Osteonecrosis has multiple causes, including thrombophilia, that are associated with osseous venous thrombosis and reduce the vascular supply to subcortical bone. Microcirculatory thrombotic occlusion promoted by both thrombophilia-hypofibrinolysis and endothelial nitric oxide synthase (eNOS) polymorphisms leads to reduced vascular supply to the femoral head, osteocyte death, collapse of the articular surface, and subsequent osteoarthritis. Microscopic evaluation of osteonecrotic femoral heads shows arteriolar and intravascular thrombosis with concurrent high levels of fibrinopeptides and fibrin degradation products.

Mutations in the T786C endothelial nitric oxide synthase gene (eNOS) are associated with osteonecrosis and Prinzmetal’s angina. Nitric oxide is necessary for bone health and ameliorates Prinzmetal’s angina. This study compared mutations of T786C eNOS in 146 patients with primary osteonecrosis, 114 patients with Prinzmetal’s angina, and 83 normal control subjects. Patients with osteonecrosis had more mutant eNOS alleles than control subjects (42% vs 22%, respectively; \( P < .0001 \)) but had the same number of mutant alleles as patients with Prinzmetal’s angina (42% vs 41%, respectively; \( P = .7 \)), who in turn had more mutant eNOS alleles than control subjects (41% vs 22%, respectively; \( P = .0001 \)). Of 146 patients with primary osteonecrosis, 65 (45%) had none of the 5 thrombophilias (Factor V Leiden heterozygosity, high levels of Factors VIII and XI, anticardiolipin antibody immunoglobulin M, and homocysteine) that otherwise distinguished patients with osteonecrosis from control subjects (\( P < .05 \)). No associations were found between eNOS hetero-homozygosity and the 5 major thrombophilias in primary osteonecrosis. Of the 65 patients who had osteonecrosis but no major thrombophilias, for 41 (28% of the total sample of 146), eNOS hetero-homozygosity was the only abnormality. Normalization of nitric oxide levels with L-arginine 9 g/d or L-citrulline 800 mg/d, both of which relieve vasospastic angina in Prinzmetal’s angina, which has the same eNOS genotype as primary osteonecrosis, may slow or stop the progression of osteonecrosis. Placebo-controlled trials of patients with primary osteonecrosis who are hetero-homozygous for the T786C eNOS mutation and have no major thrombophilias are needed to assess the safety and efficacy of this treatment.

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The authors have no relevant financial relationships to disclose.

This study was supported in part by the Lipoprotein Research Fund of the Jewish Hospital of Cincinnati.

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Received: April 26, 2017; Accepted: July 31, 2017.

doi: 10.3928/01477447-20170824-03
Nitric oxide also causes vasodilation and prevents platelet and leukocyte adhesion, playing a key role in preventing thrombosis, which is involved in the pathogenesis of osteonecrosis.\textsuperscript{7,22} Nitric oxide synthase has 3 isoforms: neuronal, induced, and endothelial (eNOS).\textsuperscript{23} The predominant nitric oxide synthase isoform in normal bone is eNOS. In osteonecrosis, eNOS mutations are believed to operate through reduced production of nitric oxide.\textsuperscript{7,20,21}

Administration of L-arginine 5 to 15 g/d restores physiologic levels of nitric oxide, augments nitric oxide-dependent signaling,\textsuperscript{24} and on the arterial side of circulation, sharply reduces vasospastic angina in patients with Prinzmetal’s angina.\textsuperscript{15,16} Administration of L-citrulline 800 mg/d, the effective precursor of L-arginine, improves endothelial function and potentiates nitric oxide-dependent arterial vasodilation in patients with vasospastic angina.\textsuperscript{19}

The goals of the current study were to assess whether eNOS mutations were more common in patients with primary osteonecrosis than in control subjects and to determine how they compared with eNOS mutations in patients with Prinzmetal’s angina, where eNOS heterogeneity is pathoetiologic.\textsuperscript{15,16} The authors were most interested in how often eNOS mutations were the only identifiable pathoetiologic for osteonecrosis, after patients with familial and acquired thrombophilia-hypofibrinolysis were excluded. Patients with primary osteonecrosis who have eNOS hetero-homozygosity as a sole identifiable etiology should benefit from L-arginine or L-citrulline therapy, similar to patients with Prinzmetal’s angina who have the same eNOS genotypes.\textsuperscript{15,16} This study also assessed what interactions, if any, occurred between eNOS mutations and thrombophilia-hypofibrinolysis in primary osteonecrosis.

