No Evidence for an Association between Persistent Measles Virus Infection and Otosclerosis among Patients with Otosclerosis in Japan

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Otosclerosis, which is characterized by disordered bone remodeling, occurs exclusively in the human temporal bone. The etiology of the disease is unknown, but a popular hypothesis is that it is caused by persistent measles virus (MV) infection. Paramyxovirus-like filamentous structures were found in otosclerotic lesions of stapes footplates from patients with otosclerosis. Although MV RNAs have been detected in otosclerotic samples by using reverse transcription-PCR, no complete MV mRNA sequence has been reported, nor has infectious virus been isolated from clinical samples. Furthermore, one study failed to obtain evidence of MV infection in otosclerotic bone samples. In this study, we tested, by three different protocols, for the presence of MV in clinical samples from patients with otosclerosis in Japan. We used a highly sensitive reverse transcription-quantitative PCR method which is able to detect viral mRNA in cells infected with MV at around one infectious unit per well. We obtained no evidence of MV infection in bone samples, primary cell cultures derived from stapes bones, or MV-susceptible cell lines (Vero/ hSLAM and II-18 cells) cocultured with bone samples or primary cell cultures derived from them. Thus, our results do not support the hypothesis that persistent MV infection is involved in the pathobiology of otosclerosis.

Antonio Maria Valsalva, an Italian anatomist and surgeon, first reported a lesion of otosclerosis in a dissected temporal bone from a patient with hearing impairment (18). Otosclerosis is characterized by disordered bone remodeling in the otic capsule. The disease is thought to start in an area anterior to the stapes footplate and the oval window niche called the fissula ante fenestram, as often observed by computed tomography (CT). In most cases, the stapes bone becomes fixed due to invasion of otosclerotic foci at the stapediovestibular joint. This fixation results in conductive hearing loss, which is often accompanied by Carhart’s notch (2-kHz bone conduction threshold dip) and negative stapedius reflex with the normal tympanic membrane. In advanced stages, otosclerosis foci can involve the cochlea and the vestibule, leading to sensorineural hearing loss and vertigo, respectively, and pathological involvement of the anterior and/or posterior pole can be observed. Otosclerosis occurs at a prevalence of 0.04 to 1% among Caucasian people (18), whereas it is uncommon among Asians and extremely rare among Africans. Recently, the incidence of otosclerosis has been increasing in Japan (44).

This disorder of bone turnover that leads to abnormal resorption and redeposition of the bone is similar to the lesion observed in Paget’s disease (PD), which has been proposed to be a chronic inflammatory disease caused by persistent measles virus (MV) infection. Although the pathogenesis of otosclerosis remains unknown, many hypotheses, including autoimmune response, viral infection, endocrine disorder, connective tissue disorder, and genetic disease (5), have been proposed. One of the most suspected environmental factors is persistent MV infection, like for PD.

Measles is an acute febrile infectious disease which remains a major cause of child deaths in developing countries. MV, a member of the Morbillivirus genus in the Paramyxoviridae family, is an enveloped virus with a nonsegmented negative-strand RNA genome. The MV genome has six genes that encode the nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. MV is serologically monotypic, but based on the sequences of the N and H genes, wild-type viruses are classified into eight clades designated A, B, C, D, E, F, G, and H. The Edmonston strain, the first MV isolate (6), is classified as genotype A and has been used in many studies, including for the development of vaccines.

The observation of filamentous structures morphologically similar to viral nucleocapsids in the endoplasmic reticulum (ER) of osteoblast-like cells of otosclerotic lesions led McKenna et al. to propose that otosclerosis might be caused by a viral infection (22). Since then, a number of studies have reported the detection of MV in samples from otosclerosis patients by using the reverse transcription-PCR (RT-PCR) method (4, 13, 14, 16, 17, 20, 26, 27) and immunohistochemical analysis (3, 21). Additionally, Arnold et al. analyzed the data for all patients who were hospitalized for otosclerosis in Germany from 1993 to 2004 (2). They concluded that there is a statistically significant decrease in otosclerosis among patients who were vaccinated against MV in comparison with the patients who were not vaccinated (2). Despite many studies that demonstrated the presence of MV in otosclerotic tissue, the complete MV mRNA sequence has never been reported, nor has the isolation of MV from otosclerotic samples been successful. Grayeli et al. reported that MV RNA was not detected in fresh otosclerotic samples in a large population of patients (n = 35) (9). The presence of different viruses was also reported in some cases (21, 37). Moreover, there is no evidence that infection of the otic capsule with MV produces cells with the otosclerotic phenotype in this tissue.

