American Journal of Pathology, Vol. 145, No. 6, December 1994

Macrophages Express Osteopontin during Repair of Myocardial Necrosis

Charles E. Murry,* Cecilia M. Giachelli,* Stephen M. Schwartz,* and Rudolf Vracko†

From the Department of Pathology, University of Washington School of Medicine, Seattle, Washington, and the Pathology and Laboratory Medicine Service,† Seattle Veterans Affairs Medical Center, Seattle, Washington*

Osteopontin is a secreted glycoprotein implicated in a variety of functions, including cell adhesion and migration. Because these functions may be of general importance in the response of tissue to injury, we examined osteopontin expression after experimental cardiac injury and human myocardial infarction. Rat hearts were injured by transdiaphragmatic freeze-thaw and examined from 1 to 28 days after injury. Osteopontin was absent from normal myocardium by immunocytochemistry, Western blotting, and in situ hybridization. On days 1 and 2 after injury, osteopontin mRNA and protein were expressed at high levels by macrophages infiltrating necrotic myocardium. Double labeling with the macrophage marker ED1, however, demonstrated that only a subset of macrophages expressed osteopontin. Western blot analysis showed a single 66-kd band in injured myocardium that was absent from control tissue. Although macrophages remained abundant in the ensuing granulation response and scar tissue formation, the expression of osteopontin was diminished on day 4 and markedly downregulated at 1 and 4 weeks after injury, with only rare cells expressing the message or protein. In a human heart with an 8-day-old myocardial infarct, there was abundant expression of osteopontin mRNA and protein in macrophages within the necrotic and granulation tissue. Transient expression of osteopontin was also observed in a subset of macrophages infiltrating lung, skin, and skeletal muscle injured during the experiment, indicating the response was not limited to the heart. Thus, synthesis of osteopontin by macrophages appears to be a generalized response in the reaction to tissue injury. Although macrophages persist in these lesions, osteopontin is dramatically downregulated as healing proceeds. These results provide the first evidence that osteopontin may be important in healing after tissue injury, possibly in cellular adhesion, chemotaxis, and/or phagocytosis. (Am J Pathol 1994, 145:1450–1462)

Myocardial infarct repair is a multistep process, wherein necrotic muscle is infiltrated by inflammatory cells, phagocytosed, and replaced initially by granulation tissue and later by dense fibrocollagenous scar tissue. The morphological features of infarct repair have long been known at the light microscopic level,[1] and have more recently been defined by electron microscopy.[2–5] From such studies we have recently learned that many so-called fibroblasts in myocardial scars have features of smooth muscle cells, including abundant cytoplasmic α-actin filaments, peripheral dense bodies, and investment by basal lamina. As such, they represent a new phenotype of muscle in the injured heart, possibly relating to the repetitive mechanical stress in the scar. Despite detailed knowledge of such structural features, we know much less about the key molecules that regulate infarct repair.

Because of the resemblance of the fibroblasts in myocardial scars to smooth muscle cells, we considered the possibility that infarct repair might be comparable to the process of neointima formation after experimental balloon angioplasty. Recent studies have shown that smooth muscle cells of the injured wall increase expression of platelet-derived growth factor A and B chains, elastin, collagen I(α1), and osteopontin, as the media is repaired and a muscular neointima is formed[6–9] (also V. Lindner et al, manuscript submitted). Osteopontin is of particular interest as a molecule that might control cell migration. This

Supported in part by NIH Grants HL26405, HL03174, HL07312, and DK47659.

Accepted for publication September 1, 1994.

Address reprint requests to Dr. Charles E. Murry, Department of Pathology, SJ-60, Room I-418, Health Sciences Building, University of Washington School of Medicine, Seattle, WA 98195.

secreted glycoprotein contains the tripeptide sequence arginine-glycine-aspartate (RGD). The RGD moiety is involved in adhesion of many matrix molecules to cell surface integrin receptors.[10] Binding of osteopontin to integrin receptors has been shown to promote cell adhesion, chemotaxis, and signal transduction in a variety of cell types.[11–13] This combination of matrix and cytokine-like effects suggests that osteopontin may play an important role in tissue repair *in vivo*. In the current study we began to compare the repair of myocardial necrosis with neointima formation by studying expression of osteopontin in necrotizing cardiac injuries.

Materials and Methods

Experimental Cardiac Injury

Adult male Sprague-Dawley rats weighing 350 to 400 g were anesthetized with methoxyflurane, and a midline abdominal incision was made. Cardiac freeze-thaw was induced by placing a 1-cm aluminum rod, precooled with liquid nitrogen, against the membranous portion of the diaphragm for approximately 12 seconds. This procedure reproducibly causes a disc-shaped region of necrosis, 2 to 3 mm deep, in the posterior wall of the heart.[14] Intervening tissues such as the diaphragm and a small amount of lung are also injured. The freeze-thaw lesion induces typical coagulation necrosis, which is followed by an acute inflammatory response and phagocytosis of necrotic debris. Granulation tissue formation begins by day 4 and progresses as necrotic debris is cleared. By 4 weeks the lesions consist of young scar tissue. The cellular events in healing of these freeze-thaw lesions are very similar to those of myocardial infarct repair. Because freeze-thaw lesions are smaller and are not transmural, however, there is not the extensive wall remodeling and compensatory hypertrophy that is typically seen with infarction. Animals (n = 4 per group) were allowed to survive for 1, 2, 4, 7, or 28 days to permit healing of the lesions. After sacrifice under anesthesia, the heart and other tissues were fixed in 3:1 methanol-acetic acid for immunocytochemistry or 3.5% paraformaldehyde for *in situ* hybridization and immunoelectron microscopy. All experiments were approved by the institutional animal care committee and were in accordance with federal guidelines.

Human Heart Analysis

Myocardial tissue was obtained from a 53-year-old male who developed a ventricular septal defect 6 days after an extensive posterior wall myocardial infarct. The patient developed cardiogenic shock but then underwent cardiac transplantation 2 days after the rupture of his interventricular septum. Multiple samples of myocardium were obtained from the infarcted region and the grossly normal anterior free wall and processed within 60 minutes of cardiectomy. Tissue samples were frozen in liquid nitrogen for RNA extraction or placed in methyl Carnoy's medium for morphological analysis.

Immunocytochemistry

After paraffin embedding, 4-μ tissue sections were deparaffinized and endogenous peroxidases blocked with 0.3% hydrogen peroxide in cold methanol for 25 minutes. After washing in phosphate-buffered saline (PBS), nonspecific binding was minimized by blocking with 1% normal horse serum for 1 hour. For rat osteopontin localization, either the monoclonal antibody MPIIIB10 (Developmental Studies Hybridoma Bank, Iowa City, IA) at a titer of 1:1000 (11 ng/ml) or the goat polyclonal antibody OP199 at a titer of 1:2000 (1 μg/ml) were used. The polyclonal OP199 antibody, raised against rat smooth muscle-derived osteopontin, has been characterized extensively in a previous report.[12] The monoclonal and polyclonal antibody preparations gave identical results, and the figures show MPIIIB10 staining. Monocyte-macrophages were identified with the monoclonal antibody ED1 (Harlan Bioproducts for Science, Indianapolis, IN), which specifically identifies all cells of the monocyte-macrophage lineage,[15] at a titer of 1:500. The primary antibody incubations were done at room temperature in PBS containing 0.1% bovine serum albumin for 60 minutes. After rinsing in PBS, horse anti-mouse biotinylated secondary antibodies in PBS and 0.1% albumin were applied at room temperature for 30 minutes. Detection was accomplished with an ABC kit from Vector Laboratories (Burlingame, CA) with diaminobenzidine as substrate. Sections were counterstained with methyl green. Double immunostaining was performed by using ED1 with diaminobenzidine detection as described, followed by OP199, using streptavidin-conjugated alkaline phosphatase with New Fuchsin substrate for detection. In one additional animal the heart was fixed in paraformaldehyde and osteopontin localized by immunoelectron microscopy by using 1), gold-labeled secondary antibody on Lowicryl-embedded thin sections; and 2), en bloc immunoperoxidase staining followed by Epon embedding and thin sectioning, as described in detail previously.[2,5]

