Vascular Inflammation and Endothelial Dysfunction in Fracture Healing

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Abstract

Angiogenesis is an important step in bone fracture healing. In this article, we report on the healing of long bone fractures, and the involvement of the vascular and the inflammatory systems in the process.

We conducted a prospective study of 20 healthy adults with traumatic long bone fracture. One week after fracture, and then 1 month later, we evaluated markers of inflammation: vascular responsiveness (brachial endothelial function and ankle brachial index) and inflammatory and cytokine levels osteopontin [OPN], E-selectin, and vascular endothelial growth factor [VEGF]).

Long bone fractures caused intense vascular and inflammatory responses, represented by high levels of OPN, E-selectin, and VEGF. In vivo measurements demonstrated severe endothelial dysfunction, which could support the idea that the vascular system is recruited to build new blood vessels that support bone regeneration.

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Osteopontin (OPN) is a secreted adhesive molecule that is thought to aid recruiting of monocyte-macrophages and to regulate cytokine production in macrophages, dendritic cells, and T-cells. As a T-helper 1 cytokine, OPN is thought to exacerbate inflammation in several chronic inflammatory diseases, including atherosclerosis. It is a potent inhibitor of mineralization, prevents ectopic calcium deposits, and is a potent inducible inhibitor of vascular calcification. Its effect is thought to be produced through its adhesive domains, particularly the arginine–glycine–aspartate sequence that interacts with several integrin heterodimers. Several studies have shown that OPN is cleaved by at least 2 classes of proteases: thrombin and matrix metalloproteases (MMPs).[1] OPN acts as a matricellular protein, facilitating adhesion and migration, and as a soluble cytokine.[2] Several in vitro studies have indicated that OPN induces adhesion, migration, and survival of several types of cells, including smooth muscle cells, endothelial cells, and inflammatory cells.[3-8] In vivo studies of OPN in disease and injury have suggested its important function in inflammation and tissue remodeling.[9,10] In particular, OPN is de novo expressed in cells that participate in renal and cardiovascular remodeling and repair.[11-16]

Given these findings, we studied the effect of long bone fractures on vascular inflammatory responses and nitric-oxide–dependent pathways and processes—the brachial endothelial function method.[17] This method allowed us to study the possible link between vascular inflammation and bone healing and to try to show in vivo that vascular activity (angiogenesis) is important in bone fracture healing.

Methods

Patients with long bone fractures were enrolled in this prospective study. They were followed up in the orthopedic outpatient clinic for 6 months and underwent a regular clinical postoperative examination, including radiographic assessment of bone recovery and fracture reconstruction. All patients and control subjects who volunteered to participate in the study signed a consent form before enrollment.

Inclusion criteria were age older than 18, admission with an acute traumatic fracture of a long bone, and agreement to take part in the study. There was no sex or age limitation. Patients were healthy otherwise. Exclusion criteria were age younger than 18, lack of cooperation, complicated trauma of more than 1 long bone, head trauma, neurologic involvement, active cancer, active smoking, type 1 or 2 diabetes mellitus, essential hypertension, hypercholesterolemia, active inflammatory disease, and immunologic disease.

Twenty healthy age- and sex-matched volunteers served as the control group. These subjects had no cardiovascular risk factors, no chronic inflammatory disease, and were nonsmokers.

Table I. Vascular Responsiveness Studies[a]

	FMD%		ABI	
	Patients	Controls	Patients	Controls
	–5.7	18	1	1.3
	–11.4	14.8	1	1.3
	–6.5	24.1	1.4	1.2
	2.6	4. 9	1	1.4
	–3.1	18.2	1.3	1.3
	6.5	18.7	1	1.3
	3.2	26.9	1.4	1.5
	–13.3	18.5	1.1	1.5
	–2.63	7.7	1	1.4
	–6.9	6.9	1.1	1.4
	2.78	17.4	1	1.4
	–11.9	7.9	1.1	1.3
	0	22.8	0.8	1.4
	3.3	11.1	1.5	1.3
	2.6	11.4	1.3	1.1
	–2.7	10	1.4	1
	5.1	23.1	1.2	1.3
	–18.9	13.5	1.1	0.8
	0	7.7	1.1	1.2
	9.1	16	1.1	1.3
Mean	–2.4	14.98	1.145	1.285
± SD	7.36	6.42	0.182	0.166
P value	.0001		.035	

[a] Percentage change in flow-mediated dilatation (diameter) is a marker of endothelial function; ankle brachial index is a marker of vascular atherosclerosis and peripheral artery disease.

