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Pathological Calcification and Replicating Calcifying-Nanoparticles: General Approach and Correlation

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ABSTRACT: Calcification, a phenomenon often regarded by pathologists little more than evidence of cell death, is becoming recognized to be important in the dynamics of a variety of diseases from which millions of beings suffer in all ages. In calcification, all that is needed for crystal formation to start is nidi (nuclei) and an environment of available dissolved components at or near saturation concentrations, along with the absence of inhibitors for crystal formation. Calcifying nanoparticles (CNP) are the first calcium phosphate mineral containing particles isolated from human blood and were detected in numerous pathologic calcification related diseases. Controversy and critical role of CNP as nidi and triggering factor in human pathologic calcification are discussed. **(*Pediatr Res* 67: 490–499, 2010)**

PATHOLOGIC CALCIFICATION RELATED DISEASES: A MAJOR HEALTH PROBLEM IN HUMANS IN ALL AGE GROUPS

The mineral phase of many kinds of hard tissue in organisms is called biologic apatite (BA). Pure hydroxyapatite (HA) has the formula $Ca_{10}(PO_4)_6(OH)_2$. BA additionally contains several other ions, mainly carbonate but also trace amounts of other anions such as HPO_4^{2-}, Cl^-, and F^-. Other cation elements are also present in minor amounts including Mg^{2+}, Na^+, and Fe^{2+}. BA is the primary mineral of normal bone and teeth (1). By definition, pathologic calcification refers to the deposition of calcium phosphates (CaP) or other calcific salts at sites, which would not normally have become mineralized. Abnormal accumulation can occur in areas of tissue damage (dystrophic calcification), in hypercalcemic or hyperparathyroid states (2). Apatite deposition is not a rare phenomenon in pediatry. Calcification is most often seen in children who had prenatal infections. It is also found in association with or as a consequence of toxic or hypoxic damage, intracranial bleed, metabolic, and hereditary diseases like mitochondrial encephalopathy, biotinidase deficiency, and in certain conditions of unknown cause (3).

Kidney and bladder stones, dental pulp stones, some gall stones, salivary gland stones, chronic calculous prostatitis, testicular microliths, calcification in hemodialysis patients, atherosclerosis, malacoplakia, scleroderma (systemic sclerosis), calcinosis cutis, calcific aortic stenosis, several malignancies, some dementias, calcific tenditis, synovitis and arthritis, diffuse interstitial skeletal hyperostosis, juvenile dermatomyositis, systemic lupus erythematosus are the most common diseases involving extraskeletal calcification (4,5). BA deposits also occur in association with inflammation in a variety of other tissues including the eye after implantation of ocular lenses (6) or the breast after breast implants (7). In each case, these deposits contribute to the morbidity and mortality of the underlying condition. Although the sizes of the mineral crystals in pathologic calcification are similar to those in bone, there is much more mineral in the deposits than there is in bone. Bone matrix proteins also accumulate associated with the deposits. The reasons for the formation of these deposits are not known. It was hypothesized that persistent inflammation is a component of BA and other HA deposition diseases. Other contributing factors are genetic, environmental, and physical chemical (5). Since inflammation has been the proposed trigger for the calcification, what is the underlying cause of the triggering inflammation?

We first consider the formation of kidney stones (KS), a major and increasing health problem. Approximately 5% of American women and 12% of men will have a KS at some time in life, at an annual cost of $2.1 billion (8). The prevalence of KS in the USA rose by 37% between 1976–1980 and 1988–1994 in both genders (9). Studies on the geographic variation in the USA in the prevalence of KS disease have shown a 50% higher prevalence in the southeast than the northwest (10), possibly associated with a changing state of dehydration related to high summertime temperatures and resulting in a low urine volume. Given the temperature rise worldwide due to the effects of global warming, it has been predicted that there could be an increase of 1.6–2.2 million lifetime cases of KS by 2050, particularly in the southeast regions of the USA (11,12). Rule *et al.* (13) have shown that KS are a risk factor for chronic kidney disease, and studies are warranted to assess screening and preventive measures for chronic kidney disease in stone formers. Urinary macromolecules in children show stronger inhibition of Ca oxalate

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Abbreviations: BA, biological apatite; **CNP,** calcifying nanoparticles; **HA,** hydroxyapatite; **KS,** kidney stone; **MV,** matrix vesicles; **NLP,** nanobacteria-like particles; **PKD,** polycystic kidney disease; **RP,** Randall's plaques; **SWL,** extracorporeal shock wave lithotripsy; **TEM,** transmission electron microscopy; **TM,** testicular microlithiasis

(CaOx) crystal growth, aggregation and adhesion than in adults. Furthermore osteopontin and calgranulin B expression is down-regulated in children due to this inhibitory effect and, thus, stone nidus formation is controlled (14). What happens in adults to make them more suseptable to KS formation? Could it be the acquisition of an infectious agent?

Another observation may be important. A strong association has been identified between the numbers of sessions of extracorporeal shock wave lithotripsy (SWL) the patient received with conversion of CaOx stones to increasing levels of CaP in recurring stones (15). The presence of CaP stones raises clinical concern, especially those stones containing brushite (calcium monohydrogen phosphate dihydrate, $CaHPO_4$), because of their increased hardness and, thus, greater resistance to communition by SWL (16). Why does SWL increase CaP KS formation? Is it possible that an infectious agent acquired over time enhances the formation of CaP rather than CaOx in recurring stones?