In Situ Hybridization

After overnight fixation in 3.5% paraformaldehyde, tissue samples were routinely dehydrated and embedded. Five-micron sections were deparaffinized, rehydrated, and post-fixed in 3.5% paraformaldehyde for 10 minutes. They were then incubated with proteinase K (1 mg/ml in 0.5 mol/L NaCl and 10 mmol/L Tris buffer, pH 8.0) for 7.5 minutes at 37 C, followed by a 10-minute wash in 0.5× standard saline citrate (SSC; 1× SSC = 150 mmol/L NaCl and 15 mmol/L sodium citrate) at room temperature. Sections were then prehybridized (50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris, pH 8.0, 1× Denhardt's solution, 10 mmol/L dithiothreitol, and 10% dextran sulfate) for 2 to 4 hours at 55 C in a humid environment. Sense and antisense [^{35}S]-labeled riboprobes were prepared from the rat osteopontin cDNA clone 2B7,[8,9] which contains the full protein coding sequence, with a transcription kit from Promega (Madison, WI) according to the manufacturer's specifications. Probe was applied to the hybridization mixture to achieve a final specific activity of 300,000 cpm/ml. The sections were hybridized overnight at 55 C. The next morning they were washed twice in 2× SSC for 10 minutes each and then immersed in a buffer containing 20 mg/ml RNAse A for 30 minutes at 37 C. Sections were again washed at low stringency in 2× SSC at room temperature, followed by a high stringency wash in 0.1× SSC at 55 C for 2 hours. After the high stringency wash, sections received two 1-minute washes in 5× SSC and then were dehydrated and dried overnight. Slides were dipped in Kodak NTB2 emulsion, dried, and exposed for 7 days.

Western Blot Analysis

Six additional animals were studied, five on day 1 after freeze-thaw injury and one sham-operated animal. After sacrifice and removal of the heart, the aorta was cannulated and perfused with PBS until the effluent was clear. The injured tissue from the posterior wall was excised with a razor blade, carefully trimmed to remove adjacent viable tissue, and frozen in liquid nitrogen. Uninjured myocardium from the anterior wall was similarly obtained and frozen. Samples were later pulverized under liquid nitrogen and extracted in a lysis buffer containing 0.05 mol/L Tris, pH 6.8, 1% sodium dodecyl sulfate, and 10% glycerol. After centrifugation, protein concentration was determined in the supernatants by using the Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical Co., Rockford, IL). Aliquots containing 30 μg total protein were boiled in sample buffer and loaded onto a 9% polyacrylamide gel. Duplicate gels were stained with Coomassie blue to verify equal protein loading. Electrophoresis under reducing or nonreducing conditions yielded identical results, and results from nonreduced gels are shown. Gels were transferred to a polyvinylidene difluoride membrane (DuPont/New England Nuclear, Wilmington, DE) with an electroblot system (Bio-Rad Laboratories, Richmond, CA). Western blotting was accomplished by using the goat polyclonal antibody OP199 at a concentration of 2.7 μg/ml. Secondary detection was accomplished with peroxidase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and a chemiluminescence kit (DuPont/New England Nuclear).

Northern Blot Analysis

Frozen myocardial samples were pulverized under liquid nitrogen and then homogenized in a solution containing 4 mol/L guanidinium isothiocyanate. Total RNA was extracted by the acid-phenol technique of Chomczynski et al.[16] Fifteen micrograms of RNA per lane were electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to Hybond-N membranes (Amersham), and immobilized by baking for 2 hours at 80 C. A [^{32}P]-labeled DNA probe was prepared with the use of a random priming kit (Amersham, Arlington Heights, IL) from the human osteopontin cDNA clone OP10. This clone was obtained from Dr. Larry Fisher (Bone Research Branch, NIH) and contains the entire osteopontin coding sequence, as well as 5′ and 3′ flanking sequences.[17] To assess potential variation in gel loading and transfer, the blots were subsequently probed with a [^{32}P]-end-labeled oligonucleotide directed to the 28S ribosomal RNA subunit, with the sequence 5′ GCG AGA GCG CCA GCT ATC CTG AGG 3′. Membranes were prehybridized for 4 hours and then hybridized overnight at 44 C. The blots were washed three times for 5 minutes each at room temperature in a low stringency buffer (2× SSC with 0.1% sodium dodecyl sulfate), followed by two 20-minute washes in a high stringency buffer (0.2× SSC with 0.1% sodium dodecyl sulfate at 65 C for the osteopontin probe and 55 C for the end-labeled 28S probe. Autoradiography was performed at −70 C with Kodak XAR film.

Results

Microscopic Evolution of Cardiac Freeze-Thaw Injury

The transdiaphragmatic freeze-thaw procedure produced uniform, disc-shaped lesions on the diaphragmatic surface of the heart, extending 2 to 3 mm into the myocardium (data not shown). Microscopically,

the early lesions (days 1 and 2 after injury) showed typical features of coagulation necrosis, with a prominent inflammatory infiltrate composed predominantly of macrophages and to a lesser extent neutrophils. By day 4 the process of organization had begun, with removal of necrotic myocytes and ingrowth of fibroblasts and capillaries, characteristic of granulation tissue. At 1 week after injury nearly all necrotic myocardium had been resorbed, and the entire lesion consisted of granulation tissue containing numerous macrophages. At 4 weeks after injury the lesions were composed largely of scar tissue. The scars were much less cellular than the granulation tissue and had retracted to occupy a smaller volume than either the original myocardium or the granulation tissue. The ultrastructural features of the injury and repair process in this model have been described in detail previously.[2–5]

Osteopontin in Cardiac Freeze-Thaw Injury

Osteopontin was not detected in a sham-operated heart or in uninjured myocardium remote from areas of freeze-thaw. In sharp contrast, abundant osteopontin protein was detected in the injured hearts at the early time points after necrotizing injury. On the first day after injury, immunostaining demonstrated that osteopontin was present in many cells located in a band-like pattern at the interface of viable and necrotic myocardium and in substantially fewer cells scattered in the deeper regions of the injured tissue (Figure 1A). Osteopontin staining was cytoplasmic and typically polarized to an eccentric perinuclear location suggestive of localization in the Golgi apparatus. Staining of an adjacent section with the macrophage marker ED1 demonstrated that most of the nucleated cells in this region were macrophages (Figure 1B), although some neutrophils were present as well. To verify that the osteopontin-producing cells were indeed macrophages, double immunolabeling was performed. All cells expressing osteopontin also expressed the ED1 antigen (data not shown). Many ED1-positive cells, however, did not express osteopontin. The osteopontin-negative macrophages were found both deep in the lesion and on the epicardial surface, where little osteopontin was detected, and at the interface of the lesion with viable myocardium, where osteopontin was abundant. By day 2 there was extensive macrophage permeation of the necrotic tissue, with loss of the dense band at the interface with viable myocardium. Osteopontin-positive cells were still present in a diffuse band at the edge of the lesion, but by this time they were also found throughout the necrotic muscle. As was seen on day 1, more cells stained with the macrophage marker ED1 on day 2 than stained with anti-osteopontin antibodies.

By day 4 after injury there was a notable decrease in the number of osteopontin-positive cells (Figure 1C), despite the fact that macrophages remained abundant components of the granulation response (Figure 1D). Macrophages containing osteopontin were most prominent around areas of residual necrotic tissue and were also scattered throughout the granulation tissue. Smooth muscle cells and endothelial cells in repairing vessels were occasionally noted to contain osteopontin protein as well (not shown). At 7 days after injury there was a further diminution in the number of osteopontin-positive cells, with only occasional cells in the injured region positive. In one heart there was a collection of osteopontin-positive cells around a focus of unresorbed necrotic myocardium. Examination of 28-day-old injuries, composed largely of scar tissue, showed only rare osteopontin-positive cells (Figure 1E). In several hearts no osteopontin was detected. Despite this, numerous macrophages were seen in the scars by ED1 staining (Figure 1F). At no time was significant osteopontin staining observed in the fibroblasts of granulation or scar tissue, and no osteopontin was detected in the extracellular space.