Patients were hospitalized in the orthopedic department and then, after discharge, were followed up in the orthopedic outpatient clinic for 6 months. Venous peripheral blood (5 mL) was drawn and centrifuged, and the serum was frozen for batch processing at the end of the study.

Vascular studies included the brachial artery method for endothelial flow-mediated dilatation (FMD) and the ankle brachial index (ABI), which was used to evaluate peripheral artery disease (PAD). These studies were performed within 3 days of admission.

Vascular Studies

Endothelial Function Measurements- Imaging studies of the left brachial artery were performed with a high-resolution ultrasound Hewlett-Packard 7.5-MHz linear array transducer after 30 minutes of rest, based on the technique reported by Celermajer and colleagues.[17] After the clearest view of the brachial artery was found, the skin was marked, and the arm was kept in the same position for the rest of the study. Baseline measurements included brachial artery diameter by pulsed Doppler, with a range gate (1.5 mm) in the center of the artery. Endothelium-dependent vasodilatation was assessed by measuring the change in diameter of the brachial artery during hyperemia created by an inflated cuff (150 mm Hg for 5 minutes) on the forearm. After cuff deflation, flow velocity was measured for the first 15 seconds, and arterial diameter was recorded continually for the next 60 seconds. Arterial diameter was measured in millimeters from the artery–blood interface on the anterior and posterior walls, coincident with the R waves on the electrocardiogram, at 2 sites along the artery for the 3 cardiac cycles, with these 6 measurements averaged. Normal endothelium-dependent dilatation is considered as a flow mediated diameter percent (FMD%) change of more than 10%.

Ankle Brachial Index (ABI) - For ABI, Doppler ultrasound was used. The apparatus, typically called the *Doppler wand* or the *Doppler probe,* was used to record the peripheral pulse. At the same time, the usual blood pressure (BP) measurement tool, the sphygmomanometer (BP cuff), was fixed over the artery, where an appendage joined the body (in this case, Doppler probe), until the pulse stopped. Afterward, all the air in the cuff was released. The respective sphygmomanometer pressure at the moment the pulse was regained was the systolic BP (SBP) reading. SBP in the posterior tibial artery of the foot and SBP in the dorsalis pedis artery of the foot were calculated, with the higher of the 2 values used as the ABPI for the leg (The formula divides P arm by P leg. Here, P leg is SBP in the lower leg, and P arm is whichever SBP is higher, that in the left arm or that in the right arm). ABPI is believed to be an effective method for noninvasive assessment of peripheral vascular disease.

ABI is the ratio of the highest ankle-to-brachial-artery pressure. An ABI between 0.9-1.4 is considered as a normal ABI. An ABI > 1.4 is abnormal and suggests calcification of the walls of the arteries and incompressible vessels, reflecting severe peripheral vascular disease. Provided there are no other significant conditions affecting the leg arteries, ABI can be used to predict severity of peripheral artery disease.

BMI and Abdominal Circumference

Body mass index (BMI), a calculated ratio between weight in kilograms and height in meters squared (kg/m^2), is a reliable index of weight relative to height and has been shown to be an important prognostic marker for survival and cardiovascular risk. Abdominal circumference (AC) is a marker of abdominal visceral fat, another important prognostic marker for cardiovascular risk.

Biochemical Analysis

Cell adhesion molecules E-selectin and vascular endothelial growth factor (VEGF) were measured with a quantitative sandwich immunoassay technique. A microplate was precoat-

Table II. Body Mass Index (BMI) and Abdominal Circumference

	BMI		Abdominal Circumference, cm	
	Patients	Controls	Patients	Controls
	28	22	59	78
	25	29	88	101
	35	29	105	99
	39	19	122	76
	29	20	97	86
	32	25	100	92
	25	37	70	110
	28	27	80	95
	31	28	116	101
	26	30	100	100
	21	17	40	68
	27	27	89	104
	30	29	97	102
	24	27	90	88
	27	31	94	108
	36	36	112	120
	27	26	94	95
	40	27	130	90
	26	27	109	92
	30	21	72	89
Mean	29.3	26.7	93.2	94.17
± SD	4.9	5.11	21.50	12.24
P-value	.115		.903	

BMI = Weight/height^2 (kg/m^2).

ed with monoclonal antibodies specific for soluble (s) E-selectin and soluble VEGF. Standards and samples were transferred into the wells, and any soluble E-selectin/VEGF present was bound by the immobilized antibodies. After any unbound substances were washed away, enzyme-linked monoclonal antibodies specific for the cell adhesion molecules were added to the wells. After a wash, a substrate solution was added to the wells, and color developed in proportion to the amount of cell adhesion molecules bound in the initial step. Color development was stopped, and color intensity was measured. The kits were manufactured by R&D Systems (Minneapolis, Minnesota).

