Bacterial diversity detected in osteoradionecrosis

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ABSTRACT

Direct microscopy, culture based studies and DNA-DNA hybridization have previously demonstrated an association between microorganisms and osteoradionecrosis. The purpose of our study was to use culture independent molecular techniques to detect bacteria in necrotic bone lesions of the mandible after radiotherapy. Bacterial DNA was extracted from six deep medullary specimens from resected mandibles, including one sample of a relapse. 16S rRNA genes were PCR amplified, cloned, transformed into Escherichia coli and sequenced to determine species identity and closest relatives. From the analysis of 438 clones, 59 predominant species were detected, of which 27% have not been cultivated. The predominant species detected from radionecrotic mandibles were Campylobacter gracilis, Streptococcus intermedius, Peptostreptococcus sp. oral clone FG014, Uncultured bacterium clone RL178, Fusobacterium nucleatum, and Prevotella spp.. The analysis demonstrated intersubject variability of the bacteria present in osteoradionecrosis. In contrast to the diverse bacterial profile detected in primary infection, only a few members of the oral indigenous flora were identified from a case of relapse. Detection of all members of the complex bacterial flora associated with osteoradionecrosis seems to be necessary to better understand the pathogenesis and to improve the therapeutic approach of the infection.
INTRODUCTION

Osteoradionecrosis (ORN) is a complex late complication and calculated risk to conventional radiation therapy used in treatment of cancer in the craniofacial skeleton. Adverse effects of radiation therapy affect normal tissue cells and the vascular system of bone, cartilage and soft tissue. Although, the complexity of the disease still leaves the definition, the etiology, and the pathogenesis of ORN unclear. (Hansen et al. 2005, Nason et al. 2007) ORN was earlier attributed to secondary infection in the traumatized irradiated tissue, with following non-healing wounds and exposed bone (Meyer 1970, Rankow 1971). Marx stated in 1983 that infection associated with ORN was only superficial and secondary, and that the microorganisms found in resections would be surface contaminants. The statement was based on a study that failed to describe microorganisms in the medullary parts of the resections (6). Støre et al. demonstrated presence of a diverse microbiota of the medullar parts of mandible both visualized by transmission electron microscopy (TEM) (7) and detected by DNA-DNA hybridization in a checkerboard assay (8). Specimens were obtained with sterile trepan burrs collecting bicortical cylinders from areas covered by mucoperiost. A diverse microbiota of bacteria and yeast was exposed. The detection of anaerobes indicates that infection might play an important role in the pathogenesis of ORN (7, 8). Hansen et al. (2006, 2006, 2006, 2007) has demonstrated an association between Actinomyces and infected osteoradionecrosis (IORN) based on analysis of irradiated bone biopsies from a high number of patients with post operative complications. Recently, Nason and Chole (2007) have described formation of biofilm in association with ORN of the temporal bone after external beam radiation. Phylogenetic analysis of complex bacterial communities in biofilms relies on detection of housekeeping genes in bacteria, like 16S rRNA, and include both the cultivable and not-yet cultivable segment of the bacterial flora. Molecular techniques
have determined the breadth of microbial diversity of the whole gastrointestinal tract in health and disease, and it is discovered that as much as 50% of the oral- (Aas et al. 2005) and 80% of the intestinal (Eckburg et al. 2005) indigenous bacterial flora consist of not cultivated phylotypes. Detection of all members of the complex bacterial communities is necessary to better understand the role of infection in the pathogenesis of ORN. The aim of this study was to use culture independent molecular techniques to detect bacteria in necrotic bone lesions of the mandible after radiotherapy.

**MATERIALS AND METHODS**

**Subjects**

Seven specimens from six consecutive cases of mandibular ORN and one specimen of a subject with relapse were included in the study. All subjects recruited for the study required a full segmental resection larger than 5 cm during the course of treatment. The participants received a conservative treatment regime of local wound care and continuous treatment with tetracycline (doxycycline 100 mg daily). In cases of clinical exacerbation this dose was doubled, or a limited supplementary regime of clindamycin, 150 mg three times daily, was prescribed.

**Bone samples**

All resections were made from the mandibular body region. Deep medullary bone specimens were obtained using sterile trepan burrs (3 mm wide), collecting bicortical cylinders from areas previously covered by mucoperiost, and at a minimum distance of 3 cm from any surface exposed by oro-cutaneous fistulas. The cortical segments were removed from the specimens and the marrow part was placed in a dental transport medium (Anaerobe Systems, Morgan Hill, CA, USA) for further analysis.
DNA extraction

The marrow part of small bone cylinders was ground on liquid nitrogen to fine powder in a sterile mortar in a laminar airflow cabinet. The powder was suspended in 50 mM Tris HCl and stored at -20°C. DNA was extracted from the bone tissue samples using the ChargeSwitch Forensic DNA Purification Kit (Invitrogen, San Diego, CA) according to the protocol of the manufacturer.