In situ hybridization was performed to determine which cells were actively synthesizing osteopontin and to examine the time course of the mRNA induction in response to injury. All sections hybridized with the control, sense osteopontin riboprobe showed very low signal (comparable with that shown below in Figure 7B). In agreement with the immunostaining results, no osteopontin mRNA was detected in uninjured myocardium with the antisense riboprobe. In contrast, there was intense hybridization in the injured tissue that followed a similar spatial and temporal course to that seen for the protein. On the first day after injury there was an intense, band-like osteopontin signal at the interface between viable and necrotic myocardium (Figure 2A, B). Positive cells were also seen throughout the injured region, but at lower density. By day 2 the signal had spread throughout the lesion but was of similar intensity. On day 4 after injury (Figure 2C, D) fewer cells expressed osteopontin message, although a very strong signal was seen over the individual positive cells. The fact that fewer cells in a given microscopic field had overlying silver grains allowed better spatial resolution, and osteopontin signal could be definitively associated with inflammatory cells and focally with cells in artery

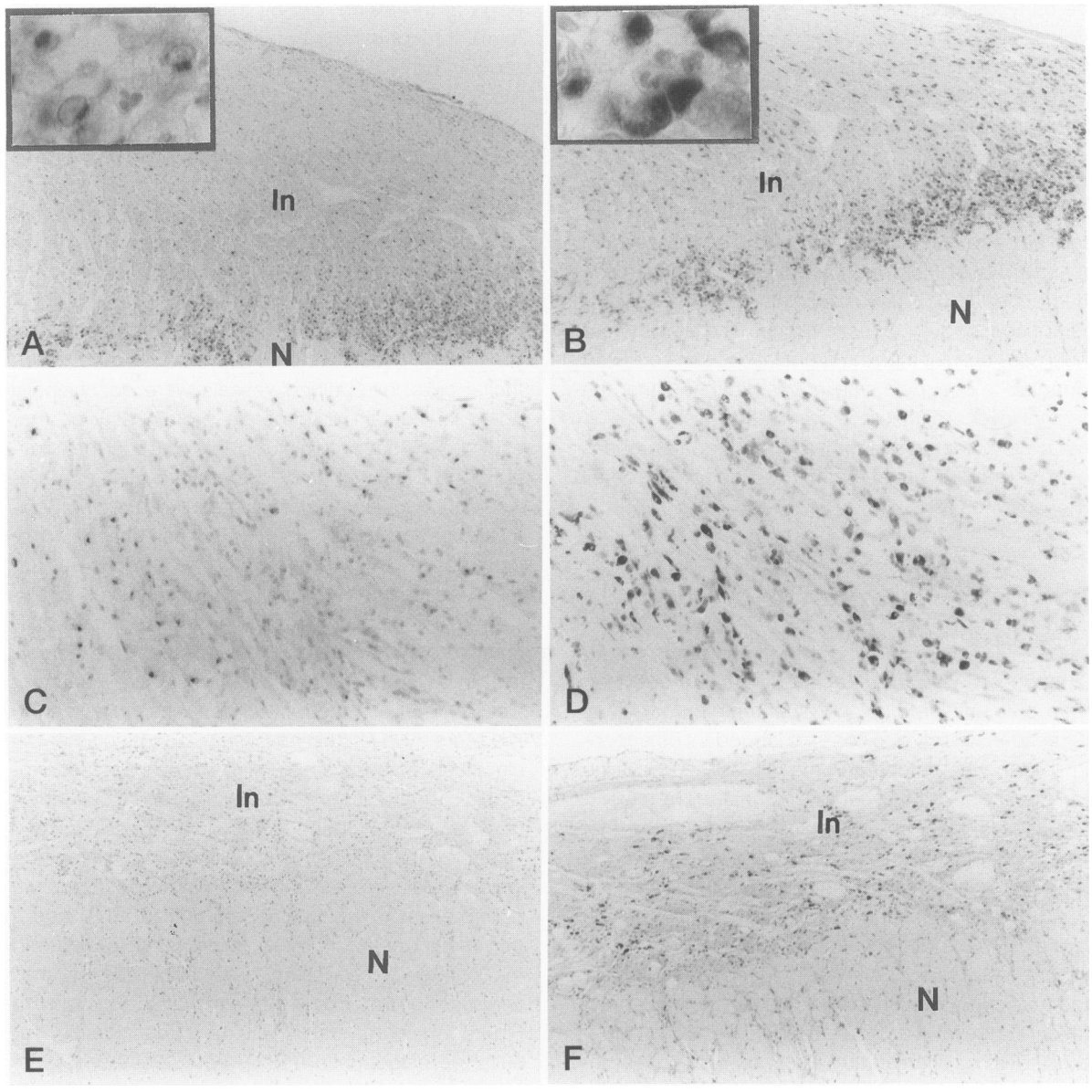

Figure 1. *Osteopontin expression and macrophage infiltration in rat heart at 1, 4, and 28 days after freeze-thaw injury.* **A:** *Day 1, osteopontin-positive cells are concentrated in a band along the interface of injured (In) and normal (N) myocardium. Scattered cells deeper in the lesion also express the protein. Inset, high power micrograph shows immunostaining concentrated in perinuclear zones, consistent with Golgi apparatus. A granulocyte is present but does not contain osteopontin.* **B:** *Day 1, ED1-positive macrophages are concentrated in a similar band at the interface of injured (In) and normal (N) myocardium. Numerous macrophages are also present deeper in the necrotic lesion. Inset, high power micrograph shows diffuse cytoplasmic immunostaining with ED1. A trio of neutrophils in the center does not contain the ED1 epitope.* **C:** *Day 4, osteopontin-positive cells are dispersed throughout the granulation tissue, but the density of positive cells is notably diminished* versus *day 1.* **D:** *Day 4, numerous ED1-positive macrophages persist in the granulation tissue.* **E:** *Day 28, virtually no osteopontin-positive cells are detected by immunostaining in the injured region (In), now composed of scar tissue. N, normal myocardium.* **F:** *Day 28, ED1-positive macrophages remain abundant components of scar tissue within the area of injury (In). N, normal myocardium. Macrophages were marked with ED1 monoclonal antibody. Osteopontin protein was stained with MPIIIB10 monoclonal antibody. All figures are magnified 323, except C and D which are ×646.*

walls. By 1 week there was a further diminution in the number of positive cells, again with strong signal persisting over individual cells. At this time osteopontin signal was associated with scattered inflammatory cells, arterial smooth muscle cells, and also focally with vascular endothelium. After 4 weeks of healing (Figure 2E, F) there was very little osteopontin signal detected, consisting of two to three cells in one heart.

Immunoelectron microscopy was performed by using two complementary methods: 1), immunogold labeling of Lowicryl-embedded thin sections; and 2), immunoperoxidase labeling of small tissue blocks fol-