**Materials and Methods**

**Patients and Control Subjects**

Study participants provided signed informed consent, and the study protocol was approved by the institutional review board of the Jewish Hospital.

Osteonecrosis was defined as primary if the patient had no known cause of secondary osteonecrosis, such as long-term use of high-dose corticosteroids or alcoholism.\textsuperscript{4,6} Patients who had Prinzmetal’s angina had well-defined vasospastic angina that was unresponsive to conventional medical therapy, as defined in previous reports.\textsuperscript{15,16} None of the patients in the current report were the subjects of the authors’ previous publications on osteonecrosis\textsuperscript{1,2} or Prinzmetal’s angina.\textsuperscript{15,16}

Control subjects for measures of coagulation and eNOS were normal, healthy subjects who did not have venous thromboembolism or osteonecrosis.

**Polymerase Chain Reaction Analysis and Measures of Thrombophilia-Hypofibrinolysis**

After patients fasted overnight, blood was drawn for polymerase chain reaction analysis in tubes containing ethylene diamine tetraacetic acid, and DNA was extracted for subsequent analysis of the T786C eNOS polymorphism. The DNA was isolated with the Capture Column (Gentra Systems, Minneapolis, Minnesota). Polymerase chain reaction analysis for the eNOS T786C mutation was conducted according to previously published methods,\textsuperscript{15,16} as were measures of thrombophilia and hypofibrinolysis.\textsuperscript{1,6}

**Statistical Analysis**

Statistical analysis was performed with SAS version 9.4 software (SAS Institute Inc, Cary, North Carolina). Case-control differences were assessed with Mantel–Haenszel chi-square analysis and chi-square analysis. Logistic regression was used to determine whether case-control differences in eNOS mutations, eNOS mutant alleles, and measures of coagulation were independent of age, race, and sex.

**Results**

Table 1 summarizes the characteristics of the cohort of 146 patients with primary osteonecrosis, 114 patients with Prinzmetal’s angina, 108 control subjects evaluated for coagulation measures, and 83 control subjects evaluated for eNOS mutations.

Logistic regression showed that, after adjustment for age, race, and sex, differences in eNOS between patients with osteonecrosis and control subjects remained significant (odds ratio, 3.61; 95% confidence interval, 1.99-6.6). Differences in eNOS between patients with Prinzmetal’s angina and control subjects were significant after adjustment for age, race, and sex (odds ratio, 2.29; 95% confidence interval, 1.18-4.45). With logistic regression for osteonecrosis vs Prinzmetal’s angina, after adjustment for age, race, and sex, eNOS genotypes did not differ.

Table 2 shows measures of thrombophilia and hypofibrinolysis for 146 patients with osteonecrosis compared with 108 control subjects. The study found 5 significant ($P<.05$) case-control differences: patients with osteonecrosis had more Factor V Leiden heterozygosity/low resistance to activated protein C, high levels of Factors VIII and XI, anticardiolipin antibody immunoglobulin M, and homocysteine ($P<.025$ for all) (Table 2).

Compared with control subjects, patients with primary osteonecrosis were more likely to have eNOS hetero-homozygosity and more mutant alleles (Table 2, Figure 1).

Patients with primary osteonecrosis and Prinzmetal’s angina had similar findings for eNOS hetero-homozygosity and mutant alleles (Figure 1). Those with Prinzmetal’s angina differed from control subjects and were more likely to have both eNOS hetero-homozygosity and mutant eNOS alleles (Figure 1).

Of 146 patients with primary osteonecrosis, 65 (45%) had none of the 5 thrombophilias that otherwise distinguished ($P<.05$) patients with osteonecrosis from
control subjects (Table 2). Of those 65 patients, 32 (49%) had eNOS heterozygosity and 9 (14%) had eNOS homozygosity vs 76 control subjects with no thrombophilias, 28 (37%) of whom had eNOS heterozygosity and 2 (3%) of whom had eNOS homozygosity (P=.0012) (Figure 2). The 65 patients who had osteonecrosis but no thrombophilia-hypofibrinolysis were more likely (38%) than control subjects (21%) to have mutant eNOS alleles (P=.0013) (Figure 2).

