Human-derived nanoparticles and vascular response to injury in rabbit carotid arteries: Proof of principle

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Abstract: Self-calcifying, self-replicating nanoparticles have been isolated from calcified human tissues. However, it is unclear if these nanoparticles participate in disease processes. Therefore, this study was designed to preliminarily test the hypothesis that human-derived nanoparticles are causal to arterial disease processes. One carotid artery of 3 kg male rabbits was denuded of endothelium; the contralateral artery remained unoperated as a control. Each rabbit was injected intravenously with either saline, calcified, or decalcified nanoparticles cultured from calcified human arteries or kidney stones. After 35 days, both injured and control arteries were removed for histological examination. Injured arteries from rabbits injected with saline showed minimal, eccentric intimal hyperplasia. Injured arteries from rabbits injected with calcified kidney stone- and arterial-derived nanoparticles occluded, sometimes with canalization. The calcified kidney stone-derived nanoparticles caused calcifications within the occlusion. Responses to injury in rabbits injected with decalcified kidney stone-derived nanoparticles were similar to those observed in saline-injected animals. However, decalcified arterial-derived nanoparticles produced intimal hyperplasia that varied from moderate to occlusion with canalization and calcification. This study offers the first evidence that there may be a causal relationship between human-derived nanoparticles and response to injury including calcification in arteries with damaged endothelium.

Keywords: arterial calcification, endothelial injury, intimal hyperplasia

Introduction

Self-replicating, self calcifying nanoparticles (NPs, formerly called nanobacteria) ranging in size of 0.08–0.5 μm which form a biofilm containing carbonate or hydroxylapatite have been isolated and propagated from mammalian blood (Kajander et al 1997) and calcified human arterial tissue and kidney stones (Miller et al 2004; Shiekh et al 2006). Although the exact nature of these nano-sized particles remains controversial, their propagation is reduced by certain antibiotics and inhibitors of oxidative metabolism (Bjorklund et al 1998; Ciftcioglu et al 2002; Kumar et al 2006). They incorporate radiolabeled uridine and phosphate into their structures and some sequences of nucleic acids have been isolated from biofilms containing nanosized structures (Kajander et al 1997; Cisar et al 2000; Miller et al 2004; Kumar et al 2006). Propagation seems to be accelerated in environments simulating microgravity (Ciftcioglu et al 2005).

In addition to calcified renal and arterial tissue, similar structures are found in human urine, renal cyst fluid (Ciftcioglu et al 2002), capsules from breast implants (Deva and Chang 1999), prostatitis (Shoskes et al 2005), gall stones (Wang et al 2006), dental plaque (Ciftcioglu et al 2003), intestinal tissue affected by inflammatory bowel disease (Meissner et al 2006), and ovarian cancer (Sedivy and Battistutti 2003). In spite of these associations, a cause and effect relationship between NPs

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and disease pathogenesis remains unclear. Therefore, in order to show a causal relationship between human-derived NPs and disease, Koch's postulates dictate that particles (organisms) must be identified in diseased tissue, isolated and propagated from diseased tissue, and inoculation of an animal with the isolates should recapitulate the disease. Therefore, this pilot study was designed to test the hypothesis that inoculation of naïve animals with different preparations of self-replicating, self-calcifying, human-derived NPs would initiate disease processes, specifically arterial calcification, in rabbits.

Methods

Nanoparticle culture and collection

Self-replicating, self calcifying NPs derived from human calcified aortic aneurysms and kidney stones (Miller et al 2004) were used in these experiments. NPs for injection were isolated from mature cultures which had formed a characteristic biofilm of calcified domes adhering to the bottom of polystyrene culture flasks. Culture flasks contained Dulbecco's Modified Eagle Medium (DMEM 10-013, Mediatech Inc, Manassas, VA) supplemented with 50 μM beta-mercaptoethanol (Sigma Chemical Co, St. Louis, MO), 10% γ-irradiated fetal calf serum (Atlanta Biologicals Inc, Lawrenceville, GA), and $CaCl_2$ (Sigma) to 3.6 mM.