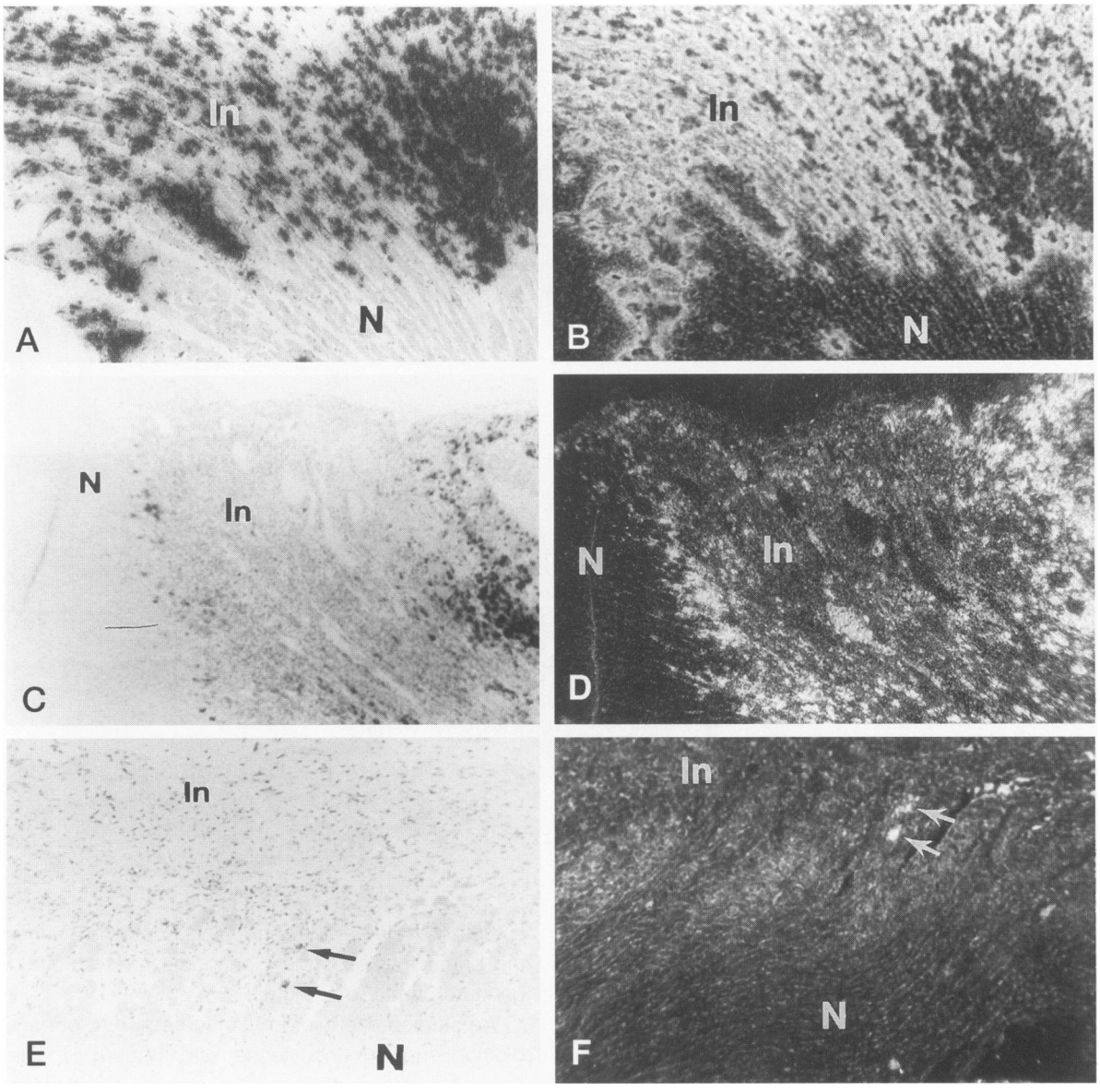

Figure 2. In situ *hybridization for osteopontin in myocardium 1, 4, or 28 days after freeze-thaw injury.* **A:** *Day 1, bright field image showing intense hybridization in cells infiltrating injured myocardium (In). Normal myocardium (N) is virtually free of signal.* **B:** *Day 1, dark field image of same section. The dark patches within the injured tissue are due to saturation of the photographic emulsion and failure to scatter incident light.* **C:** *Day 4, bright field image of interface between injured (In) and normal (N) tissue. Hybridization signal is less intense than on day 1 and more concentrated at the center of the lesion, present at the right of the photograph.* **D:** *Day 4, dark field image of same section. Osteopontin hybridization signal is present throughout the injured region but diminished relative to day 1.* **E:** *Day 28, Bright field image shows rare cells expressing osteopontin mRNA (arrows) in the injured region (In). N, normal myocardium.* **F:** *Day 28, Dark field image of same section. Positive cells indicated by arrows. All figures are magnified ×323. Osteopontin* in situ *hybridization with antisense [^{35}S]-labeled cRNA from rat probe 2B7.* In situ *hybridization with sense cRNA probes gave very low background signal, similar to that in Figure 7B.*

lowed by traditional embedding and thin sectioning. Osteopontin was located in cells morphologically consistent with macrophages (Figure 3A–D). These cells were frequently located within the residual basal lamina sheaths of necrotic myocytes. No other cell types were seen to express this protein on day 1, and no localization to the extracellular matrix was noted. Both methods showed osteopontin located in membrane-bound cytoplasmic vesicles, consistent with secretory vesicles. This was in agreement with a study of osteoblasts by Hultenby et al,[18] who also noted osteopontin localization to membranous vesicles.

Western blotting was performed on injured and control myocardium from five rats 1 day after freeze-thaw injury (Figure 4). In all five animals, a polyclonal antibody to osteopontin (OP199, also used for some of the immunostaining) detected a single broad band

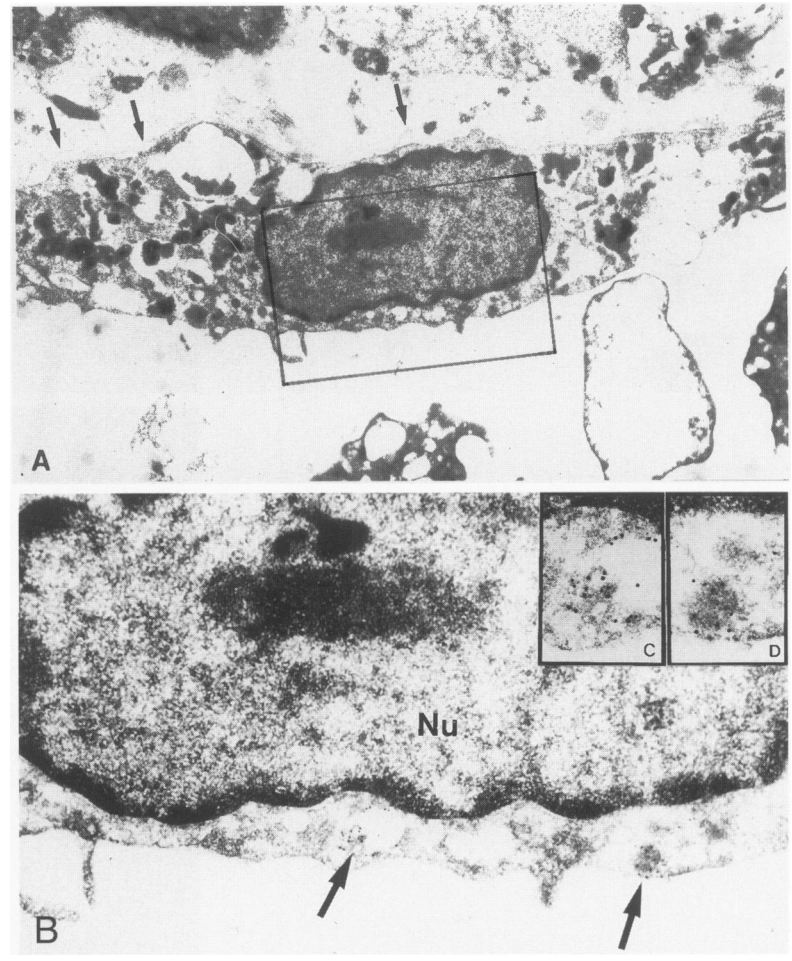

Figure 3. *Immunoelectron microscopic detection of osteopontin within a macrophage 1 day after freeze-thaw injury to rat myocardium.* **A:** *Relatively low magnification electron micrograph (×11,80) of a macrophage located within the residual basal lamina sheath (arrows) of a necrotic myocyte. Necrotic cell debris is located both within the basal lamina compartment and external to it.* **B:** *Higher power (×24,800) micrograph of region boxed in* **A**. *The macrophage contains gold particles localized to electron-dense and electron-lucent structures consistent with secretory vesicles (arrows) shown at higher magnification (×41,300) in* **C** *and* **D**. *Nu, nucleus.*