Of 146 patients who had primary osteonecrosis, 41 (28%) had eNOS heterozygosity (n=32) or homozygosity (n=9) as their only abnormality (Figure 3). Of these 41 patients, 7 had multifocal osteonecrosis, 32 had only hip involvement (9 bilateral), and 2 had only knee involvement (1 bilateral).

No associations were found (P>.05) between eNOS hetero-homozygosity and the 5 major thrombophilias in the 146 patients with primary osteonecrosis.

**DISCUSSION**

Multiple factors reduce vascular supply in osteonecrosis, as shown by increased arteriolar and intravascular thrombosis in the femoral head. As documented in this and previous studies, thrombophilia-hypofibrinolysis is a causative factor for osteonecrosis, promoting venous thrombosis, with resultant increased intraosseous venous pressure, reduced arterial perfusion, and ensuing ischemic osteonecrosis. In patients with primary osteonecrosis before joint collapse (Ficat stage I-II) and familial thrombophilia, particularly Factor V Leiden heterozygosity, anticoagulation can slow or stop the progression of osteonecrosis, presumably by reducing venous occlusion and decreasing intraosseous venous pressure and facilitating arterial inflow. In the current study, and congruent with previous publications, patients with primary osteonecrosis differed from control subjects. Those with primary osteonecrosis were more likely to have thrombophilic Factor V Leiden heterozygosity/low resistance to activated protein C as well as high levels of Factors VIII and XI, anticardiolipin antibody immunoglobulin M, and homocysteine.

Beyond differences between patients with primary osteonecrosis and control subjects for thrombophilia, those with primary osteonecrosis were more likely than control subjects to have eNOS hetero-homozygosity (P<.0001). This finding was congruent with previous publications. Moreover, a new finding was that, for 65 (45%) of 146 patients with primary osteonecrosis who had none of the 5 major thrombophilias that distinguished patients from control subjects, eNOS hetero-homozygosity was the sole probable etiology among 41 of 65 (63%) cases and was present among 28% of the cohort of 146 patients with primary osteonecrosis. The independent high frequency of T786C eNOS heterozygosity and homozygosity

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Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients With Primary Osteonecrosis</th>
<th>Patients With Prinzmetal’s Angina</th>
<th>Coagulation Control Subjects</th>
<th>Control Subjects With Endothelial Nitric Oxide Synthase Genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>146</td>
<td>114</td>
<td>108</td>
<td>83</td>
</tr>
<tr>
<td>Sex, No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>67 (46%)</td>
<td>86 (75%)</td>
<td>60 (56%)</td>
<td>43 (52%)</td>
</tr>
<tr>
<td>Male</td>
<td>79 (54%)</td>
<td>28 (25%)</td>
<td>48 (44%)</td>
<td>40 (48%)</td>
</tr>
<tr>
<td>Race, No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>135 (92%)</td>
<td>111 (97%)</td>
<td>94 (87%)</td>
<td>76 (92%)</td>
</tr>
<tr>
<td>African American</td>
<td>4 (3%)</td>
<td>1 (1%)</td>
<td>6 (6%)</td>
<td>5 (6%)</td>
</tr>
<tr>
<td>Asian</td>
<td>7 (5%)</td>
<td>2 (2%)</td>
<td>-</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>-</td>
<td>8 (7%)</td>
<td>-</td>
</tr>
<tr>
<td>Age Mean±SD, y</td>
<td>47±12</td>
<td>54±12</td>
<td>44±13</td>
<td>42±11</td>
</tr>
<tr>
<td>25th percentile, mean, y</td>
<td>38</td>
<td>45</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>50th percentile, mean, y</td>
<td>48</td>
<td>54</td>
<td>43</td>
<td>41</td>
</tr>
<tr>
<td>75th percentile, mean, y</td>
<td>57</td>
<td>63</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
in patients with primary osteonecrosis, similar to Prinzmetal’s angina, L-arginine or L-citrulline should be therapeutic for osteonecrosis, reducing arterial, arteriolar, and endothelial disease.