Calcified human-derived NPs were collected by gently washing 10 confluent flasks twice with 10 ml sterile phosphate-buffered saline (PBS). The flasks were scraped to loosen NP colonies from the flask and the scrapings collected and centrifuged (20 min, 4 °C, 20,000 g). After each spin, tubes were consolidated in 0.5 ml PBS and centrifuged again. Once all pellets had been consolidated to a single tube, the pellet was washed twice with Tris buffer (10 mM) and the final pellet was suspended in 1 ml sterile saline for injection into the animal.

Decalcified human-derived NPs were collected by gently washing 3 confluent flasks twice with 10 ml sterile PBS containing 0.5 M ethylendiaminetetraacetic acid (EDTA) and then adding 1 ml of 0.5 M EDTA to the flasks for 1 h. The flasks were then scraped and the resulting solution was centrifuged (30 min, 4 °C, 60,000 g). Pellets were consolidated, washed, and re-suspended as described above for the calcified NPs. NPs ranged in size from 50–200 nm and in some situations formed clumps (please see Miller et al 2004).

Animals and surgery

Adult male (3 kg) New Zealand white rabbits (n = 9 total) were fed LabDiet hi-fiber rabbit chow ad libitum and were maintained in individual cages (12 hr light/12 hr dark cycle). Once animals were inoculated with NPs, they were maintained in a Biohazard Level 2 safety facility. All protocols were approved by the Animal Care and Use Committee and Biosafety Committee of the Mayo Clinic.

The left carotid artery of each animal was denuded of endothelium using balloon injury. Briefly, animals were sedated intramuscularly using a mixture of ketamine (35 mg/kg, Ketaset, Fort Dodge Animal Health, Fort Dodge, IA), xylazine (5 mg/kg, VetTek, Blue Springs, MO) and acepromazine (1 mg/kg, Boehringer Ingelheim Vetmedica Inc, St. Joseph, MO). They received a prophylactic dose of 0.25 ml penicillin (Pen-aqueous, Durvet, Inc., Blue Springs, MO) IM to reduce infection related to the surgery. This antibiotic does not interfere with NP replication in culture (Ciftcioglu et al 2002). Saline (60 ml, 0.9% NaCl, Baxter Healthcare Corp, Deerfield, IL) was administered subcutaneously to prevent dehydration during the surgery. Using universal aseptic techniques, the left carotid artery was exposed and mobilized by gentle blunt dissection through a ventral midline incision in the neck. Small side branches were exposed and cauterized at least 5 mm away from the common carotid. Heparin (Baxter Healthcare Corp, Deerfield, IL) was administered (1000 U/kg) through a catheter in the marginal ear vein. The carotid artery was then temporarily occluded proximally and distally with microvascular clamps.

Monofilament suture (8–0 Ethilon; Ethicon, Somerville, NJ) was used to create a purse string suture in the anterior region of the occluded artery. A 3.0 French Fogarty (Edwards Lifesciences, Irvine, CA) angioplasty catheter was introduced into the lumen through a small incision made in the center of the purse-string suture. The balloon was inflated until the artery was distended to 150%. The catheter was withdrawn and advanced three times in order to denude the endothelium. The incision was closed with the purse-string suture. The injured area was marked by placing one small suture at the distal end of the artery where the balloon had been extended. The vascular clamps were then removed. The remaining carotid artery was not exposed, serving as the unoperated contralateral control. The surgical site was closed in three layers, the final closure being a subcuticular stitch so that the animal could not chew the sutures.

Inoculation

While still under anesthesia, and within 3 hrs after the surgery, animals were injected with one of the following solutions

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(total volume of inoculant and saline washes was 5 ml) via a catheter placed in the marginal ear vein:

1. Saline (Control; n = 2). This group was the standard for the healing response.
2. Calcified arterial-derived nanoparticles derived from 10 flasks (n = 1).
3. Calcified kidney stone-derived nanoparticles derived from 10 flasks (n = 1).
4. Decalcified arterial-derived nanoparticles derived from three flasks (n = 3).
5. Decalcified kidney stone-derived nanoparticles derived from three flasks (n = 2).