Another example of pathologic calcification occurs in cardiovascular disease. Surprisingly, cardiovascular disease is the leading cause of death in patients with chronic kidney disease. A growing body of data points to nontraditional risk factors, including disturbances in mineral metabolism, as important determinants of the extremely high cardiovascular morbidity and mortality rates in these patients. Disturbances in mineral metabolism, especially elevated calcium and phosphate levels, have been linked to vascular and valvular calcification, both of which are associated with poor prognosis in chronic kidney disease patients. New studies indicate that not only vascular intimal calcification (associated with atherosclerosis) but also vascular medial calcification are correlated with decreased survival in chronic kidney disease patients (17).

Calcification of cardiovascular prosthetic implants is a common and important problem. A variety of cardiovascular prostheses are affected clinically by calcification, including bioprosthetic heart valves, aortic homografts, and trileaflet polymeric valve prostheses. In addition, experimental studies have demonstrated calcification of artificial heart devices in ventricular assist systems in long-term calf studies. The pathophysiology of this disease process is incompletely understood (18). Is calcification on those devices form by the same mechanism of pathologic calcification in the soft tissue? Is the association of calcification-releated cardiovascular disease and KS formation closely related and perhaps caused by the same agent acquired like a bacterium or virus?

NUCLEATION AND GROWTH OF CaP IN THE HUMAN BODY

Pathologic calcification is a complicated, actively regulated process of mineralization that is similar to bone formation and remodeling (19,20). Mineralogists explain that all that is needed for crystal formation to start is nidi (nucleus) and an environment of available dissolved components at or near saturation concentrations, along with the absence of inhibitors for crystal formation (21). Bacteria or other agents producing such nidi, if present in blood and in urine, are very likely candidates to launch and accelerate pathologic calcification *in vivo* (22,23). This is clinically important since blood contains phosphate near its saturation level (24).

Matrix vesicles (MV), which are membranous structures derived from the surfaces of hypertrophic chondrocytes, are thought to initiate calcification at the mineralization front by focally concentrating calcium within their already phosphate-rich structures (25). These vesicles have high levels of alkalinephosphatase and have the ability to concentrate calcium and increase phosphorus during mineralization. Electron microscope studies have indicated that biologic calcification occurs in the MVs in two steps, the first related to the initial deposition of HA within the lumen of the MVs and the second to the propagation of mineral outside the vesicles (26).

Acidic phospholipids are present at high levels in cells at the mineralizing front in bone and in pathologic calcifications (27). Detergent pretreatment of bioprosthetic heart valves, shown to inhibit mineralization, likely acts by the detergent-mediated extraction of phospholipids and other proteolipids, which takes place under these conditions (28).

THE PREVIOUSLY UNSUSPECTED ROLE OF NANOPARTICLES

Human exposure to nanoparticles is inevitable as nanoparticles become more widely used and, as a result, nanotoxicology research is now gaining attention. Although the number of nanoparticle types and applications continues to increase, studies to characterize their effects after exposure and to address their potential toxicity are few in comparison. In the medical field in particular, nanoparticles are being used in diagnostic and therapeutic tools to better understand, detect, and treat human diseases (29). When size-dependent cytotoxicity is studied, it has been shown that the smaller the particle is more cytotoxic it gets (30), and they may act as a nidus for pathologic calcification.

Apatite is generally not toxic to human cells. However, when apatite is present somewhere other than in bones and teeth, it may cause adverse effects (31–33). Other studies have concluded that *in vivo* apatite applications are generally safe (34–37). Although these disagreements have not been completely resolved, both BA and non-BA materials have been continuously used in drug delivery and transplantation (38–41). What has not been well studied, however, is the cytotoxcity of nanophase apatite and related particles.

REPLICATING, CALCIFYING NANOPARTICLES, AND THEIR RELATION TO PATHOLOGIC CALCIFICATION

Most pathologic calcifications throughout the body contain mixtures of carbonate-substituted HA and octacalcium phosphate. According to the Merck Manuel, these ultramicroscopic crystals occur in snowball-like clumps. Those clumps can cause severe inflammation (42). Structures similar to these nanometer-sized snowballs were discovered almost two decades ago in blood and blood products (43). These structures, called calcifying nanoparticles (CNP), were detected in numerous pathologic calcification related diseases (44–47).

CNP are the first CaP mineral containing particles isolated from human blood.

CNP have the following unusual properties: bacteria-like (48), pleomorphic (49), infectious (50), self replicating (51), capable of passing sterilization filters because of their small size (80–500 nm) (52), resistance to heat and γ irradiation at doses typically fatal for conventional bacteria (53), capable of forming a calcific coating at physiologic pH and mineral concentrations (49), lacking inherent DNA (54), sensitive to certain antibiotics (55), and capable of causing the formation of lipid compounds (un published data).

CNP have now been linked to pathologic calcification related diseases such as arteriosclerosis (44–46), KS (47,56–59), gall stone (60,61), dental pulp stone formation (62–64), prostatitis (65–67), Alzheimer's (Kajander *et al.* Do Autonomously replicating sterile-filterable particles have an association with amyloid accumulation? Viruses and virus-like agents in disease. Second Karger Symposium, March 7–9, 1993, Basel, Switzerland, Abstract 41), polycystic kidney (PKD) (68,69), and cancer (70,71). CNP exert cytotoxic effects on some mammalian cells *in vitro* (72) and on living organisms *in vivo* (73).

