In Vitro Cell. Dev. Biol.—Animal 42:13–15, January and February 2006

1071-2690/06 $18.00+0.00

SELF-PROPAGATING CALCIFEROUS PARTICLES DETECTED IN A HUMAN CELL LINE KASUMI-6 (JCRB1024)

RYÔ HARASAWA,[1] HIDEYUKI TANABE, MIHARU KUREMATSU, HIROSHI MIZUSAWA, AND YOSHIMITSU SUZUKI

Department of Veterinary Medicine, Iwate University, Morioka, Iwate 020-8550, Japan (R. H.), School of Advanced Sciences, The Graduate University for Advanced Studies, Hayama, Kanagawa 240-0193, Japan (H. T.), KohjinBio Co. Ltd, Chiyoda, Sakado, Saitama 350-0214, Japan (M. K.), National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085, Japan (H. M.), and Environmental Science Center, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan (Y. S.)

(Received 7 October 2005; accepted 4 December 2005)

SUMMARY

Tiny particles were found in the medium in the presence of the human leukemia cell line Kasumi-6. The particles were separated from human cells by filtration and incubated in RPMI1640 supplemented with 10% fetal calf serum at 37 C. The particles increased in number very slowly in the liquid medium but did not reveal any biological activity. Transmission electron microscopy of the particles showed a spheroid or ovoid shape in ultrathin section. No specific polypeptides from the purified particles were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), except for bovine fetuin that adsorbed to the surface of the particles. X-ray diffractometry as well as Fourier transform infrared spectrometry suggested the particles consisted of hydroxyapatite. The mechanism of self-propagation of the hydroxyapatite particles in liquid medium is currently unknown. This type of particle has been overlooked for a long period because it is noncultivable. It will be necessary to examine its biological effects to the cultured cells.

Key words: contamination; fetuin; hydroxyapatite; Kasumi-6; nanobacteria.

Cell cultures are compromised to adventitious infections by various microbes or viruses. Mycoplasma is one of the most common contaminants in cell cultures. We recently encountered a human cell culture suspected of mycoplasma contamination because we found many tiny particles with motility by microscopic observation magnifying at ×600. This cell culture named Kasumi-6 was originally established from human bone marrow cells and subcultured in RPMI1640 medium supplemented with 20% fetal calf serums (Asou et al., 2003). We examined the Kasumi-6 cell culture extensively when deposited to the JCRB Cell Bank for presence of mycoplasmas by conventional methods such as microbiological culture (Ogata and Koshimizu, 1967), DNA staining (Chen, 1977), and polymerase chain reaction (PCR; Harasawa, 1996) but failed to find any evidence of mycoplasma contamination. No growth of microor-

[1] To whom correspondence should be addressed at Department of Veterinary Medicine, Iwate University, Morioka, Iwate 020-8550, Japan. E-mail: harasawa@iwate-u.ac.jp

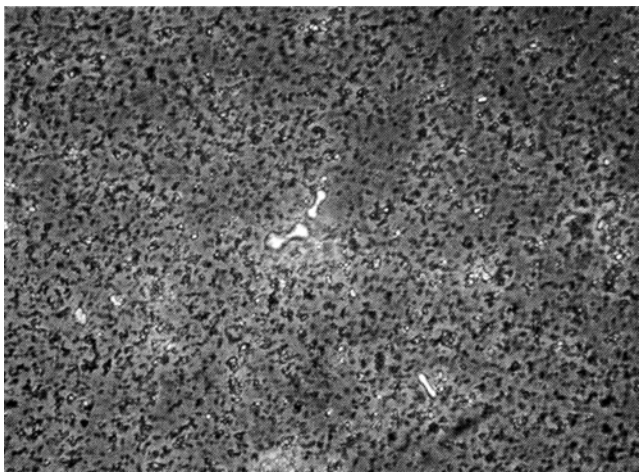

FIG. 1. Microphotograph (×600) of the SPP separated from the cultured cells of human origin. Most self-propagating particles range from 0.5 to 1.5 μm. Some particles showed a gourd-like shape. They can be propagated in duplicate every week.

