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Nanobacteria-Like Particles in Human Arthritic Synovial Fluids

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We investigated the existence of nanosize particles in synovial fluids of rheumatoid arthritis and osteoarthritis patients. These specimens were cultured under mammalian cell culture conditions (37 °C; 5% CO_2/95% air) for a long period. After about 2 months, many nanoparticles appeared and they gradually increased in number and in size. The nanobacteria-like particles exist in synovial fluids of arthritis patients. The possibility of their existence and pathogenesis in various diseases should be verified cautiously.

Keywords: nanobacteria • arthritis • synovial fluid • rheumatoid arthritis • osteoarthritis • microorganisms

Introduction

Despite many diagnostic and therapeutic developments, numerous patients all over the world suffer from diverse, severe joint diseases. It has been proposed that rheumatoid arthritis (RA) might be related to microorganism infection, and some antibiotics are suggested to be effective for these patients. Osteoarthritis (OA) is caused by cartilage degeneration and/or biomechanical disorders; however, these explanations cannot account for all of the etiologies of these conditions. Kajander and Çiftçioglu made the discovery of nanosize microorganisms, termed nanobacteria, in human blood and commercial fetal bovine serum.[1,2] They found that these biosystems were small enough to pass through 0.22-μm-pore filters, and they proliferated slowly with a generation time of about 3 days. Initial size, shape, and doubling time indicate that they are nanobacteria (NB), apatite forming biosystems implicated in various pathogenic conditions. NB have been found in urinary stones,[3,4] atherosclerotic plaques,[5,6] psammoma bodies in ovarian tumors,[7] and in bile and gallbladder mucosa of patients with cholecystolithiasis.[8] However, some scientists object to the existence of these NB.[9−11] They advocate that the DNA sequence of NB reported by Kajander et al. was indistinguishable from those of environmental microorganisms. However, currently, no conclusion has been reached.

In this study, we preserved synovial fluids under mammalian cell culture conditions from knee joints of patients. These fluids were filtered through 0.22-μm-pore sterile filters before culture. Our results show that small particles gradually appeared in these cultured products. This is the first report to indicate that human synovial fluids contain NB-like particles.

Materials and Methods

Synovial fluids were obtained from the knee joints of 6 Japanese patients after informed consent was obtained. Four of these patients suffered form RA and 2 had OA. All patients were female, and their age range was 53 to 82 (average 65.3) years old. These fluids were immediately centrifuged at 3000 rpm/min for 30 min at room temperature, and the cell-free supernatants were filtered through 0.22 μm-pore-size sterile Millex filters (Millipore, Massachusetts, USA). Then, 1 mL of synovial fluid with 9 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Maryland, USA) was infused into OptiCell (Biocrystal, Ohio, USA). By using this compact cell culture device, OptiCell, long-term aseptic cultures and frequent observations were possible. These 6 samples in OptiCells were kept in the incubator under mammalian cell culture conditions (37 °C; 5% CO2/95% air) as described by Kajander et al.[1] When kept in OptiCells, these synovial fluids were easily observed with high-powered digital optical microscope (Keyence, Osaka, Japan) without any fixation procedure. The medium in OptiCells tended to evaporate very slowly, so additional DMEM was added by monitoring the total weight.

Results

After about 2 months of culture, nanoparticles appeared in the synovial fluids from all the patients to greater or lesser degrees. These nanoparticles gradually increased in number and in size, and macroscopically they made the medium cloudy. The films of OptiCells became unevenly obscured and appeared to form a geographic pattern (Figure 1). Microscopically, these particles were distributed unevenly on the surfaces, therefore their density probably varied considerably from place to place. Typically, the particles had a spherical or hemispherical and varied in sizes of less than 1 or 2 μm, and some of them made clusters, but others existed independently (Figures 2 and 3). In some cases, these particles adhered to the inner surfaces of OptiCells, and in other cases, they were located on the inner surfaces and appeared to make small movements, which may be Brownian movement. Partially, a large number of particles clustered forming groups with a diameter larger than 10 μm (Figure 4).