Tissue collection

At 35 days post-operative, animals were anesthetized and carotid arteries were removed, fixed in formalin, embedded in paraffin, sectioned, and examined histologically. Adjacent sections (5 micron) from each artery were evaluated by light microscopy for general anatomical features such as intima:media ratio, presence of a fibrous cap, and calcification using elastin van Giesen, hematoxylin and eosin, and von Kossa stains, respectively. Slides were viewed on an Axioplan 2 upright microscope equipped with a Plan-Neofluar 5×, 0.15 na objective lens (Zeiss, Thornwood, NY). Images were digitized with an AxioCam HRc camera (Zeiss) mounted to the microscope with a 0.63 × c-mount and interfaced through KS400 image analysis software (Zeiss). Lumen, intima, and media borders were interactively selected and the intimal and medial areas were calculated with the KS400 software.

Histological analysis

The extent of myointimal hyperplasia was evaluated based on intima:media ratios and a qualitative analysis of the condition of the internal elastic lamina and media in arterial cross-sections. One section per stain was evaluated from each animal (three sections/animal).

Results

All animals remained in good health throughout the study and none showed anorexia or signs of neurological deficits (eye drooping, immobility, etc). The uninjured artery from all rabbits in all groups showed normal anatomy with a single layer of endothelial cells, intact internal elastic lamina and media, and an adventitia devoid of inflammatory cells (Figure 1, upper left panel). The injured artery of animals injected with saline showed eccentric intimal hyperplasia (Figure 1, upper right panel); the intima:media ratios for the injured arteries from the two animals injected with saline were 0.39 and 1.33.

The lumen of the injured artery of each rabbit inoculated with calcified arterial- or kidney-stone-derived NPs was filled with acellular and cellular material which was canalized with blood vessels. The media layer lost cohesion even though the internal elastic lamina remained intact (Figure 1; middle panels).

The extent of vascular healing and intimal hyperplasia in injured carotid arteries of rabbits injected with decalcified arterial-derived NPs was variable. In one animal, there was minimal intimal hyperplasia consistent with that observed in the injured arteries of animals injected with saline (Figure 2, left panel). The art ery from another rabbit inoculated with decalcified arterial-derived NPs had greater myointimal hyperplasia than observed in saline-injected animals and the internal elastic lamina was discontinuous (Figure 2, middle panel). In the third rabbit injected with decalcified arterial-derived NPs, the denuded artery occluded (with canalization) and a plaque, which stained positive for calcium phosphate, formed along the intima-medial border where the internal elastic lamina was disrupted (Figure 2, right panel and Figure 1, bottom left panel). Injured arteries from the two animals inoculated with decalcified NP of kidney stone origin had milder injury responses similar to the two animals inoculated with decalcified NP of aneurysmal origin shown in the left and center panels of Figure 2.

Diffuse staining for calcium phosphate by von Kossa stain was identified within the occluded injured artery of the animal injected with calcified NPs of kidney stone origin (Figure 1, lower right panel). A defined area of calcification was present in the occlusion of one animal injected with decalcified NPs of aneurysmal origin (Figure 1, lower left panel).