The incidence of KS formation and recurrences may continue to rise every year. Thus, new approaches in treatment and prevention could have a huge economic effect apart from benefits in terms of reduced morbidity. Over seventy years ago, Randall examined the papillae of cadaveric renal units and demonstrated that interstitial crystal plaques in the papillary tip were common in stone formers (74). These crystals were composed not of CaOx, the most common solid phase found in patients with nephrolithiasis, but of CaP. He believed that the CaP crystals serve as a nucleation surface for CaOx. Recently, Matlaga *et al.* (75) analyzed Randall's plaques (RP) and confirmed that they are formed of spherical CaP deposits with a multilaminated internal morphology. With the CNP detection methodologies we developed (59), important features of CNP and their triggering effect on nephrolithiasis have been suggested (47,59). In our study, there was evidence of a link between detection of CNP and presence of RP. Although causality was not demonstrated, the results we obtained suggest that further studies with negative control samples should be made to explore the etiology of RP formation, thus leading to a better understanding of the pathogenesis of stone formation. Additionally, this hypothesis may bring an explanation to the reason of the increase on CaP KS formation after SWL.

Another urinary disease, PKD, is the most common autosomal dominant lethal disease in humans. Interestingly, a higher prevalence of kidney calcifications is observed in PKD than in the normal population (76). It has been proposed that the currently known cellular toxicities, tissue distribution, and pharmacology of CNP are plausibly related to the known pathology and pharmacology of PKD (68). Hjelle *et al.* (68) evaluated 13 PKD cyst fluids and detected CNP antigen positivity in each sample as well as in liver cystic fluid from affected individuals. This may explain the reason of high rate calcification problem in patients with PKD.

The presence of prostatic calculi in younger men is associated with both inflammation and symptoms of chronic pelvic pain syndrome (77). Biopsies, urine, and prostatic secretion cultures fail to demonstrate bacterial pathogens, however inflammation is often detected in chronic prostatitis (78). The core of prostatic calculi is typically CaP (79), which is the hallmark of CNP action. Wood and Shoskes (66) proposed that there is a potential role of CNP in chronic prostatitis. Recent clinical research targeting these agents has proven effective in treating some patients with refractory category III prostatitis (chronic pelvic pain syndrome) (67). In that research, CNP antigen or antibody was found in 60% of serum and 40% of urine samples. In 10 patients who underwent transrectal ultrasound after therapy, prostatic stones were decreased in size or resolved in 50% (67).

Testicular microlithiasis (TM) is an uncommon pathologic condition of unclear etiology, which is characterized by calcium deposits within the seminiferous tubules. Zhang *et al.* has studied infertility patients with and without TM. Their results have showed that CNP may be linked to the development of TM, which may provide a potential target for the diagnosis and treatment of infertility with TM (80).

Mechanisms mediating vascular calcification remain incompletely understood. Some have hypothesized the potential role of CNP in arterial calcification (44,45). Miller *et al.* cultured nano-sized particles from calcified but not from noncalcified aneurysms. These particles were stained with CNP-specific MAb, recognized by a DNA-specific dye and incorporated radiolabeled uridine, and, after decalcification, they appeared via electron microscopy to contain cell walls (45). Puskas *et al.* propagated CNP- like spherical particles from 26 of 42 sclerotic aorta and carotid samples and confirmed their nature by dot immunoblot by using CNP-specific MAb, light microscopy, and transmission electronmicroscopy (TEM). [3H]L-aspartic acid was incorporated into high molecular weight compounds of demineralized particles. PCR amplification of 16S rDNA sequences from the particles was unsuccessful using traditional protocols (44). Identification of CNP-like particles at the lesion supports but does not by itself prove the hypothesis that these agents contribute to the pathogenesis of atherosclerosis, especially vascular calcifications. Specific therapies targeting these particles has demonstrated reduced plaque formation, regression of plaques, and improved lipid profiles (81). The potential of anti-CNP treatments are controversial and await larger clinical trials. Epidemiologic studies have implicated antibodies made by the body against CNP to be a strong independent risk factor for coronary artery calcification. Also Hu *et al.* (82) has cultured CNP-like material from calcified cardiac valves with rheumatic heart disease.

Apatite deposition is not a rare phenomena in pediatry. In an infant with idiopathic arterial calcification of infancy, prenatal diagnosis of arterial calcification was made by ultrasonography and allowed initiation of therapy *in utero*. Etidronate therapy produced apparent radiographic and ultrasonographic improvement in the degree of vascular calcification (83). Etidronate is a compound, which inhibits CNP replication (55).

Originally, Sedivy and Battistutti (84) reported that CNP promoted crystallization of psammoma bodies in ovarian can-

cer. Hudelist *et al.* soon verified the 100% concordance between the expression of CNP and the presence of psammoma bodies in malignant ovarian tumors. In their research, several lines of evidence suggest the involvement of these organisms in the process of biomineralization. Therefore they have concluded that CNP infection of malignant ovarian tissue contributes to mechanisms leading to the formation of calcified deposits known as psammoma bodies (70).