OPN level was measured with the methodology used with the ELISA (enzyme-linked immunosorbent assay) kit. Levels of inflammatory markers and OPN were measured the first week of admission and 1 month later. Results were compared with each other.

Statistical Analysis

Student *t* test was used to detect differences between measures of endothelial function in the first week of fracture, and 1 month later in patients with full recovery and fracture healing. Paired *t* test was used to compare levels of inflammatory markers and OPN, on admission and 1 month later, that could support the results of the endothelial function and vascular studies. We sought a "recovery trend" by comparing marker levels over time and fracture recovery rates using the paired *t* test. All clinical data were compared with those of the age- and sex-matched healthy control subjects. Student paired *t* test was used to compare patients with healthy control subjects.

Results

Mean (SD) age of the 20 patients (13 men, 7 women) was 40.63 (11.13) years. These patients' endothelial dysfunction was severe, a mean (SD) of –2.5% (7.5%), in spite of the fact they were relatively young and had no known risk factors for cardiovascular disease. They were nonsmokers with no diabetes mellitus, hypertension, or hypercholesterolemia, and their long bone (18 tibia, 2 fibula) fractures occurred in traumatic events (Table I).

Mean (SD) age of the 20 control subjects (10 men, 10 women) was 41.86 (11.34) years. These healthy volunteers also had no risk factors for cardiovascular disease. Their endothelial function, which was normal, a mean (SD) of 15.0% (6.5%), differed significantly (P = .0001) from that of the patients with fractures (Table I).

Table I lists the vascular parameters studied. Percentage change in FMD represents the endothelial dysfunction/function of patients versus control subjects. Normal percentage change in FMD is more than 10%. All patients had severe endothelial dysfunction, whereas control subjects had normal function. Table I also describes ABI, which evaluates PAD and atherosclerosis. ABI was significantly (P = .035) lower in patients, a mean (SD) of 1.145 (0.182), than in control subjects, a mean (SD) of 1.285 (0.166), but this difference was not clinically important, as ABI between 0.9 and 1.4 is considered normal.

Table II shows no difference between patients and control subjects in their clinical characteristics of BMI and AC.

Biochemical parameter levels were available for 16 patients with fully healed fractures. One week after fracture, ELISA demonstrated patients' OPN levels were high, a mean (SD) of 77.19 (52.10) ng/mL; over the next month, they decreased (P = .001) to a mean (SD) of 30.73 (17.49) ng/mL (Table III). A study found that the mean (SD) OPN level of normal volunteers was 6.19 (2.69) ng/mL.[18] E-selectin levels also started high, a mean (SD) of 28.15 (14.88) ng/mL, and decreased (P = .01), to a mean (SD) of 21.50 (10.75) ng/mL (Table III). VEGF levels started high, a mean (SD) of 449.81 (191.38) pg/mL, and remained high (P = .5), a mean (SD) of 400.81 (323.19) pg/mL, even with recovery of the fractured bones (Table III).

Discussion

In this study, young patients with long bone fractures exhibited a

Table III. Inflammatory Markers[a]

	Osteopontin, ng/mL		E-Selectin, ng/mL		VEGF, pg/mL	
	M1	M2	M1	M2	M1	M2
	66.25	51.42	38.12	33.89	386	390
	75.17	45.00	25.80	14.71	788	610
	42.57	39.62	24.28	15.70	443	578
	99.05	15.27	12.00	8.36	697	56
	31.87	26.32	40.63	36.90	393	157
	30.10	14.12	50.28	40.05	267	202
	103.77	17.07	53.03	25.95	391	234
	16.67	25.10	17.86	8.08	483	539
	49.87	16.67	15.26	19.17	240	220
	46.87	27.97	8.26	7.37	221	150
	125.7	55.57	26.43	27.66	409	440
	48.75	13.30	12.22	14.87	301	188
	123.75	53.82	33.26	27.61	298	363
	93.75	52.50	43.77	33.42	738	1434
	53.80	0	9.50	15.44	762	342
	227.10	37.97	39.78	14.85	380	510
Mean	77.19	30.73	28.15	21.50	449.81	400.813
SD±	52.11	17.49	14.88	10.75	191.38	323.19
P-value	.001		.01		.50	

Abbreviations: M1, first measurement; M2, second measurement (1 month after first); VEGF, vascular endothelial growth factor.