Discussion

Results of this study, while preliminary, provide the first evidence of a causal relationship between human-derived NPs and arterial disease processes including accelerated development of intimal hyperplasia and calcification. Evidence has been published to support a causal relationship between NPs and black pigment gall stones (Wang et al 2006) and renal lithogenesis (Ciftcioglu et al 2005; Shiekh et al 2006). However, the gall stone study utilized direct injection of NPs into the target organ. Intravenous injection of kidney-derived NPs localized to the kidney in the nephrolithiasis study (Shiekh et al 2006). The present study extends these observations to demonstrate that an intravenous injection of kidney stone- and arterial-derived

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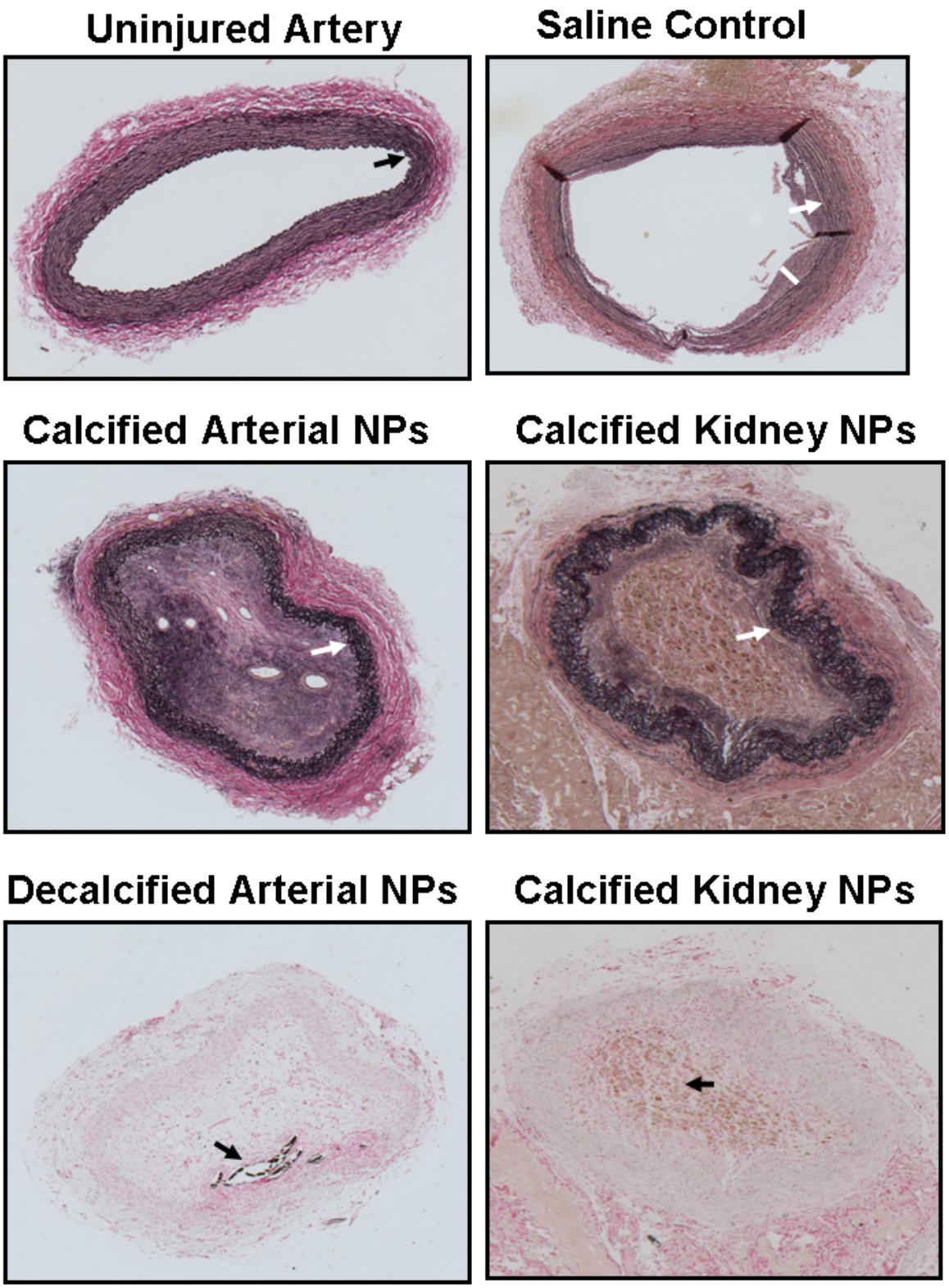