In addition, CNP have been shown to be detected at higher rates in serum of patients with gallstone disease (60), and mitral valve calcification (85), dental calculus, and periodontitis (46,62,63). Others have suggested that CNP may contribute to the development of peripheral neutropathy in HIV-positive patients (86,87) and even osteoporosis (88). All these hypothetical approaches require further investigation.

DETECTION METHODS FOR CNP AND THEIR *IN VITRO* EFFECT ON MAMMALIAN CELL CULTURES

Methods to diagnose CNP in biologicals, cells, tissues, blood, and urine include immunodetection with CNP-specific MAb, electron microscopy, and culture techniques (89). Because CNP pass through 0.22-μm pore size filters, which exclude most common microbes, filtration is often used to clean up fluid specimens before culture for CNP (89). Replication can be measured by particle counting and OD at 650 nm (55). It has been also shown that growth of the CNP could be detected by specific methods, such as ELISA and turbidity (43).

Calcified and noncalcified forms of CNP have been observed as free, cell-attached, and internalized particles in mammalian cell cultures *in vitro* (43,72). In our experiments, we have chosen six different fibroblast lines as experimental models, because they are the most ubiquitous cells in the animal body and might be most accessible in wound tissue for invading pathogens with the exception of professional phagocytic leukocytes (72). We have shown that CNP were bound as clusters on the cell surfaces within 15 min. It is concluded that CNP are internalized either by receptor-mediated endocytosis or by a closely related pathway within 12 h (72). We showed that cytotoxicity was dependent on CNP concentration and exposure time. Dying cells always contained numerous ingested CNP. Hybridomas and many lymphocytes were found to be affected by CNP, but considerably higher doses were needed (72).

IN VIVO EFFECTS OF CNP (ANIMAL EXPERIMENTS)

Åkerman *et al.* reported that radiolabeled (^{99m}Tc) viable CNP accumulated in the kidney and appeared in urine after 15 min of their i.v. injection into rabbits. This could be due to the fact that kidneys are the preferred sites for this agent, unlike other known nanoparticles, and the presence of injured epithelium or a nucleus in the kidney/urinary tract provides a preferable niche for CNP to adhere and grow, resulting in biocrystallization (73). A control study in rabbit was performed administering a similar dose of ^{99m}Tc-labeled albumin cross-linked particles or tin-technetium nanoparticles as nanocolloids. Nanocolloids were not targeted to kidneys as CNP were (72). Shiekh *et al.* too observed that CNP, when injected i.v. into rats, were localized in kidneys. In their research they observed regions of chronic inflammation infiltrated in the cortex and medulla, which could be due to damage induced by CNP. The research team has also shown CNP adhering to the surface of the epithelium and their penetration into the epithelial cells (58).

In a small study, Garcia Cuerpo *et al.* (90) found that translumbar, percutaneous intrarenal injection of CNP (isolated from KS) into rats resulted in KS formation. In addition, Shiekh *et al.* has examined CNP's role in biocrystallization and *in vivo* effects on kidney pathology. CaOx monohydrate assay was carried out in the presence of CNP to study biocrystallization. Wistar rats were given an i.v. injection of CNP and the kidneys were examined for pathologic changes. The assay showed accelerated biocrystallization of CaOx in the presence of CNP, indicating them to be efficient candidates for biomineralization. Histopathological studies revealed bacteria induced renal tubular calcifications and various manifestations of infection (58). Their studies confirm that CNP may be involved in the pathogenesis of renal tubular calcification. Such findings are required to prove Koch's postulates linking CNP to other pathologic calcification related diseases.

Schwartz *et al.* designed an experiment to test the hypothesis that the systemic delivery of planktonic forms of CNP derived from calcified, diseased human tissue, or bovine blood are transmissible particles that exacerbate arterial response to injury. New Zealand White rabbits in which the endothelium was mechanically removed from one carotid artery were injected i.v. with either saline (control), lipopolysaccharide, HA crystals. HA crystals exposed to culture media, or planktonic forms of bovine- or human-derived CNP. They have shown that the systemic administration of planktonic forms of human-derived CNP exacerbated arterial response to injury distinct from that of bovine-derived CNP and other inflammatory agents (91).

BIOLOGIC CLASSIFICATION OF CNP

Despite their potential role in major medical health problems, CNP have not been classified in any taxonomic group due to limited information on their biologic characteristics. Although the biologic characterization of CNP is yet to be fully understood, the precipitation and growth of CaP readily occurs in systems containing trace amounts of CNP, but not in identical control systems lacking CNP (51,54).

Chemical analysis using energy-dispersive x-ray microanalysis (EDX) of these mineral layers shows Ca and P peaks (43). The effectiveness of CNP biomineralization is remarkable: apatite formation *in vitro* stopped only when the calcium level decreased by 50% from 1.8 to 0.9 mM, and the phosphate levels fell to near zero (47). Our results indicate that the CNP CaP phase can be formed at pH 7.4 consistent with human physiologic phosphate and calcium concentrations. This can be also monitored by ^{85}Sr incorporation and provides a unique model for *in vitro* studies on calcification (43,47,92).