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</tr>
<tr>
<td>3-11</td>
<td>Frozen in TRIzol</td>
<td>OS</td>
<td>L</td>
<td>F</td>
<td>56</td>
<td>Unilateral</td>
<td>Normal</td>
<td></td>
<td>11</td>
<td>3-1</td>
<td>Frozen in Tissue</td>
<td>OS</td>
<td>OS</td>
<td>Stapedectomy</td>
<td></td>
<td></td>
<td></td>
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a Samples from the same patient are marked with the same letter.
b OS, otosclerosis; TS, tympanosclerosis; Chole, cholesteatoma.
c R, right; L, left.
d F, female; M, male.
e FAF, low density at fissula ante fenestram in computed tomography.
f CN, Carhart's notch in pure-tone audiometry.
g A, normal; As, ossicular fixation, tympanosclerosis, or otitis media with effusion.
h NE, not examined.

**Table I.** Clinical data for all patients.
fectious virus) in stapes bones from patients with otosclerosis in Japan using highly sensitive detection methods.

**MATERIALS AND METHODS**

**Patients.** The clinical data for all patients, which were obtained from medical files, are given in Table 1. There were 5 male patients (6 ears) and 17 female patients (21 ears). The mean age was 51.05 years (range, 23 to 67). Preoperative vestibular symptoms such as vertigo were not reported in any cases. Preoperative diagnosis was made by clinical course and examinations of pure tone audiometry (PTA), stapedius reflexes (SR), tympanometry, and CT of the temporal bone. The grade of otosclerosis was considered to be extensive (with associated sensorineural hearing loss) in 17 ears. The definitive diagnosis of otosclerosis was made during surgery by the condition of the stapes bones and their immobility. All patients showed fixation of the stapes bone. During surgery, the involved stapes bones were removed. The removal of the whole stapes bone, called a stapedectomy, was performed in 22 cases (81%). The removal of its superstructure, called a stapedotomy, was performed in 5 cases (19%). Approval by the ethics committee of Kyusyu University Hospital and the patient consent were obtained for these samplings and all experimental procedures.

**Cells and viruses.** The characteristics and culture conditions of the Vero/hSLAM (33) and II-18 (38) cell lines were as described previously. The MV used in this study was a recombinant virus, based on the wild-type IC-B strain, expressing enhanced green fluorescent protein (IC323-EGFP) (10). The virus was grown on Vero/hSLAM cells, and virus titers were determined by plaque assay on Vero/hSLAM cells. cDNAs prepared from MV isolates of genotypes D3, D4, D5 (two stocks isolated in 2000 and 2008), D8, D9, G3, and H1, were kindly provided by Y. Nakatsu, K. Komase, and M. Takeda (National Institute of Infectious Diseases, Tokyo, Japan). These cDNAs were synthesized from RNAs directly extracted from the genome of the Edmonston tag strain (31, 34) was also used in the QPCR assay. cDNA derived from the IC-B (genotype D3, isolated in 1990 in Japan) and Japan during the period from 1999 to 2011. We also prepared cDNAs from MV isolates of different MV genotypes. We prepared cDNAs from MV isolates of the D3, D4, D5 (two stocks isolated in 2000 and 2008), D8, D9, G3, and H1 genotypes. These were the prevailing MV genotypes in the D3, D4, D5 (two stocks isolated in 2000 and 2008), D8, D9, G3, and H1 genotypes. These were the prevailing MV genotypes in Japan during the period from 1999 to 2011. We also prepared cDNA from the IC-B (genotype D3, isolated in 1990 in Japan) and Edmonston (genotype A) strains. Our primer pair could efficiently amplify the N mRNA in RNA extracted from cells infected with IC323-EGFP even at less than 1 IU/well (Fig. 1).

**RESULTS**

**Comparison between clinical and histological diagnoses.** We first examined whether stapes bones from patients with clinically diagnosed otosclerosis in our institute had the same diagnosis histologically, because samples of stapes bones from patients to be used in our study would be diagnosed only clinically. Clinical diagnosis was perfectly matched with histological diagnosis in patients who underwent surgery for stapedial fixation at Kyushu University Hospital during 2003 to 2009 (the period before our study was initiated) (Table 2).