Figure 1 Representative light micrographs of sections (5 μm) of an uninjured (upper left panel) carotid artery from a rabbit inoculated with human arterial-derived nanoparticles, and the injured carotid artery of a rabbit inoculated with saline (control; upper right panel); calcified human arterial-derived (middle left panel) and calcified human kidney stone-derived (middle right panel and bottom right panel) nanoparticles; decalcified human arterial-derived nanoparticles (bottom left panel). All tissue was collected 5 weeks post-inoculation and sections are stained with either elastin van Giesen stain (upper and middle panels) or von Kossa stain (brown-black; lower panels). Sections are shown at the lowest (5X) magnification in order to show the entire artery. Uninjured arteries of rabbits in each treatment group are indistinguishable from that shown. Arrows indicate internal elastic lamina, bar indicates intimal thickening.

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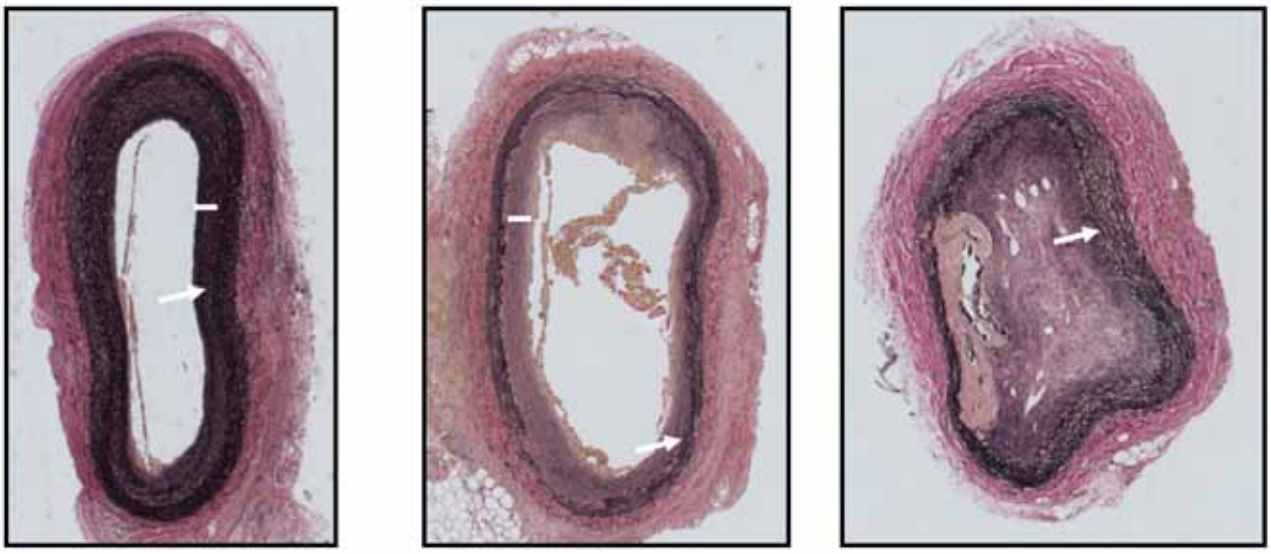


Figure 2 Representative light micrographs of sections (5 μm) of injured carotid arteries of three rabbits inoculated with decalcified human arterial-derived nanoparticles. All tissue was collected 5 weeks post-inoculation and stained with an elastin van Giesen stain. Sections are shown at the lowest (5X) magnification in order to show the entire artery. Uninjured arteries in these rabbits are indistinguishable from that shown in Figure 1, upper left panel. Injured arteries from rabbits injected with decalcified human arterial-derived nanoparticles exhibited a spectrum of vascular healing, ranging from disruption of the internal elastic lamina with minimal development of intimal hyperplasia (left panel), disruption of the internal elastic lamina and greater intimal hyperplasia than developed in the saline-injected rabbits (middle panel), to complete occlusion with canalization and calcification (right panel). Arrows indicate internal elastic lamina, bars indicate intimal thickening.

