
Nanobacteria promote crystallization of psammoma bodies in ovarian cancer

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Sedivy R, Battistutti WB. Nanobacteria promote crystallization of psammoma bodies in ovarian cancer. *APMIS* 2003;111:951–4.

Serous papillary adenocarcinomas of the ovary are often associated with microcalcifications, namely psammoma bodies. Archebacteria such as nanobacteria are, for example, involved in kidney stone formation. Nanobacteria deserve close scrutiny as their cytotoxicity and ability to cross the placenta present a potential clinical risk. In this study we investigated whether nanobacteria are associated with psammoma bodies in ovarian cancer. We identified in all seven carcinomas with multiple psammoma body nanobacterial antigens in histological specimens and in the ascitic fluid. Control cases of adenocarcinomas without such calcifications did not present nanobacterial antigens. This finding indicates that apart from non-malignant conditions, nanobacteria could also be found in cancer. Hence, nanobacterial infection may be of clinical importance as these bacteria are related to microcalcifications in ovarian cancer.

Key words: Nanobacteria; psammoma bodies; infection; cancer; ovary.

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Bio-mineralization is involved in many different diseases resulting in a variety of calcifications. The formation mechanisms of these calcifications, however, often remain widely speculative. One such phenomenon is the well-known appearance of psammoma bodies (PB), which are detectable in benign and malignant conditions, e.g. in the choroid plexus, meningioma, thyroid tumours, schwannoma, somatostatinoma, and ovarian tumours. PB are microcalcifications appearing histologically as basophilic, onion peel-like, laminated apatite crystals (Fig. 1A). A particular example of PB in cancer is serous papillary cystadenocarcinoma of the ovary, which is to a greater or lesser extent associated with those calcifications. In some instances, the huge number of PB is so remarkable that these carcinomas

have been called psammoma carcinomas. Similarly, a psammomatous variant of meningioma is known. The reason why some tumours contain more and some contain fewer PB remains obscure. Regarding pathogenesis, osteopontin, an extracellular matrix protein, which is expressed in a variety of malignant tumours, is consistently co-localized with non-corpuseular calcified deposits. Those deposits are progressively transformed to PB, which has been described for serous papillary cystadenocarcinomas (1). Recently, bio-mineralization processes were found to be closely related to archebacterial infections with nanobacteria and were associated with kidney stone formation (2, 3). We speculated whether nanobacteria might be involved in the pathogenesis of PB found in ovarian cancer. Therefore, we investigated tissue specimens and the ascitic fluids of 14 patients (7 study cases, 7 control cases) with serous papillary adenocarcin-

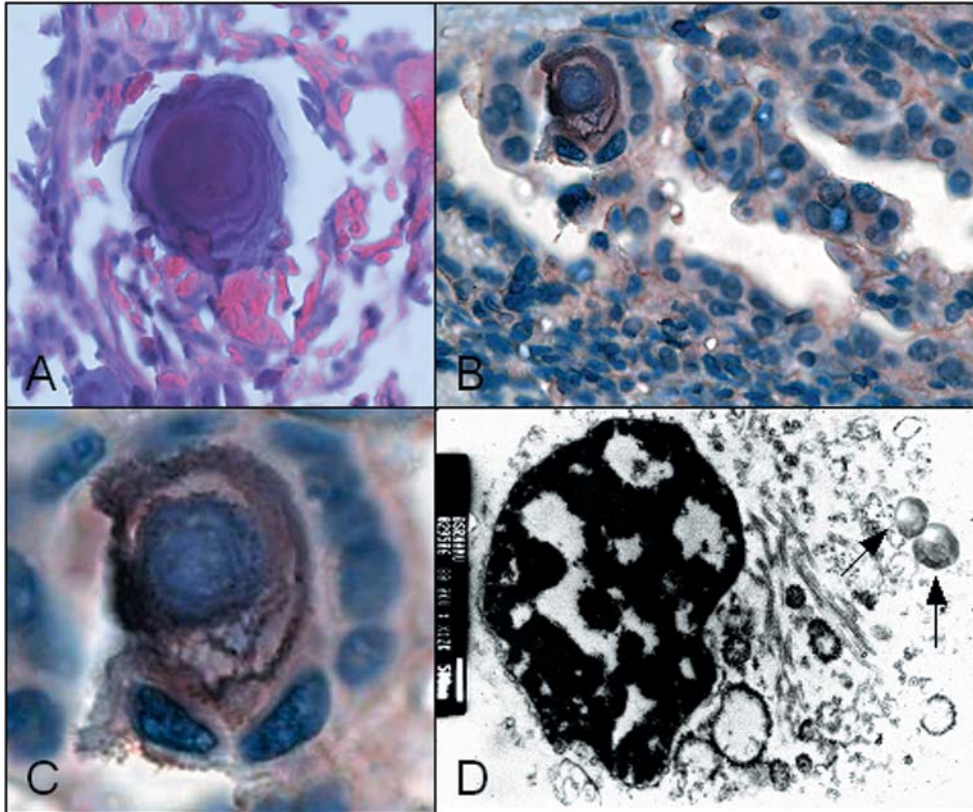


Fig. 1. Histology of PB. Haematoxylin staining (A; original magnification $\times 400$) and immunohistochemistry (B, C; original magnification $\times 100$; $\times 400$). Electron microscopy. Arrows indicate coccoid-shaped nanobacteria (C; original magnification $\times 12000$).