EVIDENCE FOR SELF-REPLICATION OF CNP

To enhance our understanding of the self-replicating nature of CNP, we investigated their growth using inverted light microscope and BioStation IM time-lapse imaging system (51). The results are in conjunction with previous findings of metabolic activity (54) antibiotic sensitivity (55), antibody specificity (72), morphologic aspects, and infectivity (49,50), all concomitantly validate CNP as live-like self-replicators (51). We found a systematic increase in both the cell-like nanoparticles and the apatite coatings and apatite "igloos" (Fig. 1*A*). The preferred formation of igloos over apatite-coated cell-like particles occurred when the serum or protein in the nutrient solution was depleted. With sufficient protein, the igloos did not readily form but the cell-like nanoparticles (Fig. 1*B*) rapidly increased along a flattening growth curve (51). Although the mechanism for the formation of both the cell-like nanoparticles and the apatite crystals and igloos may be entirely nonbiologic, it mimics in many ways the life cycle of typical bacteria. As long as serum proteins, calcium, and phorphorous are present, new cell-like nanoparticles continue to form and apatite continues to precipitate as coatings on these nanoparticles. Igloos continue to form in serum protein depleted media. Controls lacking an initial charge of CNP do not form either the cell-like nanoparticles or the apatite precipitates, even under identical conditions of chemistry and environmental conditions (43,51). Controls salted with inorganic apatite dust particles or albumin addition also do not produce the formation and growth of either the CNP or new apatite precipitates of any type. The "life cycle" of CNP is more complex than previously realized, and includes the formation of pleomorphic cell-like nanoparticles, their acquisition of precipitated apatite coatings built up from extremely small particles of apatite, the formation under a depleted protein environment of much larger apatite structures or igloos, the production of new cell-like nanoparticles within the igloos, the release of these nanoparticles, and a repeat of this cycle (see Fig. 1*A*). In the case of abundant protein in the media, the igloo phase may be bypassed and the cell-like nanoparticles may be reproduced by budding or by fission (51). This "life cycle" is reproducible, relatively invariant, and predictable and has been observed over more than three decades in "cultures" inoculated with a small amount of initial CNP identical to those on file with the German cell bank (DSM no. 5819–5821; Braunschweig), although some of the details are still being elucidated.

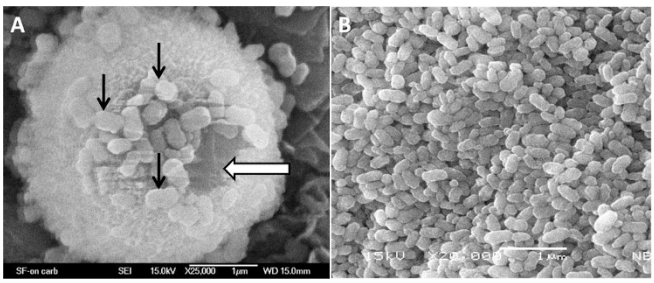

Figure 1. Scanning electron microscopic image of CNP cultured in serum-free condition (*A*), and in serum containing condition (*B*). On the left (*A*), an "igloo" is seen. White arrow shows the igloo opening, and black arrows show the small CNP released from the igloo. On the *B,* coccobacillar shaped small CNP "cells" are seen. Bars, 1 μm.

CNP BIOFILM

CNP also produce a slime-like biofilm, which may contribute to the "stickiness" of the CNP complex (cell-like vesicles and associated apatite precipitates) in human tissue, circulatory systems, the obstruction of blood flow in arteries (43,45), and on catheter or stent-like artificial prosthesis (unpublished data). This biofilms links together the cell-like nanoparticles and provides anchoring to fixed structures such as arterial walls or kidney tissue. Detailed TEM imaging shows that this biofilms is usually present along with the cell-like vesicles and the precipating apatite (55). The formation of this induced apatitic biofilm formation is dependent on the presence of oxygen (43,93). Additional studies have shown that the formation and growth of this biofilm can be prevented with several antibiotics and antimetabolites, and by high gamma irradiation at sterilizing doses (55,90). Schwartz *et al.* (94) has shown that CNP biofilm retains some characteristics of conventional bacterial biofilm and requires protein-calcium interactions, although extracellular RNA is not required.

FORMATION OF APATITE

Previous studies have shown that CNP form two distinct forms of apatite. The early-formed apatite consists of extremely small (10's of nanometer) flakes, plates, and crystals, which coat the cell-like organic nanoparticles. Under conditions of serum or protein depletion, these apatite crystals coalesce to form much larger structures (1–10 μm) which are usually concentrically zoned in texture (igloos) (51). These larger structures often contain a central cavity which typically contain multiple cell-like nanoparticles. In some cases, the igloo is partially open allow the egress of these cell-like particles which can then repeat the cycle (43,51). These subspherical units or igloos were identified in most human KS examined (47,95,96). Fourier Transform Infrared Spectroscopy of CNP revealed the mineral as almost identical to bone and apatite KS mineral (54).