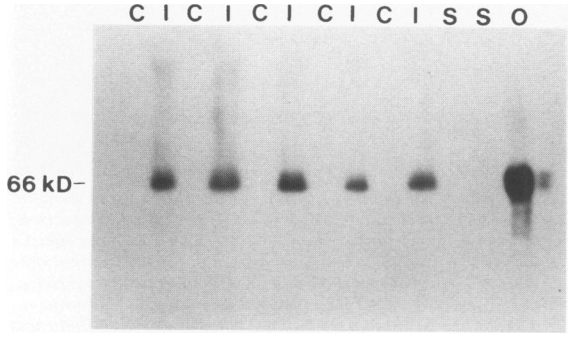

Figure 4. *Western analysis of osteopontin in control and injured rat myocardium. Osteopontin was detected as a broad band at 66 kd in the injured regions (I) of five hearts examined 1 day after freeze-thaw. No protein was detected in the uninjured control regions (C) of the same hearts or in hearts subjected to a sham procedure (S). O, osteopontin standard purified from cultured rat pup aortic smooth muscle cells.*

at an apparent molecular weight of 66 kd in the injured tissue, which was absent in uninjured myocardium from the same hearts. No band was present in samples from a sham-operated animal. The bands from injured myocardium were indistinguishable from samples of osteopontin purified from cultured rat pup smooth muscle cells. Control blots from which either the primary or secondary antibodies were omitted gave no bands (data not shown).

We studied one injured human heart by Northern blotting and immunocytochemistry (Figure 5). The 8-day-old infarct contained a mixture of necrotic muscle, inflammatory cells (macrophages and neutrophils), and granulation tissue. The anterior wall appeared grossly normal but had patchy, focal collections of macrophages microscopically. Although the normal tissue expressed detectable levels of osteopontin, there was a marked increase in the osteopontin message in the infarcted human tissue as determined by Northern analysis (Figure 5A). Immunostaining (Figure 5B) demonstrated numerous osteopontin-positive cells in the granulation tissue, morphologically consistent with macrophages. A few scattered inflammatory cells in the non-infarcted anterior wall were also noted to contain osteopontin (not shown) and were likely the source of osteopontin message detected by Northern analysis from this region.

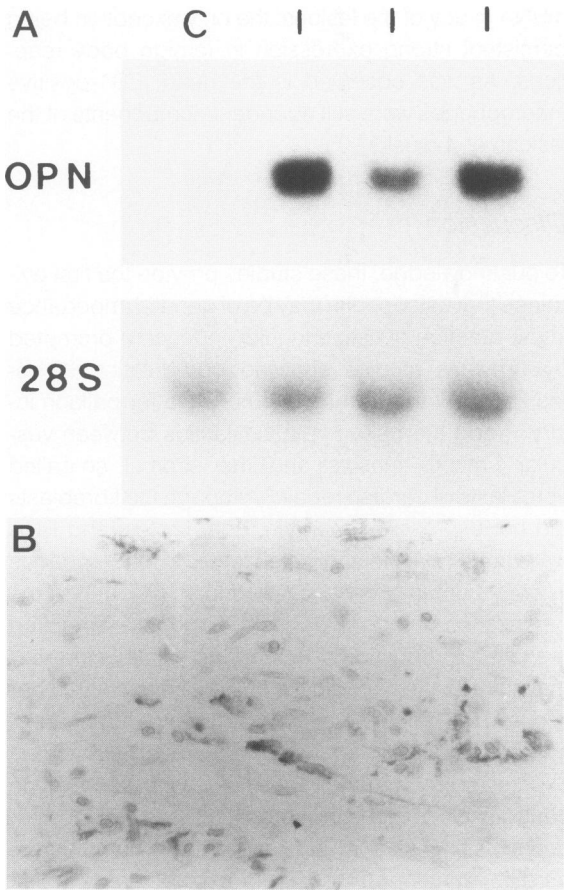

Figure 5. *Detection of osteopontin in human heart 8 days after infarction.* A: *Northern analysis demonstrated a faint osteopontin (OPN) signal in control myocardium (C) and marked upregulation of osteopontin message in three samples of infarcted myocardium (I) after a 3-hour autoradiographic exposure. The signal in control myocardium was more apparent with longer exposure. Hybridization with a 28S ribosomal RNA probe confirmed equal loading of lanes.* B: *Immunostaining with antibody LF7 localized osteopontin protein to cells consistent with macrophages in granulation tissue. Magnification, ×1300.*

Osteopontin in Skin, Pulmonary, and Skeletal Muscle Wounds

The surgical approach for inducing cardiac freeze-thaw injury produced incisional wounds in the abdominal wall, including the skin and rectus abdominus muscle. Additionally, freeze-thaw injury was also induced in the diaphragm and small amounts of intervening lung tissue. To determine whether the patterns of osteopontin expression seen in the heart were unique to myocardium or part of a generalized injury response, we performed immunocytochemical and *in situ* hybridization analysis of osteopontin expression in these tissues as well. All injured tissues contained a prominent inflammatory infiltrate. The incisional skin wounds were infiltrated initially by a mixture of neutrophils and macrophages on days 1 and 2, which converted to predominantly macrophage infiltrates by 1 and 4 weeks. The skeletal muscle injuries, both incisional and freeze-thaw as well as the pulmonary freeze-thaw injury, contained predominantly macrophages at all time points (data not shown).

In uninjured skin and skeletal muscle no osteopontin protein or mRNA were observed. Focal osteopontin protein and mRNA expression were seen in bronchial epithelium. Notably, no osteopontin was detected in alveolar macrophages in uninjured lung. Osteopontin mRNA and protein also were absent from cells of the monocyte-macrophage lineage in normal spleen, liver, lymph node, and thymus (data not shown).

In general, the expression patterns of osteopontin in the lung, skin, and skeletal muscle paralleled that in the heart. On days 1 and 2, numerous infiltrating macrophages expressed osteopontin message and protein (as shown for the skin in Figure 6A). As in the heart, more cells stained with the macrophage marker ED1 (Figure 6B) than stained with osteopontin antibodies, indicating that induction was limited to a subset of macrophages. Osteopontin expression was not detected in neutrophils in the skin exudate. There

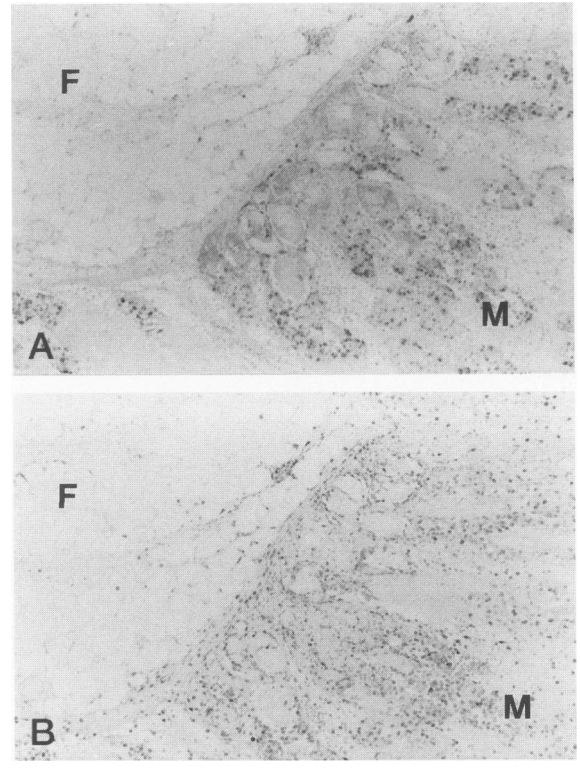

Figure 6. *Osteopontin and macrophages in skin wound 1 day after injury.* A: *Osteopontin staining was present predominantly in macrophages in necrotic skeletal muscle (M). Little staining was present in subcutaneous fat (F). Immunostaining with MPIIIB10. Magnification, ×323.* B: *Adjacent section showing macrophages diffusely infiltrating necrotic skeletal muscle (M) and fat (F) in subcutaneous wound. Immunostaining with ED1. Magnification, ×323.*

was, however, abundant mRNA and protein expression in macrophages involved in foreign body reactions to suture. In the lung, both macrophages and regenerating bronchiolar epithelium were synthesizing osteopontin. By day 4 after injury, osteopontin expression was notably diminished in all injured tissues, despite no appreciable decline in the number of ED1-positive macrophages. A striking finding at this time was an intense *in situ* hybridization signal in some regions of injured skeletal muscle (Figure 7). Protein was also detected, although the intensity was less impressive. The injured muscle had signal localized at regular intervals along the cell periphery, often in a perinuclear distribution. This could represent expression by satellite cells. By 1 week (not shown) there was a further decline in the number of cells containing osteopontin protein. mRNA signal was also diminished but to a lesser extent than seen for protein. Focal collections of osteopontin-positive macrophages were present in the basal lamina sheaths of skeletal muscle and in foreign body reactions to sutures. Numerous macrophages persisted in all injured regions at this time. At 4 weeks after injury (not shown) only very rare cells contained osteopontin protein or mRNA in any of the lesions, the only exception being persistent strong expression in foreign body reactions. As was observed in the heart, ED1-positive macrophages were still abundant components of the lesions at 4 weeks.