omas of the ovaries using immunohistochemistry, electron microscopy, ELISA, and infrared spectroscopy.

MATERIALS AND METHODS

The specimens originated from 14 patients with serous papillary cystadenocarcinoma of the ovaries at the University of Vienna between 1999 and 2001. These women were aged 43 to 62 years (mean age: 52.5 ± 7.3 years), and none of them had any other infectious disease. Patients were chosen consecutively, control cases were age and sex matched. Study patients presented bilateral serous cystadenocarcinomas, two of them well, two moderately, and three poorly differentiated with numerous PB and malignant ascites. Control cases comprised seven women with serous papillary cystadenocarcinoma of the ovaries without PB but with malignant ascites. Of the control cases, three were well, three moderately and one poorly differentiated. Two additional samples were taken from the ovaries of one 32-year-old and one 65-year-old woman as further controls for im-

munohistochemistry. Two additional samples from ascitic fluid caused by high hydrodynamic pressure due to chronic heart failure were taken for ELISA controls. All tissues were embedded in paraffin blocks, while small fragments of fresh tissue were kept for transmission electron microscopy (TEM), fixed in glutaraldehyde, and processed using standard protocols. Ascitic fluid was used for cytological diagnosis and the residues were taken for ELISA.

ELISA: A commercial monoclonal antibody kit (ELISA; Cat. no. E1001; Nanobac Oy, Kuopio, Finland) was used to detect nanobacterial antigens in ascites from the same patients with ovarian adenocarcinomas with psammoma bodies. A 1:5 dilution of the sample was used according to the manufacturer's recommendations. Positive controls were also obtained from Nanobac Oy.

Infrared spectroscopy: A dried sample of 2 mg was carefully mixed with 300 mg dry KBr and the pellet was placed in a magnetic holder. The system was purged with dry air for 1 h to remove water vapour from the sample compartment. Polarized fourier transformed infrared spectra of 2000 scans at 8 cm^{-1} were obtained using a Nicolet 5DX spectrometer with a DTGS detector and a solid transmission

sample compartment. Spectrum analyses were performed using standard Nicolet and Microcal Origin software.

Immunohistochemistry: According to routine protocols all ovarian cancer tissues were embedded in paraffin, sectioned (4–5 μm), dewaxed, and microwaved for 4 \times 5 min in 0.01 M Na-citrate buffer (pH 6.0). The slides were first immersed in 1.6% hydrogen peroxide in methanol for 30 min and then in blocking solution (0.01 M Tris, 0.1 M MgCl_2 , 0.5% Tween-20). Immunostaining was performed with a mouse monoclonal antibody against nanobacteria (Nanobac Oy, P.O. Box 1188,70211 Kuopio, Finland) at a dilution of 1:100. Staining was performed using biotinylated secondary antibodies binding to avidin-biotin reagents coupled to peroxidase, and incubation using 3,3'-diaminobenzamide (all reagents from DAKO, Glostrup, DK). Positive controls were also obtained from Nanobac Oy. In negative controls, the primary antibody was omitted.

RESULTS

Immunohistochemically, we found positively stained small rims of PB for nanobacterial antigens (Fig. 1B, C) in all cases from the study group. Occasionally, a positive reaction was found in tumour cells of some papillae near the top. All other parts of tissues and all control cases were negative. In addition, nanobacteria were identified in TEM by their typical appearance (Fig. 1D), which was shown by Kajander (2). Nanobacteria appeared as coccoid cell-like structures in all cases but only in a few cells and were found in tumour cells near the top of the papillae, similar to the immunohistochemical location. Using an ELISA kit we found a strong reaction with nanobacterial antigens in all seven

samples of the ascites. Applying infrared spectroscopy of isolated PB, hydrogen apatite was identified as the chemical substrate of those deposits (Fig. 2). In control tissues of regular ovaries and in ovarian cancers without PB we were not able to detect nanobacteria by any method.

DISCUSSION

In this study we identified, in all seven PB-rich carcinomas, nanobacterial antigens in histological specimens and in the ascitic fluid. Further, we detected nanobacterial antigens by electron microscopy in the cytoplasm of tumour cells adjacent to the PB and near the top of the papillae. Hydrogen apatite was identified as the chemical substrate of those deposits.