In one interesting study, we examined CNP cultures in High Aspect Rotating Vessels designed at the National Aeronautics and Space Administration's Johnson Space Center, which are designed to simulate some aspects of microgravity (97). CNP cultured in that system multiplied 4.6 times faster than under stationary conditions and 3.2 times faster than in shaker flask incubation. Interestingly, the results demonstrated that the degree of apatite crystal formation on the cell-like nanoparticles, and the properties of the apatite are strongly affected by the gravity and other specific culture conditions used (97). Although some researchers believe that microgravity does not affect crystal formation and biomineralization (98), it has been shown that long periods in a microgravity environment does cause loss of bone, and enhance KS formation-like biomineralization disorders in astronauts (99–102). Our data from the experiments would support the hypothesis that reduced gravity may enhance the development of KS by enhancing the growh of CNP (103).

THE CONTROVERSY

CNP were originally called "nanobacteria" because they behaved like known bacteria yet were very small (43). That terminology prompted a search for nucleic acid. (RNA or DNA) (104–107). DNA fragments identified early subsequently were shown to be contamination or DNA absorbed by the particles from their environment (107). As of the present time, no undisputed DNA has been associated with these particles. The original term nanobacteria was therefore dropped by our group as misleading and controversial. We have subsequently used the terms CNP. As we have learned more about their life cycle, we now realize that they consist of a membrane-enclosed vesicle or cell-like particle which has the ability to cause or catalyze precipitation of extremely small HA particles or crystals on the membrane surfaces, and to combine the precipitated apatite into more complex layered concentric structures, which we call igloos because they are often hollow containing newly formed membrane-enclosed vesicles (51). In one version of the life cycle, the hollow igloo is breached and releases the cell-like nanoparticles, which continue to reproduce and precipitate new apatite. In another version of the life cycle, the cell-like nanoparticles precipate fresh apatite on their membrane surfaces but do not form igloos. In both versions, a biofilm may link the nanoparticles together, link them to the igloo, or form an anchor against fluid movement and dispersion. The term CNP refers only to the membrane-enclosed cell-like particle. The associated apatite (both membrane coatings and igloos) and biofilms are separate, but the whole association is termed by us the CNP complex. No previous definition made clear that the membrane-enclosed cell-like particles are not apatite but can exist alone, but are closely associated with both a precipitated apatite coating and with a much larger apatite igloo. Previous definition did not always include the biofilms as separate and distinct part of the complex.

The very small size (50–200 nm) raises the critical question of whether these particles can contain sufficient DNA to operate as a true replicating and metabolizing cell. Maniloff's work suggests that to contain the DNA and proteins needed to function, a cell must be at least 140 nm across (108). However, recently it has been shown that a genome constructed to encode 387 protein coding and 43 structural RNA genes could sustain a viable synthetic cell, a *Mycoplasma laboratorium,* which can shrink its size below that limitation (109). CNP are also incredibly resistant to heat and other methods that would normally kill bacteria, which makes some scientists wonder if they might be an unusual form of crystal rather than organisms (53). Cisar *et al.* (107) and Martel *et al.* (105) presented alternative theories for the experimental findings of earlier CNP studies. They stated that biomineralization previously attributed to CNP may be initiated by nonliving macromolecules and transferred on "subculture" by self-propagating microcrystalline apatite. We are unaware of any report showing the nucleation and growth of inorganic apatite under physiologic conditions. In addition, for inorganic crystallization to continue over prolonged periods of time, conditions of nonequilibrium must exist and be maintained.

Martel *et al.* (105) have investigated a purported similar material which they termed nanobacteria-like particles (NLP) in their research. It is not clear what the relationship of these NLP is to the original culture deposited by us in the German cell bank, so it is not possible to directly compare results. Cisar *et al.* also cultured similar particles from serum and human saliva sources and named them nanobacteria with no test comparing them to the original material deposited by us in the cell bank (107). His team verified our findings, and also confirmed the extreme difficulties in performing PCR, but finally suggested his opinion that the culturable particles cannot be bacteria, since they were too small, were not inhibited with a respiratory poison, nucleic acids could not be detected with standard procedures and their protein patterns revealed only few proteins, much less than one would expect from a common bacterium. CNP could not be lysed with lysozyme, proteinase K, several other proteinases, lipases, amylases, alkali, ultrasound, X-press, detergents or solvents (93). In our experiments we needed to use acid or EDTA-like chelators before the analysis, which were the factors for structural change in nucleic acid. It has been our experience that CNP actually inhibit the amplification of added exogeneous classical bacterial DNA by polymerase chain reaction methods. We currently conclude that no convincing evidence exists for the presence of inherent nucleic acid within CNP. PCR analysis using universal primers practically impossible and worthless.

Cisar *et al.* has not sequenced any proteins. They did not do any DNA work besides staining with Hoechst 33258, where they got the same weakly positive result than we did. Contrary to Dr. Cranton's claims (110), Cisar *et al.* did not do a PCR phylogenetic analysis using 16S rRNA sequences (107).

As pointed out by Dr. Cranton, apatite can be formed under super-saturating concentrations of calcium and phosphate *via* several mechanisms (110). To our knowledge, CNP-mediated calcification is the only mechanism to make apatite at nonsaturating levels of calcium and phosphate. Cisar *et al.* did not follow saturation degree analysis in his studies although saliva is known to be highly supersaturated with calcium and phosphate.