[a]Osteopontin is an adhesion molecule that characterizes bone activity in vascular and inflammatory processes; E-selectin is an adhesion molecule that characterizes endothelial cell activation; VEGF is a growth factor that characterizes blood vessel involvement and angiogenesis.

strong inflammatory response within the first week of trauma. This was demonstrated by severe endothelial dysfunction (accompanied by an intense vascular response) and by increased inflammatory marker (OPN, E-selectin) levels, which decreased during 1 month of recovery. We confirmed that bone fractures caused secretion of VEGF at high levels for months after injury.

To learn more about the association between vasculature and bone formation, Sanada and colleagues[19] analyzed forearm brachial artery endothelial function in postmenopausal women. Endothelial function was worse in women who had osteoporosis than in women who did not have osteoporosis. To determine the physiologic time course of angiogenic cytokines during fracture healing, Weiss and colleagues[20] collected serum samples from patients with long bone fractures. Fifteen patients had a fracture nonunion, and 15 had successful healing and union. Basic fibroblast growth factor, platelet-derived growth factor, and VEGF levels were measured over 24 weeks. Comparison with healthy uninjured control subjects showed that serum concentrations of all 3 cytokines were increased in both patient groups (union, nonunion), but were significantly higher in the union group than in the nonunion group, 2 weeks and 4 weeks after injury. In another study,[21] the VEGF levels of patients with long bone fractures were measured over 6 months. Of the 114 patients, 103 had a physiologic fracture healing, and 11 had delayed union. Thirty-three healthy volunteers served as the control group. VEGF concentrations were significantly higher in both groups of patients than in the control group. However, VEGF concentrations were higher in patients with impaired fracture healing than in patients with a physiologic healing. This difference was not statistically significant.

Role of OPN in Bone Healing

OPN is highly expressed in the inflammatory cells associated with many diseases, including cancer,[18] arterial restenosis,[22] myocardial infarction,[23] stroke,[24] and with wound healing.[23,25] OPN appears to regulate macrophage infiltration during the inflammatory response, is expressed by macrophages, and is one of the potent macrophage chemotactic stimuli.[23] Wound healing studies have also indicated that OPN is expressed during the acute inflammatory phase at very high levels in infiltrating leukocytes.[24, 25] Other studies in bone wound healing suggest that OPN is a positive regulator of phagocytic activity.[26, 27] Bone development requires recruitment of osteoclast precursors from surrounding mesenchyme, thereby permitting the key bone growth events, such as marrow cavity formation, capillary invasion, and matrix remodeling.[27] MMP-9 is specifically required for invasion of osteoclasts and endothelial cells into the discontinuously mineralized hypertrophic cartilage that fills the core of the diaphysis. MMP-9 stimulates the solubilization of unmineralized cartilage by MMP-13, a collagenase highly expressed in hypertrophic cartilage before osteoclast invasion.[28] Hypertrophic cartilage also expresses VEGF, which binds to extracellular matrix and is made bioavailable by MMP-9. MMP-9 and VEGF have specific and critical roles in early bone development.[28] OPN protein is abundantly expressed in a very restricted zone at the endosteal interface of bone and hematopoietic tissue. Osteoblasts within this zone have been proposed as key components of the hematopoietic stem cell (HSC) niche and implicated in regulating HSC numbers in vitro and in vivo.[29-33]

Within the hematopoietic niche, the main source of OPN is likely osteoblasts that line bone surfaces within the bone marrow cavity.[29] Osteoblasts have been shown to stimulate HSC expansion,[34] potentially through a parathyroid hormone–dependent mechanism that results in up-regulation of Kagged1 and subsequent increased signaling through its cognate receptor Notch1 on HSCs.[35,36] On the other

hand, data show that direct interaction of HSCs and OPN produced by osteoblasts resulted in β1-integrin–mediated inhibition of cell proliferation.[33]

We have shown that, in the first few days after a long bone fracture, there is an intense vascular response represented by severe endothelial dysfunction (in otherwise healthy young subjects) accompanied by intense inflammatory and vascular responses, as demonstrated by high OPN and E-selectin levels, which decreased after a few weeks (parallel to fracture recovery). VEGF levels were found to be high within the first days of fracture and continued at high levels through 1 month after fracture, even though all patients had a full recovery as demonstrated by routine clinical tools. Bone fracture seemed to be an intense vascular trigger, represented by high levels of OPN and E-selectin and high levels of VEGF. In vivo measurements have shown an unexpected severe endothelial dysfunction, which could support the idea of recruitment of the vascular system (maybe through high OPN levels secreted from fractured bone) to build new blood vessels that will support the healing process of the fractured bone.

Authors' Disclosure Statement

The authors report no actual or potential conflict of interest in relation to this article.

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