In the pathogenesis of dystrophic calcifications and stone formations the final common pathway is the formation of crystalline calcium phosphate mineral in the form of an apatite. Kajander and co-workers presented an additional pathway for pathological calcification and stone formation in nanobacteria-infected fibroblasts (2). For many malignant cells, receptors were detected for nanobacterial adherence that could introduce nanobacteria into the tumour cells with subsequent calcification (4). Our observation of nanobacteria in tumour cells near the top of papillae without presenting a PB may support that suggestion. A limitation of our study is that immunohistochemical staining of calcified material may yield false-positive or -negative reactions. For that reason we used a standard method of EM with fresh tissue. But as well as the standard method we also used an

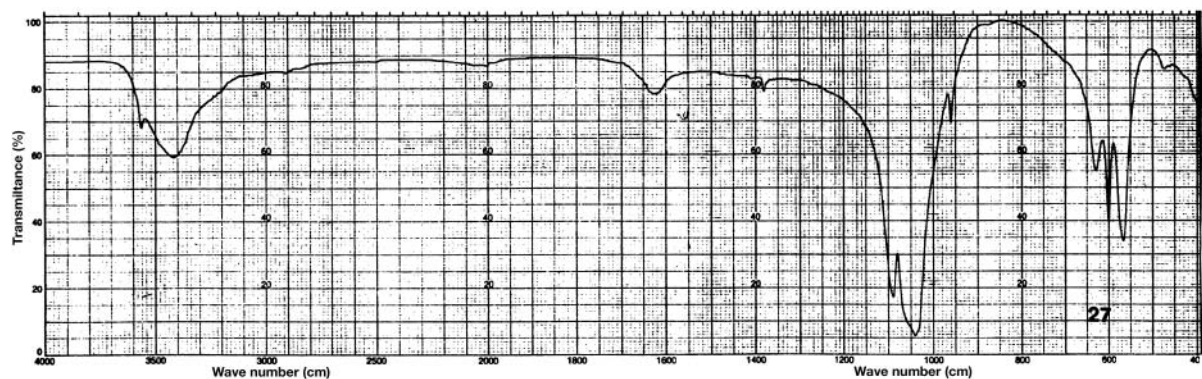


Fig. 2. Infrared spectroscopy: the wave number between 400 and 4000 corresponds to the chemical structure of hydroxylapatite.

electron diffraction method, which determines chemical composition of an unknown crystalline specimen. This is made possible, under favourable circumstances, by comparing the d-spacings of the unknown specimen with those determined from known standard specimens by electron diffraction analyses (EDA). These d-spacings are compiled in tables of crystallographic data, particularly the A.S.T.M. index, and the International Tables for X-ray Crystallography.

After obtaining the EDA results, we continued with Infrared Spectroscopy, to check the results of EDA. With a combination of EDA and infrared spectroscopy it is possible to obtain the real chemical composition of the crystalline specimen in the tissue. Based on the work of Majno G. Joris (5), the work of Kajander & Ciftcioglu (2) and the results of our investigation we came to the conclusion first that hydroxylapatite is the substance of these deposits and second that the structures diagnosed by EM were nanobacteria.

Nanobacteria are novel microorganisms that are not detected using current sterility testing methods (6). They can, however, be detected with new culture and immunotechniques. Nanobacteria are commonly found in bovine and blood products, and, thus, in cell cultures and antigens, including vaccines derived from these. In this connexion, Ciftcioglu and co-workers mentioned the possibility that nanobacteria may be present in vaccines made with cell cultures or in gammaglobulin or other antibody preparations, which, therefore, should be checked. Furthermore, nanobacteria deserve close scrutiny since they present a potential risk because of their cytotoxic properties and ability to cross the placenta (6). Nanobacteria are responsible for biomineralization, and have been associated with various diseases, such as kidney stone formation (2). Although an ascending infection is plausible, it is less clear how nanobacteria might have reached the ovaries in our cases.

We suspect an oral route of infection since nanobacteria were recently identified in beef (7). Nanobacteria multiply very slowly and, if in

fact they are pathogenic in humans, they might be expected to cause slow chronic immunogenic disorders. So far, there is no known chronic bacteraemia that is not harmful.

In conclusion, our findings indicate that nanobacteria are involved in the pathogenesis of PB. If a patient contracts bacteraemia and develops a tumor likely to form PB, nanobacteria could be responsible for this. When is a tumour likely to form PB? As PB always appear in papillary or whorl formations, we believe this histological structure supports the formation of PB. As microcalcifications are in general a common feature of several malignant tumours, it would be very interesting to explore whether nanobacteria are also involved in other carcinomas.

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