In the study by Martel *et al*., electron microscopic images from so called NLP morphologically look like the serum protein precipitation formed in long-term serum cultures we have described earlier (89) and are not comparable with our earlier findings (Figs. 2 and 3). Therefore, we find their entire report not comparable with our findings, and the immunostaining tests that they have pursued in their research cannot be comparable with the results we and other laboratories obtained in earlier years. As they have claimed, if the MAb raised for CNP would cross react with a major component of serum, in every experiment containing serum component, the results would be positive. We would recommend using negative controls with no primary antibody and nonspecific MAb in every immunostaining experiments to eliminate false-positive possibilities. Vali *et al.* (111) have also shown that nanoforms contain apatite-protein complexes and immuno electron microscopy reveals protein antigens in proximity to apatite suggesting a novel form of protein-associated mineralization.

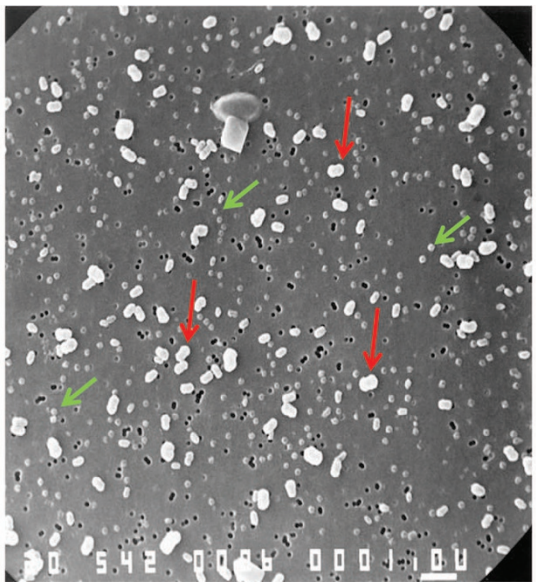

Figure 2. Scanning electron microscopic image of a filter (with 100 nm pore size) having CNP (coccobacillar, shown with red arrows) and protein precipitation (smaller and spherical, shown with green arrows) on it. Bar is 1 μm.

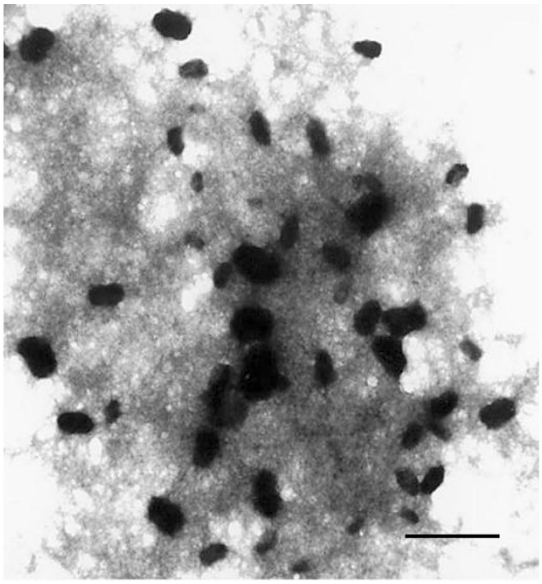

Figure 3. Transmission electron microscopic image (negative staining) of CNP and protein precipitation mixture. CNP is seen as electron dense dark particles. Protein precipitation looks like a gray cloud. Bar is 500 μm.

The claims that CNP must be a nonbiogenic (albumin, fetuin-A, and apolipoprotein A1) crystallization still cannot explain the biogenic-like properties of CNP. Some examples of those properties are

1. CNP cannot be cultured from every serum sample although each serum contains albumin, fetuin, and apolipopretein.
2. TEM examination of CNP cultures have always shown a close association of apatite with submicrometer vesicles (cell-like bodies) enclosed within membraneous structures (51). We and others have always found the presence of these membranous vesicles remaining after the apatite has been chelated and dissolved away with acid or EDTA (51,112,113). Nonbiogenic apatite has larger crystals that are easily dissolved in acidic solution but CNP dissolves very slowly (43).
3. When inorganic apatite is added to culture medium (DMEM), it dissolves but CNP introduced to identical media begins and continues to self propagate (51).
4. CNP can be cultured in serum-free conditions and be passaged for years with no serum addition (43,51). Therefore, CNP cannot be simple serum protein-derived vesicles with associated crystal formation.
5. Inorganic apatite crystals do not stimulate immune response and cause pathologic calcification as CNP do (50,112,114).
6. CNP has specific staining properties (72,113). Other components of the serum or growth media do not stain using the same stain.
7. CNP are cytotoxic and they have a specific route *in vivo* (72,73). Serum proteins do not have these properties. Serum protein coated inorganic apatite crystals have been cultured and injected to animals but their route in animals was different (73,112,114).
8. CNP have and release endotoxin (72) and thereby stimulate and mediate chronic local inflammatory reactions in atherosclerotic plaque (44). Other suggested explanations (albumin, fetuin-A, and apolipoprotein A1, inorganically precipitated apatite particles) lack this property.
9. CNP growth could be prevented with tetracycline, high doses of aminoglycoside antibiotics, EDTA, cytosine arabinoside, 5-fluorouracil, and gamma-irradiation (55).
10. CNP structures have been isolated from many diseased tissues and when found, are always similar to other structures from other unrelated patients (44,47,59,60).
11. The metabolic potential of CNP was confirmed using a tetrazolium salt detecting dehydrogenase activity (113), and S-methionine incorporation (54). Also, β-mercaptoethanol, known to enhance growth of certain microorganisms and mammalian cells, promoted CNP metabolism and growth (113).
12. Polarized light was shown to reduce CNP biofilm formation indicating a light induced metabolic process within the CNP complex (115). Apparently, CNP have metabolic activity, which clearly differentiates them from inorganic crystal formation. Alternatively, the behavior of these nanoparticles closely mimics metabolic activity using a mechanism not understood despite decades of research.