The T786C genotype in patients with primary osteonecrosis is the same as in Prinzmetal’s angina. In addition, L-arginine 9 g/d ameliorates symptomatic vasospastic angina among patients with Prinzmetal’s angina and L-citrulline 800 mg/d increases flow-mediated dilation of the brachial artery among patients with vasospastic angina. To the extent that nitric oxide plays an important etiologic role in osteonecrosis, in a fashion similar to Prinzmetal’s angina, L-arginine or L-citrulline should be therapeutic for osteonecrosis, reducing arterial, arteriolar, and endothelial disease.

Table 2

Coagulation Disorders Among 146 Patients With Primary Osteonecrosis Compared With 108 Control Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Factor V Leiden/Low</th>
<th>Factor VIII</th>
<th>Factor XI</th>
<th>ACLA IgG</th>
<th>ACLA IgM</th>
<th>MTHFR</th>
<th>Factor V Leiden/AF46</th>
<th>Factor V Leiden/AF45</th>
<th>Factor V Leiden/AF44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disorder in 108 normal control subjects, No./total</td>
<td>2/108 (2%)</td>
<td>3/108 (3%)</td>
<td>26/104 (25%)</td>
<td>31/107 (29%)</td>
<td>7/103 (7%)</td>
<td>3/101 (3%)</td>
<td>6/108 (6%)</td>
<td>2/108 (2%)</td>
<td>5/105 (5%)</td>
</tr>
<tr>
<td>Disorder in 146 patients with primary osteonecrosis compared with control subjects, No./total</td>
<td>13/139 (9%)</td>
<td>8/136 (6%)</td>
<td>38/130 (29%)</td>
<td>30/132 (23%)</td>
<td>35/128 (27%)</td>
<td>14/128 (11%)</td>
<td>2/132 (2%)</td>
<td>6/130 (5%)</td>
<td>20/108 (19%)</td>
</tr>
<tr>
<td>p = .014</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: ACLA, anticardiolipin antibody; eNOS, endothelial nitric oxide synthase; GA, heterozygous mutant; AA, homozygous mutant; IgG, immunoglobulin G; IgM, immunoglobulin M; Lp(a), lipoprotein (a); MTHFR, methylenetetrahydrofolate reductase; NS, not significant; PAIG, plasminogen activator inhibitor gene; PTG, prothrombin gene mutation; RAPC, resistance to activated protein C.
Along with venous occlusion, this disease promotes primary osteonecrosis. The use of L-arginine 5 to 15 g/d restores physiologic levels of nitric oxide and augments nitric oxide-dependent signaling. It is therapeutic for Prinzmetal’s angina and may be therapeutic for primary osteonecrosis if initiated before joint collapse occurs (Ficat stage I-II).

Limitations
A limitation of this report is that the study did not examine other polymorphic sites of eNOS that have been associated with osteonecrosis, including 4b/a and G894T. A strength of the study is the inclusion of a large cohort of patients with primary osteonecrosis with concurrent assessment of thrombophilia-hypofibrinolysis along with comparison of the T786C eNOS genotype with patients with Prinzmetal’s angina and control subjects.

A placebo-controlled clinical trial of L-arginine or L-citrulline is needed to determine whether treatment with L-arginine 9 g/d or L-citrulline 800 mg/d substrates for eNOS conversion to nitric oxide that are effective for the treatment of Prinzmetal’s vasospastic angina would stop the progression of primary osteonecrosis before joint collapse (Ficat stage I-II).

The T786C eNOS mutation is common among patients with primary osteonecrosis, as it is among patients with Prinzmetal’s angina, where the mutation is a known pathoetiology. For primary osteonecrosis, T786C eNOS hetero-homozygosity is the only identifiable etiology for 28% of patients. A blinded, placebo-controlled clinical trial is needed to determine whether treatment with L-arginine 9 g/d or L-citrulline 800 mg/d substrates for eNOS conversion to nitric oxide that are effective for the treatment of Prinzmetal’s vasospastic angina would stop the progression of primary osteonecrosis before joint collapse (Ficat stage I-II).

REFERENCES