Amplification of 16S rRNA genes

The 16S rRNA genes were amplified under standardized conditions using a universal forward primer (5′-GAG AGT TTG ATY MTG GCT CAG-3′) and a universal reverse primer (5′-GAA GGA GGT GWT CCA RCC GCA -3′) (Paster 2001). PCR was performed with the GeneAmp PCR systems 9700 (ABI, Foster City, CA). One microliter of DNA template were added to a reaction mixture (final volume, 50 µl) containing 20 pmol of each primer, 40 nmol of deoxytriphosphates, and 1 U of Platinum Taq polymerase (Invitrogen). In a hot-start protocol, the samples were preheated at 95°C for 4 min, followed by amplification under the following conditions: 95°C for 45 s, 60°C for 45 s, and 72°C for 1.5 min, with an additional 15 s for each cycle. A total of 30 cycles were performed, which was followed by a final elongation step at 72°C for 15 min. The results of the PCR amplification were examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

Cloning procedures

Cloning of PCR-amplified DNA was performed with the TOPO TA cloning kit (Invitrogen) according to instructions of the manufacturer. Briefly, transformation was done with competent Escherichia coli TOP10 cells. The transformed cells were plated onto Luria-Bertani agar plates supplemented with kanamycin (50 µg/ml), and the plates were incubated overnight at 37°C. Colonies were transferred to 70 µl of 10 mM Tris HCl. Correct sizes of the inserts were
determined in a PCR with an M13 forward primer and an M13 reverse primer (Invitrogen). Prior to sequencing of the fragments, the PCR-amplified 16S rRNA fragments were purified and concentrated with use of the QIAquick PCR purification kit (Qiagen, Valencia, CA).

16S rRNA gene sequencing

Purified DNA from the PCR was sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS, GeneAmp PCR systems 2700 and 9700; ABI). The protocol and primers used for sequencing have been described previously (Paster 2001). The sequencing reactions were run on an ABI 3730 DNA sequencer (ABI).

16S rRNA gene sequencing and data analysis of unrecognized inserts

The number of sequenced clones per sample ranged from 45 to 84, with an average of 62.6 clones. A sequence of approximately 500 bases was obtained first to determine identity or approximate phylogenetic position. For identification of closest relatives, the sequences of the inserts were compared to the 16S rRNA gene sequences of over 100,000 sequences in the Ribosomal Database Project (Cole et al 2005), more than 188,000 sequences in the Greengenes databases (http://greengenes.lbl.gov/) and NCBI GenBank databases (http://www.ncbi.nlm.nih.gov/). The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes and Cantor (1969). Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which data were available for 90% of the strains tested. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (1987). TREECON, a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer 1994). We are aware of the potential creation of 16S rRNA chimera molecules assembled during the PCR (Liesack 1991). Chimeric sequences were identified by using the Chimera Check program in the Ribosomal Database Project, by treeing analysis, or by base signature analysis.
Species identification of chimeras was obtained, but the sequences were not examined for phylogenetic analysis. Published nucleotide sequences are available for electronic retrieval in the EMBL (http://www.ebi.ac.uk/embl/), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and DDBJ (http://www.ddbj.nig.ac.jp/) nucleotide sequence databases under accession numbers included in Figure 1.

RESULTS AND DISCUSSION

Scanning electron microscopy (Støre 2005) demonstrated presence of bacteria in marrow spaces of the mandible and the following study based on DNA-DNA hybridization (Støre 2005) from our group detected the cultivable bacterial flora present in ORN specimens. In this study, eight specimens from six subjects with ORN were selected for further analysis with culture independent molecular techniques to detect both cultivated and not cultivated bacteria. From a total of 438 clones, a diverse bacterial flora of 59 taxa was detected from eight specimens of ORN. Six phyla were represented in the diverse bacterial flora, including Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Spirochaetes and Bacteroides, of which 27% of the bacteria present have not been cultivated.

The introduction of molecular techniques and the opportunity of using housekeeping genes, like 16S rRNA, to detect and identify microbes has manifested the human microbiota as a biofilm of complex microbial communities. In the last decade, culture independent techniques have underlined the need to include all members of the bacterial flora to understand the role of bacteria in health and disease. Despite the known challenges related to DNA extraction from bone tissue, Fenollar et al. (2006) demonstrated bacterial diversity, including not cultivated phylotypes, from an impressing large set of 525 samples of infected bones and joints. The bone and joint samples were
collected from the whole body (Fenollar et al. 2006). Their results confirmed findings of previous studies (Støre et al. 2005, Lewis et al. 1978) suggesting that anaerobes are underestimated and may play a central role in polymicrobial infections in bone. From the data of the ORN specimens in the present study we also recognize a predominance of anaerobes (Figure 1). In accordance to our data, Fenollar et al. (2006) also detected in their study several human pathogens not previously reported or rare in bone and joint infections, like *Streptococcus anginosus*, *Peptostreptococcus micros*, *Peptoniphilus lacrimalis*, *Porphyromonas asaccharolytica*, and *Prevotella buccalis* (Figure 1).

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**Figure legends**

Figure 1. Bacterial profiles of mandibular osteoradionecrosis. Subject #1 represented with two separate specimens (1a and 1b) from one lesion and subject #2 with primary infection (2p) and relapse (2r). Distribution and levels of bacterial species/phylotypes among the six subjects is shown by the columns of boxes to the right of the tree as either not detected (clear box), <10% of the total number of clones assayed (shaded box), or ≥10% of the total number of clones assayed (darkened box). 10% was chosen arbitrarily. GenBank accession numbers are provided. Marker bar represents a 5% difference in nucleotide sequences.
Bacteria in osteoradionecrosis

Figure 1.