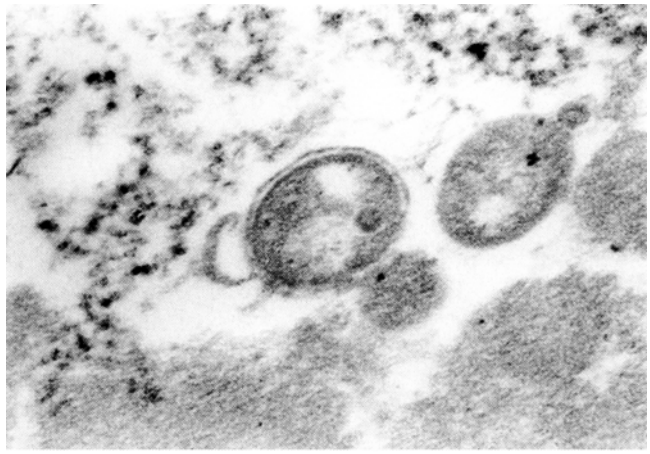

FIG. 2. Electron-microphotograph (×50,000) of ultrathin section of the self-propagating particles (SPP). The SPP was surrounded by a thick wall.

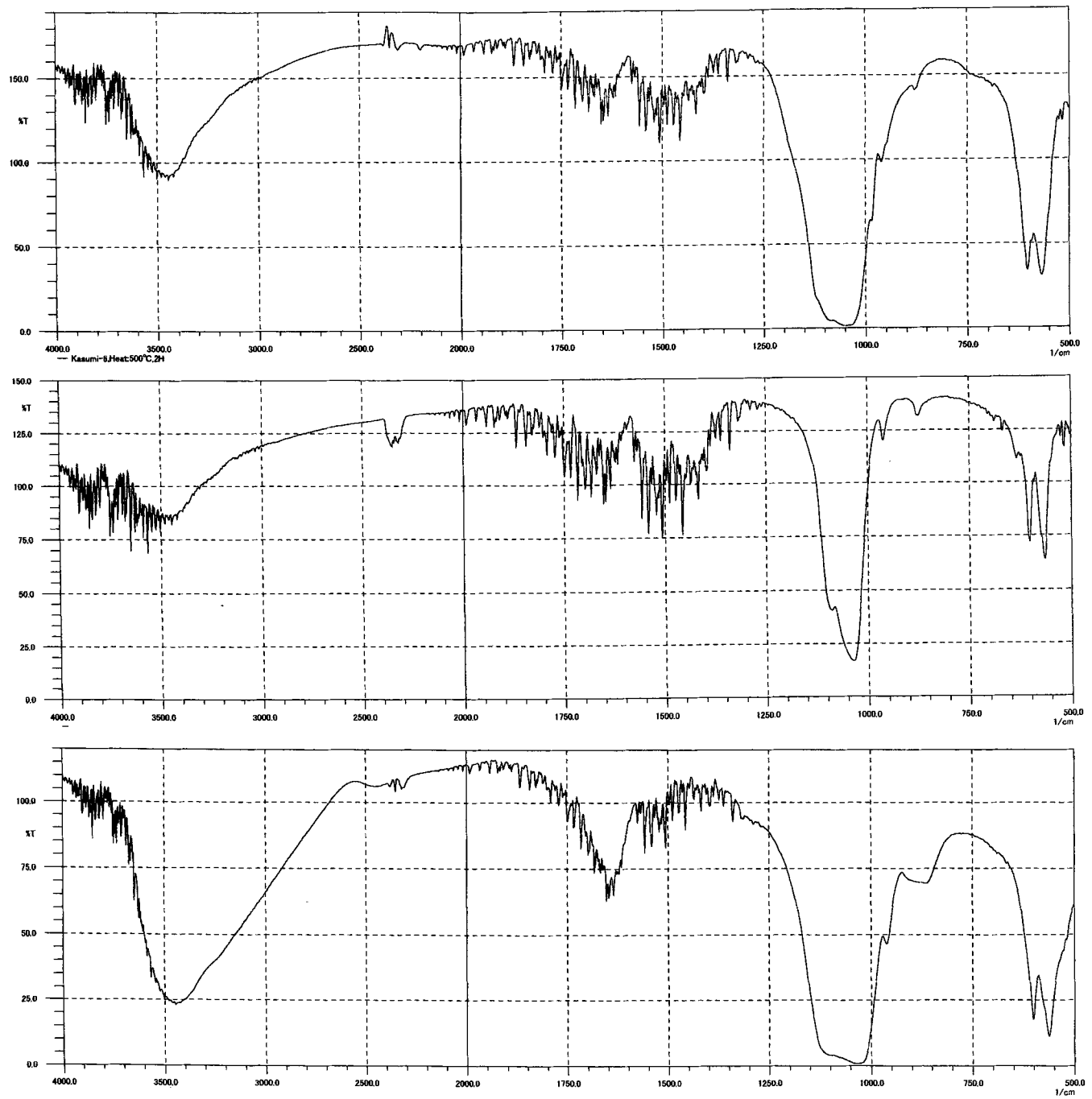

FIG. 3. Fourier transform infrared spectrometry of the self-propagating particles (SPP) (top), hydroxyapatite (middle), and calcium phosphate (bottom). The SPP were heated at 500 C for 2 h prior to analysis.

ganisms was apparent in a mycoplasma medium, and the result of DNA staining with Hoechst 33258 (0.1 μg/ml) was not convincing. No significant amplification was obvious by the PCR.

The tiny particles seemed to propagate in the medium in the cell line Kasumi-6. The size of the particles was almost constant, but the number of the particles increased slowly in the liquid medium. We froze the Kasumi-6 cell culture at −80 C overnight and filtered the thawed cell culture through a membrane filter with a pore size of 800 nm to eliminate the cells and cell debris of human origin. The filtrate was added to the fresh RPMI1640 medium supplemented with 10% fetal calf serums and was further incubated at 37 C. The isolated *tiny* particles seemed to be viable since they increased in number daily in the cell-free medium supplemented with fetal calf serums but were nonculturable on conventional agar plates of enriched medium in an atmosphere of aerobic or anaerobic condition. The size of the self-propagating particles (SPP) ranged from 0.5 to 1.5 μm in the liquid medium, which was almost equivalent to or less than the sizes of ordinary bacteria. Some of the SPP