Discussion

The etiologies of various joint diseases remain largely unexplained. Mechanical stimulation of microparticles and

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Figure 1. Right side; the OptiCell with synovial fluid from the RA patient. Synovial fluid was cultured with DMEM for about 22 months, and microparticles increased in size and caused the films to become obscured unevenly and resulted in a geographic pattern. Left side; the control OptiCell, which was filled with only DMEM, and not cultivated.

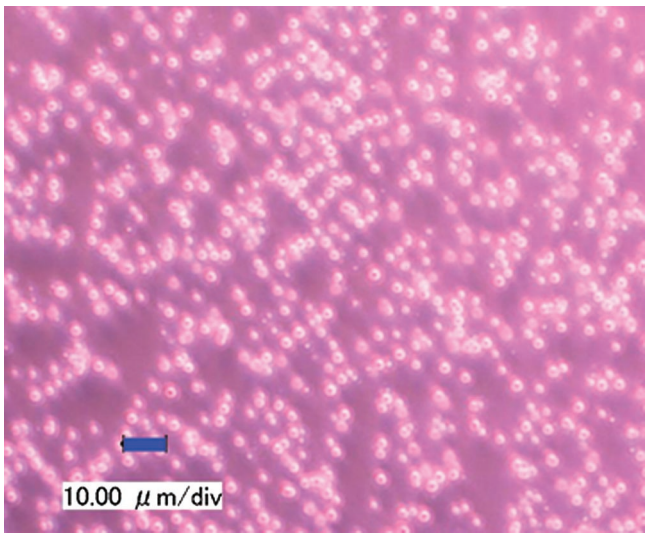

Figure 2. Microscopic photograph of microparticles of 10% synovial fluid from patients with RA after about 18 months of culture. Particles adhered to the inner surface of OptiCell. Blue bar = 10 μm. Original magnification, ×1000.

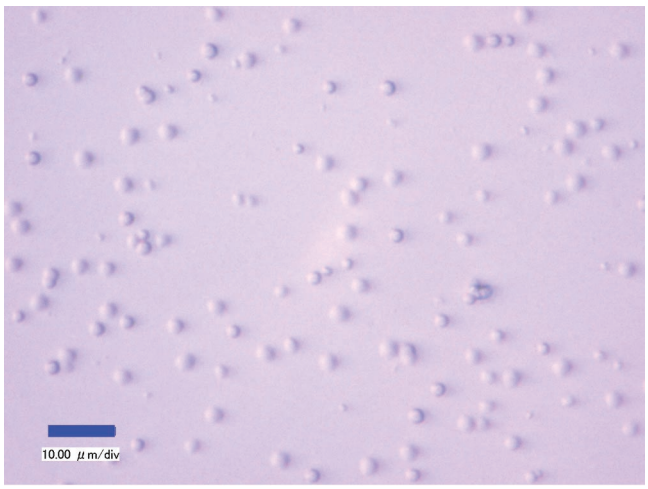

Figure 3. Microparticles from the same patient as in Figure 2 (after 22 months of culture). The size of these microparticles appeared to be less than about 2 μm. They were sphere or hemisphere and varied in size. Blue bar = 10 μm. Original magnification, ×3000.

crystals or immunological reaction could be one of the causes of these joint disorders. If self-proliferating nanoparticles exist in mammalian synovial fluids and membranes, then they may have an effect on many joint diseases. In our experiment, synovial fluids of RA and OA patients contained nanoparticles measuring less than 0.22 μm, and these particles gradually grew in size and in number with culture in vitro. Some of them were observed to move, perhaps by Brownian movement, and some of them made large clusters. We propose that these proliferating microparticles from human synovial fluids in our laboratory could be nanosize microorganisms. With quantification by means of Hechst 33258, the fluorescence intensities of all the cultured synovial fluids with DMEM were shown to increase significantly. However, PCR on DNA with 16S rRNA gene primers failed to produce any amplicons. For further investigation, we are planning a large study with synovial fluids from more patients with OA or RA and from younger patients of post-traumatic arthritis as control groups.

