eNOS T-786C mutation             | 22        | 6 (27%)   | 8 (36%)   | 8 (36%) | \(\chi^2 = 14.3; \text{df} = 2; P = .0008\) | 45% vs. 17% | 16 (73%); 16 (73%) | 16 (73%); 16 (73%) | 16 (73%); 16 (73%) |
| Control                          | 44        | 15 (34%)  | 19 (43%)  | 10 (23%) | \(\chi^2 = 4.2; \text{df} = 2; P = .13\) | 45% vs. 48% | 15 (69%); 15 (69%) | 15 (69%); 15 (69%) | 15 (69%); 15 (69%) |

- eNOS, Endothelial nitric oxide synthase; TT, homozygous mutant; TC, heterozygous; CC, wild-type normal; RR, risk ratio; CI, confidence interval.
specificity of the stromelysin 5A6A mutation (homozygosity versus nonhomozygosity) was 82%, with 36 out of 44 healthy controls free of eNOS homozygosity.

DISCUSSION

In the present report, patients with biopsy- and imaging-defined NICO differed from matched normal control subjects in being more likely to have the eNOS T−786C polymorphism. This mutation is associated with reduced NO production. Homozygosity for the eNOS T−786C polymorphism was found in 6 out of 22 NICO case subjects (27%), versus 0% of 44 healthy normal control subjects. This finding was generally similar to our previous study of idiopathic osteonecrosis of the hip, where homozygosity for the mutant eNOS allele was present in 8 (22%) of 36 patients versus 1 (3%) of 36 race/gender–matched control subjects.

Nitric oxide production is impaired by the T−786C eNOS single nucleotide polymorphism, with a substitution of the nucleotide thymine by cytosine at a locus 786 base pairs upstream of the eNOS gene. There are 3 main NO synthases in bone cells (eNOS, bNOS, and iNOS) of which eNOS is the predominant constitutive isoform expressed in normal adult bone, mainly in osteoblastic lineage cells. Nitric oxide, which is vasoactive, is produced in the vascular endothelium, and its production is controlled to a large degree by eNOS and stromelysin genes. Nitric oxide is an osteocytic signaling molecule which regulates bone mass and bone turnover through effects on osteoblast-osteoclast activity. Nitric oxide inhibits osteoclastosis, and its generation by eNOS is enhanced by mechanical stimuli and estrogen. Nitric oxide release is impaired by eNOS T−786C and stromelysin-1 5A6A mutations, leading to vasoconstriction, platelet aggregation, thrombosis, reduced angiogenesis, and bone formation. Nitric oxide plays a role in bone angiogenesis, thrombosis, and turnover, all probably related to the pathogenesis of osteonecrosis and other forms of chronic
ischemic bone disease. Impaired NO release mediated by the T→H11002 polymorphism could produce ischemically damaged bone and bone marrow, with multiple intraosseous intravascular thrombi, and neural degeneration with subsequent production of jawbone pain, as evidenced by the present patients with NICO. Severe chronic pain characterized all 22 patients, requiring opiate and/or fentanyl analgesia in 73%.

Although skepticism has been expressed in some review articles about NICO and its treatment, with some rejecting the very existence of the entity, the 22 patients in this study had chronic facial pain and well-defined chronic ischemic bone disease by pathologic review of excised tissue using the same histopathologic criteria as osteonecrosis of long bones.

A limitation of the present study is sample size. Although case-control comparisons revealed highly significant differences in eNOS T→786C genotypes between NICO case subjects and healthy control subjects, our sample size calculations suggest that future studies include at least 58 NICO patients. Future studies of the eNOS T→786C polymorphism in osteonecrosis of the jaws should also include patient control subjects with atypical face pain affecting the jawbones and patients with periapical granulomas or radicular cysts.

The eNOS T→786C polymorphism affecting NO production may contribute to the pathogenesis of NICO. Our finding of an increased frequency of homozygosity for the T→786C eNOS mutation (27%) in subjects with NICO, and high specificity, with none of 44 control subjects having eNOS T→786C homozygosity, is congruent with our recent finding of eNOS T→786C mutation in osteonecrosis of the hip. We speculate that provision of the amino acid L-arginine, an NO precursor, might be therapeutic in osteonecrosis patients with homozygosity for the eNOS T→786C mutation, whether the osteonecrosis is found in the hip or in the jaws.

REFERENCES


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