NPs accelerates a pathological process initiated by other stimuli, in this case, mechanical endothelial denudation. This systemic exposure to NPs might be comparable to that resulting from an environmental exposure as could occur in humans. The systemic route of administration, and perhaps an immune response to the NPs, may explain some of the variability in the vascular healing response observed in animals inoculated with the human-derived NPs. Whether NPs would accelerate the response to vascular injury caused by other means, ie, endothelial dysfunction due to hyperlipidemia, remains to be determined.

Variability in vascular responses in animals exposed to human-derived NPs may also have resulted from the way inoculants were prepared. Calcified NPs were derived from combined scrapings of biofilm from 10 flasks and, even with several washing of the scraped biofilm, some proteins in the culture media may remain adhered to hydroxyapatite shells of the NPs, initiating an immune response. Future experiments could investigate the contribution of inorganic hydroxyapatite as a carrier of mitogenic proteins and stimuli of inflammation (Nadra et al 2008).

To eliminate the potential contribution of the hydroxyapatite shell, NPs were decalcified. However, variability in vascular remodeling was still evident. Thus, the response to injury observed in NP-inoculated animals does not appear to be mediated only by the calcium phosphate shell and/or nonspecifically associated proteins.

These experiments have several shortcomings. First, the number of animals in this pilot study was small. However, there were clear differences in responses of animals injected with saline compared with those injected with NPs and the intimal hyperplasia in saline-injected rabbits was comparable with that seen in other studies of endothelial damage in rabbits (Orlandi et al 2002), rats (Chen and Mehta 1996), and pigs (Shimokawa et al 1989). Second, the inoculant was not standardized beyond using material initially derived from the same number of culture flasks. This method does not take into account differences in the quality of the cultures or number of viable NP colonies in each flask. Differences in NP preparations could account for some of the variability in responses among animals. In the future, methods to standardize inoculants need to be developed including evaluating responses to free floating, "planktonic" NPs rather than those isolated from established biofilm. Furthermore, complete removal of the hydroxyapatite shell from the decalcified NPs could not be verified using the current process. Therefore, reliable methods of isolating or producing uncalcified NPs must be developed. Finally, it is not known whether inoculated animals developed an immune-type response to the NPs that would be comparable to that of animals exposed to other infectious agents (eg, *Chlamydia pneumonia cytomegalovirus*) implicated in arterial disease (Mattila et al 1993; Muhlestein 1998; Epstein et al 2000; Burnett et al 2001; Muhlestein and

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Anderson 2003). Future experiments will need to explore the immunological responses evoked by NPs.

In spite of these short comings, results of this study provide exciting proof-of-principle that human-derived, self-replicating, self-calcifying NPs accelerate vascular wound healing and may lead to pathological calcification. Although the exact chemical, physical and biological nature of these human-derived NPs remains to be elucidated (Martin et al 1965; Miller et al 2004; Benzerara et al 2006), these results support the need for ongoing investigation of the medical and pathophysiological consequences of NPs in humans. Regardless of whether or not NPs represent a pleiotropic form of bacteria or biofilm (Domingue and Schlegel 1977; Akerman et al 1993; Domingue and Woody 1997; Costerton et al 1999; Cisar et al 2000; Vali et al 2001), the cumulative evidence from this and other studies (Shiekh et al 2006; Wang et al 2006) suggest that human-derived NPs participate in disease processes. Even if NPs are ultimately found not to be a life form in the classic sense, these experiments suggest a novel mechanism of disease propagation, namely that endogenous or exogenously derived calcified NPs can deliver pathogenic factors to a diseased segment of vessel and stimulate atherogenesis. Determining when and how humans may be exposed to these particles and the inter-individual variability in response to such an exposure could signify a paradigm shift in the diagnosis and treatment of some diseases, including arterial calcification.

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