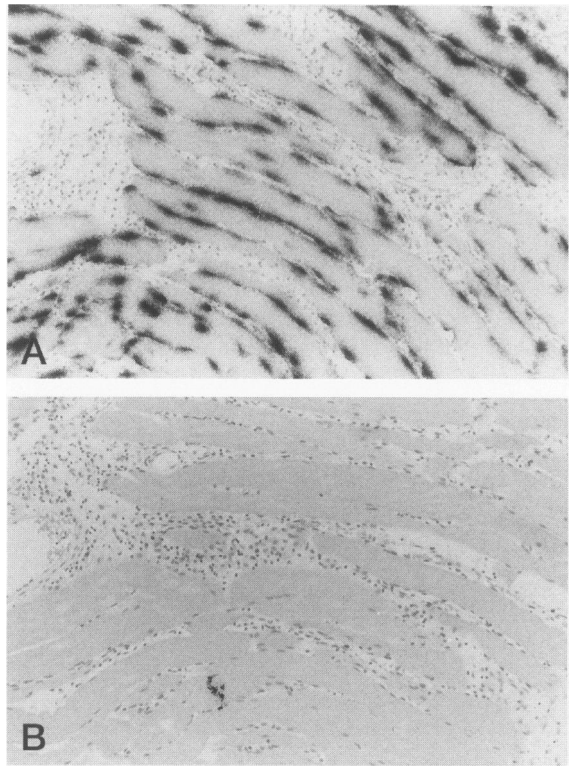

Figure 7. *Osteopontin expression in regenerating skeletal muscle 4 days after incisional wound.* **A:** *Antisense cRNA probe to osteopontin showed extensive hybridization to the perinuclear regions of regenerating skeletal myocytes, possibly involving satellite cells.* **B:** *Sense cRNA probe to osteopontin demonstrates very low background signal in adjacent section.*

Discussion

To our knowledge, these studies provide the first evidence that osteopontin may be of general importance in the reaction of tissue to injury. We were prompted by previous studies showing upregulation of osteopontin by arterial smooth muscle after balloon injury[8,9] and the phenotypic similarities between vascular smooth muscle and the stromal so-called fibroblasts of cardiac repair.[5] Although the fibroblasts did not express osteopontin, both protein and RNA were present at strikingly high levels in macrophages in early human and rat cardiac lesions. Despite the persistence of macrophages throughout the healing response, production of osteopontin diminished dramatically as the necrotic lesions evolved toward scar tissue. This response was not unique to the heart or freeze-thaw injury, because high levels of osteopontin expression by macrophages also were seen in the early stages of repair in injured lung, skeletal muscle, and incisional skin wounds as well as in human myocardial infarction. Osteopontin expression was also induced transiently by injury in other cell types, including skeletal myocytes, arterial smooth muscle cells, endothelial cells, and bronchiolar epithelial cells.

Although first described as a component of bone matrix,[19] osteopontin is now known to be more widely expressed. For example, in normal adult tissues it is present in the epithelium and secretions of many exocrine glands, renal tubular epithelium and urine, peripheral nerves, decidualized endometrium, and placenta.[20–27] We have also observed increased expression during corpus luteum formation (unpublished observations). Notably, osteopontin is not present in normal skin or skeletal or cardiac muscle and is restricted to bronchial epithelium in lung (24 and this study). The function of osteopontin is best understood in bone, in which it appears to anchor osteoclasts to the hydroxyapatite matrix via integrin-RGD adhesion as well as signal bone resorption by these cells.[28,29] Expression of osteopontin by osteoblasts is increased by vitamin D3 and reduced by parathyroid hormone,[30–33] consistent with a role in calcium homeostasis. Osteopontin production increases rapidly during repair of simple bone fractures[34,35] and repair of mechanical marrow ablation.[36]

Recent studies also have shown that osteopontin expression is increased in vascular and renal disease. Experimental balloon injury greatly stimulates its production by arterial smooth muscle cells during medial repair and neointima formation,[8,9] consistent with a role in smooth muscle migration or proliferation. In human atherosclerosis, osteopontin is synthesized at high levels in plaque smooth muscle cells, macrophages, and endothelial cells of the plaque *vasa vasorum*. Osteopontin is also found at high levels in plaque matrix, particularly in regions of calcification[9,37,38] (also O'Brien et al, manuscript submitted). In the rat kidney, osteopontin protein and mRNA are elevated in angiotensin II-induced tubulointerstitial nephritis.[39]

Osteopontin expression has been shown to be upregulated in parallel with induction of cell proliferation in several circumstances. Treatment of cultured smooth muscle cells with several different growth factors induces osteopontin expression (see below).[40] Tumor-promoting phorbol esters induce osteopontin production in mouse epidermis,[41,42] and H-*ras* transformation of cultured fibroblasts is associated with increased osteopontin production.[43] We did not measure cell proliferation in this study, and therefore cannot address a potential link between osteopontin and proliferation directly. The predominant cell producing osteopontin in the heart was the tissue macrophage, and to our knowledge, macrophage proliferation in healing heart lesions has not been studied. It is conceivable that macrophages proliferate in heart lesions, however, inasmuch as these cells have been shown to proliferate in atherosclerotic plaques.[44,45] At this point, the significance of osteopontin induction with cell proliferation is not clear.

The factors that induce osteopontin in wound macrophages are not known. *In vitro*, monocytic cells increase osteopontin expression in response to lipopolysaccharide, phorbol ester, crude lymphokines, and interleukin-1[46,47] (also Giachelli and Urieli-Shovall, unpublished observations). All of these agents induce macrophage activation, ie, they stimulate the cell to perform more complex, differentiated functions such as phagocytosis and tumor killing.[48,49] In addition to the calcitropic hormones mentioned above, a variety of peptide ligands such as basic fibroblast growth factor,[9,33] transforming growth factor-β[9,50–53] angiotensin II,[9] and, in one report, platelet-derived growth factor[40] have been shown to increase osteopontin expression in cells other than macrophages. To the best of our knowledge, the effects of these agents on macrophages have not been investigated. It seems likely, however, that some of these growth factors are present in the injured tissue. Transforming growth factor-β, for example, is increased in myocytes adjacent to infarct zones.[54]

What is the function of macrophage-derived osteopontin in wound healing? This central question cannot be answered definitively with available data. On the basis of what is known about macrophages in wound repair and osteopontin in other systems, however, three related functional properties seem possible: 1), cell adhesion and migration; 2), chemotaxis; and 3), phagocytosis. These properties could involve macrophages only or might also affect other cells in wound repair, eg, fibroblasts, smooth muscle cells, or endothelial cells.