ARE CNP LIVE OR DEAD?

We summarized the properties of CNP that resemble the characteristics of living bacteria and also point out the differences that set them aside as a different and distinct type of small cell-like objects. We raise the question of whether they should be considered living entities. We conclude that CNPs do not fit the typical definition of life, but we suggest that perhaps the typical definition is too restrictive, and a broader definition is required, which encompasses the properties of CNPs.

One property of living systems is their ability to pass on to subsequent generations some information that restricts the new structure and properties to those of the earlier generation. Mutations are allowed, but basic properties should be passed on to subsequent generations. Bullard *et al.* put to test the "crystals-as-genes" hypothesis. For crystals to resemble genes, there must be more inheritance than mutation in successive generations. However, despite the greatest of care taken to not expose the fresh crystal seeds to atmosphere, and even in the absence of cleavage, new hillocks, "mutations" proliferated so that the detailed structure and morphology of the new crystals differed considerably from the original structure (116). An antibody analog that recognized external structural morphology of the parent crystals would not recognize morphology of the subsequent crystals. By contrast, for more than a decade, CNP have been cultured and passaged under physiologic conditions similar to mammalian culture conditions, without any change in their growth characteristics and specific MAb recognizing epitopes (43,51), a property analogous to the morphology of the crystal surfaces of Bullard *et al.*

There is truly no universally agreed definition of life. Although the theme of DNA has permeated so deeply in the scientific world, lately, there have also been some contrary publications questioning the concept of the gene as the unit of life (117,118). The theory that life could have started with very simple heterotrophic primordial cells is currently gaining recognition (119). Such could be the case with CNP. These particles consist of an organic membrane surrounding a vesicle. This membrane seems to have the ability to induce apatite precipitation and the formation of complex external structures embodied by the concentric layered larger precipitates including the igloos. The membrane-encased cell-like nanoparticles seem to have the ability to self-replicate while maintaining a complex set of characteristics which are passed on to subsequent generations (51). One hypothesis for the origin of life is that it started with a simple membrane-enclosed vesicle, which allowed or promoted simple chemical reactions across the or through a membrane so that the interior chemistry was somewhat different from the exterior chemistry (119). The membrane may also have had catalytic properties so that it promoted chemical reactions not normally found in its surrounding environment. At some point, the membrane-enclosed vesicle split or budded and a new vesicle was formed. If one of the chemical reactions induced at the membrane surface consisted of precipitation of a salt such as CaP, the system might resemble what we now call CNP. Continuation of this process would lead to growth, increase in complexity, and eventually to the formation of simple DNA. If life began in such a system, the early cell-like version would lack DNA, but would still be considered a living system. Perhaps the CNP can be considered a modern-day analog of such a primitive system, and could be considered a primitive form of life lacking nucleic acid, but still possessing self-replication, membrane-induced chemical reactions, the ability to maintain distinctive characteristics over multiple generations, and the ability to exist and grow over a range of environmental conditions. It is evident that CNP involved in pathologic biomineralization with their distinctive characteristics defy the old standard scientific expectations and definitions of life. Perhaps, an expanded definition of life is appropriate.

CONCLUSIONS

CNP and their associated apatite precipitates and biofilms make up an extremely interesting complex found in many humans. In some ways, it might be considered to be an analog of a possible hypothetical early primitive life form, which developed and reproduced without the benefit of DNA. However, naming an agent as nanoparticles or nanobacteria, living or nonliving but self-replicating, has relatively little meaning with respect to causing disease. The fundamental importance is that these self-replicating special particles that we call CNP are found in blood and in pathogenic calcification and their properties of promoting ready crystallization and growth of Ca minerals are well established. These self-replicating particles may induce calcification and stone formation *in vivo* because: CNP a) have been detected in human blood, b) are transported from blood into urine and bile as living organisms, c) are renotropic, d) cause apoptotic cell death, e) are present in human stone-isolates, and tissues with calcification, and f) clearly cause KS formation in rats within one month when injected in an intrarenal route. CNP may be the potential reason of poorly understood inflammation that is detected in many pathologic calcification related diseases.

Because CNP have been detected in blood and "sterile" blood products, they should be of interest to the biopharmaceutical industry. For example, CNP have been isolated and cultured from the cultured nasopharyngeal carcinoma epithelia HNE1 cell supernatant (120). Safety of vaccines produced in cell culture by using (bovine) serum or serum-derived materials in culture of the cells, sterilized by filtration is an issue needing thorough risk analysis, and method validation (121).

Only continued research will reveal the nature of CNP and their impact on health and disease. CNP are a good model system to use in developing drugs to alter the likely diverse pathways involved in tissue calcification. The evidence that CNP exist in the human body and are closely associated with many kinds of disease is now overwhelming. Although interesting, future investigations of CNP should not be side tracked by controversies over whether they are alive or not; their potentially very detrimental effect on human health should be the focus of future effort.

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