showed a gourd-like shape (Fig. 1). The SPP did not propagate in serum-free medium. No such particle was evident in the mock-infected control RPMI1640 medium supplemented with fetal calf serum for at least 3 wk.

We tried to isolate DNA from the SPP pellets collected by centrifugation of the cell-free cultures at 10,000 × *g* for 10 min at 4 C but failed. The white pellet of the SPP was not soluble by proteinase K or SDS. We used several procedures, such as the phenol-chloroform method as well as the column method, using commercial kits such as QIAGEN genomic-tip 500/G (catalog no. 10262) with genomic buffer set for bacteria (catalog no. 19060) and TaKaRa Dr. GenTLE for yeasts (code no. 9084).

The pellet of SPP was washed three times with Dulbecco's PBS solution without calcium and magnesium and then subjected to 10% SDS-PAGE and to fluorescent X-ray analysis in energy-dispersive X-ray spectrometer EDV-700HS (Shimadzu Corp. Inc., Kyoto, Japan). SDS-PAGE produced a single band of about 66 kDa under a reduced condition and about 54 kDa under an intact condition (data not shown). The band was transferred to polyvinylidene fluoride membranes and used for determination of amino acid sequence at the N-terminus. Thirteen amino acid residues at the N-terminus were revealed to be IPLDPVAGYKEPA, which were identical to bovine α-2-HS-glycoprotein (fetuin). Fetal calf serum used for medium supplement seems to be most probable source of the bovine fetuin. The pellet of SPP was found to consist of calcium and phosphorus by fluorescent X-ray analysis (data not shown).