The best described role for osteopontin is as an extracellular adhesion molecule and a substrate for cell migration. A wide range of cells, including macrophages, endothelial cells, and smooth muscle cells have the capacity to bind to it via RGD-dependent mechanisms.[12,55,56] The most extensively studied receptor for osteopontin is the integrin complex $\alpha v\beta 3$, which also binds vitronectin. Recent studies from our laboratory have demonstrated that, in addition, osteopontin can bind to smooth muscle cells via the $\alpha v\beta 5$ and $\alpha v\beta 1$ complexes (Liaw et al, manuscript submitted). The receptors for osteopontin on macrophages have not yet been defined, although macrophages have been shown to make $\alpha v\beta 3$.[57] Some investigators have proposed that osteopontin may function as an autocrine adhesion factor for tumor cells, promoting their adhesion during invasion and metastasis.[11,42] A similar function for macrophage-derived osteopontin can be envisioned, for which infiltrating cells elaborate a matrix molecule that facilitates their adhesion, retention, and migration within the injured tissue. (For a theoretical discussion of integrins and cell migration, the reader is referred to the recent article by Tooney et al.[58])

Chemotactic properties of osteopontin have been observed in multiple cell types. It is produced by normal T cells in response to concanavalin-A stimulation[59] and when injected subcutaneously induces macrophage accumulation.[56] Deficient production of osteopontin has been implicated in rickettsial susceptibility in mice.[59] In a rat model of tubulointerstitial nephritis, Giachelli et al[39] have shown that macrophages clustered around tubules that had elevated osteopontin production. In addition to its effects on macrophages, osteopontin has recently been shown to be chemotactic for cultured smooth muscle cells[12] and chemokinetic for endothelial cells.[60] Chemotactic activity is RGD dependent, suggesting it is mediated by integrins (Giachelli, unpublished observations). Thus, osteopontin could function as a chemotactic factor, recruiting additional macroph-

ages into the lesion or, alternatively, attracting fibroblasts and endothelial cells for formation of granulation tissue.

A third possibility the function of for osteopontin is facilitation of phagocytosis. Although this idea has not been tested directly, there is at least circumstantial evidence from the current study. Osteopontin was expressed at high levels when abundant necrotic myocardium was present, and its levels declined as this tissue was resorbed. In several hearts studied 4 and 7 days after injury, osteopontin-positive macrophages were definitely clustered around foci of residual necrotic muscle. In skin wounds osteopontin expression similarly declined with removal of necrotic tissue, and at 1 and 4 weeks the predominant site of synthesis was in macrophages involved in foreign body reactions to suture, a site where attempts at phagocytosis are likely to persist. There is also precedent in the literature for a matrix molecule functioning like an opsonin. Fibronectin enhances phagocytosis of antibody-coated streptococci by macrophages and neutrophils via RGD-dependent mechanisms.[61] Furthermore, phagocytosis of apoptotic cells by macrophages occurs via the $\alpha v\beta 3$ receptor. The ligand for this process has not yet been defined, but it requires an RGD motif.[57,62]

Virtually all known information about osteopontin suggests its function resides in the extracellular space. Despite this, we were unable to demonstrate osteopontin protein in the extracellular matrix. All immunostaining was cytoplasmic. We do not know with certainty why no extracellular protein was detected. Our working explanation is that extracellular levels are below the threshold of detection by immunocytochemistry, whereas intracellular stores are readily detectable. It seems less likely that epitopes could be masked in the extracellular space, inasmuch us we found identical staining patterns with both monoclonal and polyclonal antibodies. It is possible that osteopontin has an unrecognized function as an intracellular molecule, but again, there is no evidence to support this notion. These possibilities will be addressed by future studies that neutralize extracellular osteopontin.

The generalized and abundant expression of osteopontin after injury suggests to us that this protein may be widely important in tissue repair and remodeling. In most normal and pathological states in which osteopontin is made, the tissue is in flux (eg, cell migration, proliferation, death, or matrix remodeling), whereas most static tissues (eg, heart, brain, or skeletal muscle) do not synthesize it. If osteopontin is required for cell and tissue turnover, it might be possible to enhance healing or control inflammation by augmenting or blocking its effects.

Acknowledgments

The authors are indebted to Thanh Nguyen for performing immunocytochemistry and assistance with animal surgery; to Joe Su, Patti Polinsky, Donna Lombardi, and Mike Garvin for assistance with *in situ* hybridization; and to Manuela Almeida for performing the Western analysis.

References

1. Mallory GK, White PD, Salcedo-Salgar J: The speed of healing of myocardial infarction: a study of the pathologic anatomy in 72 cases. Am Heart J 1939, 18:647–671
2. Vracko R, Cunningham D, Frederickson RG, Thorning D: Basal lamina of rat myocardium: its fate after death of cardiac myocytes. Lab Invest 1988, 58:77–87
3. Vracko R, Thorning D, Frederickson RG, Cunningham D: Myocyte reactions at the borders of injured and healing rat myocardium. Lab Invest 1988, 59:104–114
4. Vracko R, Thorning D, Frederickson RG: Fate of nerve fibers in necrotic, healing and healed rat myocardium. Lab Invest 1990, 63:490–501
5. Vracko R, Thorning D: Contractile cells in rat myocardial scar tissue. Lab Invest 1991, 65:214–227
6. Majesky MW, Reidy MA, Bowen-Pope DF, Wilcox JN, Schwartz SM: Platelet-derived growth factor (PDGF) ligand and receptor gene expression during repair of arterial injury. J Cell Biol 1990, 111:2149–2158
7. Majesky MW, Giachelli CM, Schwartz SM: Rat carotid neointimal smooth muscle cells re-express a developmentally regulated phenotype during repair of arterial injury. Circ Res 1992, 71:759–768
8. Giachelli CM, Bae N, Lombardi DM, Majesky MW, Schwartz SM: Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes *in vitro* and is identical to osteopontin (secreted phosphoprotein I, 2aR). Biochem Biophys Res Commun 1991, 177:867–873
9. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM: Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 1993, 92:1686–1696
10. Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992, 69:11–25
11. Chambers AF, Hota C, Prince CW: Adhesion of metastatic, ras-transformed NIH 3T3 cells to osteopontin, fibronectin, and laminin. Cancer Res 1993, 53:701–706
12. Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM: Osteopontin promotes vascular cell adhesion and