NB are some of the smallest cell-wall microorganisms isolated from human and cow blood and commercial cell culture serum. They produce biogenic apatite on their cell envelope. Recently, NB were isolated from several human organs and tissues. Khullar et al.[4] observed the presence of apatite forming, ultrafilterable, gram-negative coccoid microorganisms in 62% of the renal stones from 65 patients in north Indian populations. Puskas et al.[5] propagated NB-like particles from 26 of 42 sclerotic aorta and carotid samples and confirmed the results using dot immunoblot, light microscopy, and transmission electron microscopy. Miller et al.[6] examined a total of 16 calcified human arteries and cardiac valves, and they isolated nanometer-scale particles similar to those described as NB from human kidney stones. NB were also detected in 8 out of 8 ovarian cancers with psammoma body samples but none of the 10 without psammoma bodies.[7] Hudelist et al.

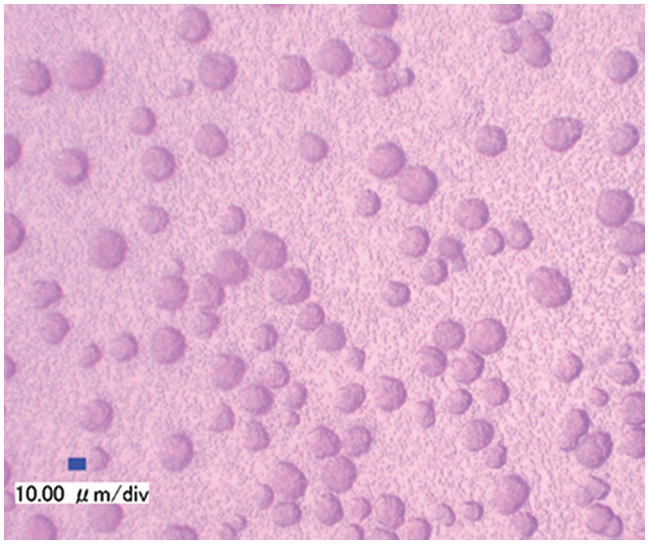

Figure 4. From the same patient as in Figure 2 (after 22 months of culture). Partially many particles appeared in large clusters with diameters greater than 10 μm. Blue bar = 10 μm. Original magnification, ×450.

analyzed gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR), and nanobacterial mRNA was detectable in all of the 4 tissues that contained psammoma bodies. Wen et al.[8] investigated NB in serum, bile, or gallbladder mucosa of healthy Chinese and patients with cholecystolithiasis, and found NB in the bile of 61.4% of patients with cholecystolithiasis by bacterial culture, immunohistochemical staining, and transmission electron microscopy. Here, we report on the detection of NB-like particles in synovial fluid, a blood-poor condition, as that of calcium apatite nanoparticles in perineural tissues reported by King.[12] NB seem to exist even in tissues with low blood circulation/perfusion.

Although NB have been recognized by the scientists who conducted the above-mentioned studies, other scientists have objected to the existence of NB. Cisar et al.[9] have disputed the existence of NB, and they indicated that the DNA sequences of NB reported by Kajander et al. were indistinguishable from those of environmental microorganisms. Drancourt et al.[10] searched for NB from 10 aseptically removed upper urinary tract stones to confirm the reported data. Although they found nanoparticles in 4 of 4 stones similar to those described, they failed to reveal NB by Gimenez staining, Hoechst staining, and specific monoclonal immunofluorescence. In the report by Barr et al.,[11] biofilm composed of aggregates of calcium and phosphate crystals formed in serum samples of some domesticated animals under long-term cell culture conditions. However, PCR assay failed to amplify 16S rRNA gene sequences of NB. More data should be verified before the existence of nanosize microorganisms is disproved. By investigating these NB-like particles in particular, unknown etiologies of some refractory diseases might be elucidated to develop an innovative therapeutic strategy in the future.

Conclusion

NB-like microparticles exist in synovial fluids of arthritis patients.

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