The white pellet of SPP was further fixed with glutaraldehyde. Thin sections prepared from the fixed pellets of SPP were stained with uranyl acetate and lead nitrate and examined by electron microscopy at 80 kV (Fig. 2). The SPP seemed to be homogeneous and was surrounded by thick walls.

The white pellet of SPP was then heated at 500 C for 2 h and analyzed in an X-ray diffractometer RINT2200 (Rigaku Corp. Inc., Tokyo, Japan) and in a Fourier transform infrared spectrophotometer FTIR 8600PC (Shimadzu Corp. Inc., Kyoto). Spectrums obtained from the X-ray diffractometry suggested the SPP were hydroxyapatite or calcium phosphate when commercial hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ (lot no. SDR3008; Wako Pure Chemical Ind., Tokyo, Japan) and calcium phosphate tribasic $[(Ca_3(PO_4)_2)_3Ca(OH)_2]$ (lot no. SDN0964, Wako Pure Chemical Ind.) were used as references (data not shown). Fourier transform infrared spectrometry distinguished hydroxyaapatite and calcium phosphate by a specific spectrum at 1640 cm^{-1}. The spectrum of the SPP was similar to that of hydroxyapatite (Fig. 3). A trace amount of organic compounds, such as fetuin, was removed by heating at 500 C for 2 h.

These accumulated data indicate that the contaminants found in Kasumi-6 cell cultures are hydroxyapatite particles adsorbed by bovine fetuin. Fetuin, a negative acute-phase reactant, is known to have affinity to apatite (Price et al., 2004) and may promote an increase in the numbers of hydroxyapatite particles by unknown mechanisms. Although the SPP was similar to the large type of the alleged nanobacteria (Kajander and Ciftcioglu, 1998; Cisar et al., 2000), no biological feature was obvious. Although nanobacteria also called nannobacteria were originally found as geological objects by scanning electron microscopy (Folk, 1993), their biological features are also unknown.

References

Asou, H.; Gombart, A. F.; Takeuchi, S.; Tanaka, H.; Tanioka, M.; Matsui, H.; Kimura, A.; Inaba, T.; Koeffler, H. P. Establishment of acute myeloid leukemia cell line Kasumi-6 from a patient with a dominant-negative mutation in the DNA-binding region of C/EBPα gene. Genes Chromosomes Cancer 36:167–174; 2003.

Chen, T. R. *In situ* demonstration of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. 104:255–262; 1977.

Cisar, J. O.; Xu, D.-Q.; Thompson, J.; Swaim, W.; Hu, L.; Kopecko, D. J. An alternative explanation of nanobacteria-induced biomineralization. Proc. Natl. Acad. Sci. USA 97:11511–11515; 2000.

Folk, R. L. SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks. J. Sediment. Petrol. 63:990–999; 1993.

Harasawa, R. PCR: application of nested PCR to detection of mycoplasmas. In: Razin, S.; Tully, J. G., ed. Molecular and diagnostic procedures in mycoplasmology, vol. 2. New York: Academic Press; 1996:75–79.

Kajander, E. O.; Ciftcioglu, N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc. Natl. Acad. Sci. USA 95:8274–8279; 1998.

Ogata, M.; Koshimizu, K. Isolation of mycoplasmas from tissue cell lines and transplantable tumor cells. Jpn. J. Microbiol. 11:289–303; 1967.

Price, P. A.; Williamson, M. K.; Nguyen, T. M. T.; Than, T. N. Serum levels of the fetuin-mineral complex correlate with artery calcification in the rat. J. Biol. Chem. 279:1594–1600; 2004.