spreading and is chemotactic for smooth muscle cells *in vitro*. Circ Res 1994, 74:214–224
13. Miyauchi A, Alvarez J, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallone A, Ross FP, Teitelbaum SL, Cheresh D, Hruska KA: Recognition of osteopontin and related peptides by an $\alpha v\beta 3$ integrin stimulates immediate cell signals in osteoclasts. J Biol Chem 1991, 260:20369–20374
14. Vracko R, Thorning D: Freeze-thaw injury of rat heart across an intact diaphragm: a new model for the study of the response of myocardium to injury. Cardiovasc Res 1985, XIX:76–84
15. Dijkstra CD, Dopp EA, Joling P, Kraal G: The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in rat recognized by monoclonal antibodies ED1, ED2 and ED3. Adv Exp Med Biol 1985, 186:409–419
16. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
17. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW: cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics 1990, 7:491–502
18. Hultenby K, Reinholt FP, Oldberg A, Heinegard D: Ultrastructural immunolocalization of osteopontin in metaphyseal and cortical bone. Matrix 1991, 11:206–213
19. Franzen A, Heinegard D: Isolation and characterization of two sialoproteins present only in bone calcified matrix. Biochem J 1985, 232:715–724
20. Yoon K, Buenaga R, Rodan GA: Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun 1987, 148:1129–1136
21. Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BLM: Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by *in situ* hybridization. J Cell Biol 1988, 106:441–450
22. Mark MP, Prince CW, Gay S, Austin RL, Butler WT: 44-kDal bone phosphoprotein (osteopontin) antigenicity at ectopic sites in newborn rats: kidney and nervous tissues. Cell Tissue Res 1988, 251:23–30
23. Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG: Inhibition of calcium oxalate crystal growth *in vitro* by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 1992, 89:426–430
24. Brown LF, Berse B, Van De Water L, Papadopoulos-Sergiou A, Perruzzi CA, Manseau EJ, Dvorak HF, Senger DR: Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol Biol Cell 1992, 3:1169–1180
25. Kohri K, Suzuki Y, Yoshida K, Yamamoto K, Amasaki N, Yamate T, Umekawa T, Iguchi M, Sinohara H, Kurita T: Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin. Biochem Biophys Res Commun 1992, 184:859–864
26. Worcester EM, Blumenthal SS, Beshensky AM, Lewand DL: The calcium oxalate crystal growth inhibitor protein produced by mouse kidney cortical cells in culture is osteopontin. J Bone Mineral Res 1992, 7:1029–1036
27. Waterhouse P, Parhar RS, Guo X, Lala PK, Denhardt DT: Regulated temporal and spatial expression of the calcium-binding proteins calcyclin and OPN (osteopontin) in mouse tissues during pregnancy. Mol Reprod Dev 1992, 32:315–323
28. Reinholt FP, Hultenby K, Oldberg A, Heinegard D: Osteopontin: a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 1990, 87:4473–4475
29. Flores ME, Norgard M, Heinegard D, Reinholt FP, Andersson G: RGD-directed attachment of isolated rat osteoclasts to osteopontin, bone sialoprotein and fibronectin. Exp Cell Res 1992, 201:526–530
30. Prince CW, Butler WT: 1,25-dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Collagen Rel Res 1987, 7:305–313
31. Noda M, Rodan GA: Transcriptional regulation of osteopontin production in rat osteoblast-like cells by parathyroid hormone. J Cell Biol 1989, 108:713–718
32. Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT: Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (Spp-1 or osteopontin) gene expression. Proc Natl Acad Sci USA 1990, 87:9995–9999
33. Rodan SB, Wesolowski G, Yoon K, Rodan GA: Opposing effects of fibroblast growth factor and pertussis toxin on alkaline phosphatase, osteopontin, osteocalcin, and type I collagen mRNA levels in ROS 17/2.8 cells. J Biol Chem 1989, 264:19934–19941
34. Alberius P, Johnell O: Repair of intra-membranous bone fractures and defects in rats: immunolocalization of bone and cartilage proteins and proteoglycans. J Cranio-Max Fac Surg 1991, 19:15–20
35. Ohta S, Yamamuro T, Lee K, Okumura H, Kasai R, Hiraki Y, Ikeda T, Iwasaki R, Kikuchi H, Konishi J, Shigeno C: Fracture healing induces expression of the proto-oncogene c-*fos in vivo*: possible involvement of the Fos protein in osteoblastic differentiation. FEBS Lett 1991, 284:42–45
36. Suva LJ, Seedor G, Endo N, Quartuccio HA, Thompson DD, Bab I, Rodan GA: Pattern of gene expression following rat tibial marrow ablation. J Bone Mineral Res 1993, 8:379–388
37. Hirota S, Imakita M, Kohri K, Ito A, Morii E, Adachi S, Kim H, Kitamura Y, Yutani C, Nomura S: Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques: a possible association with calcification. Am J Pathol 1993, 143:1003–1008
38. Ikeda T, Shirasawa T, Esaki Y, Yoshiki S, Hirokawa K:

Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest 1993, 92:2814–2820
39. Giachelli CM, Pichler R, Lombardi D, Denhardt DT, Alpers CE, Schwartz SM, Johnson RJ: Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. Kidney Int 1994, 45:515–524
40. Gadeau A, Campan M, Millet D, Candresse T, Desgranges C: Osteopontin overexpression is associated with arterial smooth muscle cell proliferation *in vitro*. Arterioscler Thromb 1993, 13:120–125
41. Craig AM, Smith JH, Denhardt DT: Osteopontin, a transformation-associated cell adhesion phosphoprotein, is induced by 12-O-tetradecanoylphorbol 13-acetate in mouse epidermis. J Biol Chem 1989, 264: 9682–9689
42. Craig AM, Bowden GT, Chambers AF, Spearman MA, Greenberg AH, Wright JA, McLeod M, Denhardt DT: Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. Int J Cancer 1990, 46:133–137
43. Chambers AF, Behrend EI, Wilson SM, Denhardt DT: Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, *ras*-transformed NIH 3T3 cells. Anticancer Res 1992, 12:43–48
44. Gordon D, Reidy MA, Benditt EP, Schwartz SM: Cell proliferation in human coronary arteries. Proc Natl Acad Sci USA 1990, 87:4600–4604
45. O'Brien ER, Alpers CE, Stewart DK, Ferguson M, Tran N, Gordon D, Benditt EP, Hinohara T, Simpson JB, Schwartz SM: Proliferation in primary and restenotic coronary atherectomy tissue: implications for antiproliferative therapy. Circ Res 1993, 73:223–231
46. Miyazaki Y, Setoguchi M, Yoshida S, Higuchi Y, Akizuki S, Yamamoto S: The mouse osteopontin gene: Expression in monocytic lineages and complete nucleotide sequence. J Biol Chem 1990, 265:14432–14438
47. Zhang Q, Wrana L, Sodek J: Characterization of the promoter region of the porcine *opn* (osteopontin, secreted phosphoprotein 1) gene: identification of positive and negative regulatory elements and a "silent" second promoter. Eur J Biochem 1992, 207:649–659
48. Adams DO, Hamilton TA: The activated macrophage and granulomatous inflammation. Cell Kinetics of the Inflammatory Reaction. Edited by OH Iversen. New York, Springer-Verlag, 1989, pp 151–167
49. Yu S, Koerner TJ, Adams DO: Gene regulation in macrophage activation: differential regulation of genes encoding for tumor necrosis factor, interleukin-1, JE, and KC by interferon-γ and lipopolysaccharide. J Leukocyte Biol 1990, 48:412–419
50. Noda M, Yoon K, Prince CW, Butler WT, Rodan GA: Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type β transforming growth factor. J Biol Chem 1988, 263:13916–13921
51. Noda M, Rodan GA: Type β transforming growth factor regulates expression of genes encoding bone matrix proteins. Connect Tissue Res 1989, 21:71–75
52. Kubota T, Zhang Q, Wrana JL, Ber R, Aubin JE, Butler WT, Sodek J: Multiple forms of SPPI (secreted phosphoprotein, osteopontin) synthesized by normal and transformed rat bone cell populations: regulation by TGF-β. Biochem Biophys Res Commun 1989, 162: 1453–1459
53. Wrana JL, Kubota T, Zhang Q, Overall CM, Aubin JE, Butler WT, Sodek J: Regulation of transformation-sensitive secreted phosphoprotein (SPPI/osteopontin) expression by transforming growth factor-β. Biochem J 1991, 273:523–531
54. Thompson NL, Bazoberry F, Speir EH, Casscells W, Ferrans VJ, Flanders KC, Kondaiah P, Geiser AG, Sporn MB: Transforming growth factor β-1 in acute myocardial infarction in rats. Growth Factors 1988, 1:91–99
55. Butler WT: The nature and significance of osteopontin. Connect Tissue Res 1989, 23:123–136
56. Singh RP, Patarca R, Schwartz J, Singh P, Cantor H: Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages *in vitro* and its effect upon macrophages *in vivo*. J Exp Med 1990, 171:1931–1942
57. Savill J, Dransfield I, Hogg N, Haslett C: Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature 1990, 343:170–173
58. Tooney PA, Agrez MV, Burns GF: A re-examination of the molecular basis of cell movement. Immunol Cell Biol 1993, 71:131–139
59. Patarca R, Freeman GJ, Singh RP, Wei F, Durfee T, Blattner F, Regnier DC, Kozak CA, Mock BA, Morse HCI, Jerrells TR, Cantor H: Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene: Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. J Exp Med 1989, 170:145–161
60. Liaw L, Lindner V, Schwartz SM, Giachelli CM: Osteopontin is synthesized by regenerating endothelium and stimulates endothelial migration. FASEB J 1994, in press (abstract)
61. Hill HR, Augustine NH, Williams PA, Brown EJ, Bohnsack JF: Mechanism of fibronectin enhancement of group B streptococcal phagocytosis by human neutrophils and culture-derived macrophages. Cell 1993, 61:2334–2339
62. Fadok VA, Savill JS, Haslett C, Bratton DL, Doherty DE, Campbell PA, Henson PM: Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J Immunol 1992, 149:4029–4035