**High sensitivity of the RT-QPCR method.** We examined the sensitivity of our RT-QPCR method for detection of MV RNA. Vero/hSLAM cells were infected with serially diluted MV (IC323-EGFP) at 1/4 PFU per well to 40 PFU per well, followed by medium replacement and addition of the fusion block peptide (100 μg/ml) (35) at 2 h after incubation to block the second-round infection by progeny viruses. After 36 h of incubation, infectious units (IU) were determined by counting EGFP-expressing cells, the number of which was consistent with the PFU inoculated (Fig. 1). Total RNA was extracted from the infected cells, followed by RT-QPCR. To detect viral RNA, we used a pair of primers against the N gene, which is known to be most abundantly expressed in MV-infected cells (12). Our RT-QPCR method could detect the N mRNA in RNA extracted from cells infected with IC323-EGFP even at less than 1 IU/well (Fig. 1).

**Next, we examined whether the primer pair used could detect different MV genotypes.** We prepared cDNAs from MV isolates of the D3, D4, D5 (two stocks isolated in 2000 and 2008), D8, D9, G3, and H1 genotypes. These were the prevailing MV genotypes in Japan during the period from 1999 to 2011. We also prepared cDNA from the IC-B (genotype D3, isolated in 1990 in Japan) and Edmonston (genotype A) strains. Our primer pair could efficiently amplify the N genes from all these MV strains and isolates (Fig. 2).

**Analysis of otosclerosis samples.** MV infects immune cells and polarized epithelial cells by using SLAM and nectin 4, respectively, as receptors (28, 41). Vaccine strains of MV can also use CD46, and streptococcal infections (10), previously reported (8). After 3 to 4 weeks of incubation, primary cells migrated out of bone samples and reached maximal cellular growth. At this point, the primary cells were trypsinized, and stapes bones were used for RNA extraction. These primary cells were then cocultured with 60% confluent Vero/hSLAM or II-18 cells in a 10-cm dish and observed for 3 to 5 weeks. Another portion of the primary cells was placed in a 6-cm cell culture dish and allowed to grow to confluence for 2 to 3 additional weeks, and total RNA was extracted. In the third protocol, bone fragments were pulverized and total RNA was extracted directly from bone fragments by using TRIzol reagent. The presence of MV RNA was analyzed using the RT-QPCR method.

**TABLE 2** Comparison between clinical and histological diagnoses for patients who underwent surgery at Kyushu University Hospital during 2003 to 2009

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. male/</th>
<th>Mean age, yr (range)</th>
<th>No. (%) with histologically diagnosed otosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otosclerosis (47)</td>
<td>7/40</td>
<td>41.1 (17–77)</td>
<td>47/47 (100)</td>
</tr>
<tr>
<td>Tympanosclerosis (4)</td>
<td>2/2</td>
<td>58.25 (54–63)</td>
<td>0/4 (0)</td>
</tr>
</tbody>
</table>
and is highly susceptible to MV. We considered the possibility that MVs in otosclerotic tissues might be defective, mutant viruses that exhibit altered tropism. Therefore, we used both Vero/hSLAM and II-18 cells for coculture experiments. Defective MV strains from patients with subacute sclerosing panencephalitis have been shown to infect Vero/hSLAM cells efficiently (11, 30).

Patient samples were examined by one of the three protocols. The first protocol was performed for patients 1-1 to 1-5 (Table 1). Patient 1-4 was diagnosed with tympanosclerosis. Bone fragments were directly cocultured with 90% confluent Vero/hSLAM and II-18 cells and observed for 7 to 12 days. However, no CPE was observed. Furthermore, viral RNA was not detected in cocultured cells by RT-QPCR (Fig. 3).

The second protocol was performed for patients 2-1 to 2-11 (Table 1). Patient 2-8 was diagnosed with choresteatoma. Fresh bone fragments were collected immediately after surgery and incubated in McCoy’s 5A medium in a 10-cm dish. During the incubation, the cells grew in a centrifugal pattern from the bone (Fig. 4A). If MV is present in primary cell culture as previously reported (8), coculture of primary cultured cells with cells permissive for MV could facilitate the isolation of MV. However, Vero/hSLAM (Fig. 4B) and II-18 (Fig. 4C) cells cocultured with primary cells did not show any CPE such as syncytium formation. Similarly, no CPE was observed in any primary cell cultures. Furthermore, the N mRNA was not detected in any primary cell cultures (Fig. 4D) or cultured stapes bones (Fig. 4E) by using RT-QPCR.

The third protocol was performed for patients 3-1 to 3-11 (Table 1). We assessed total RNAs directly extracted from fresh frozen bones. The RNA quantity was assessed by amplifying /H9252-actin, which was detected in all samples by using RT-QPCR. However, the MV N mRNA was not detected in any samples (Fig. 5).

DISCUSSION

Many hypotheses have been proposed to explain the cause of otosclerosis, including persistent MV infection. In the present study, we could not detect MV in otosclerotic samples by using coculture experiments with highly susceptible cell lines and a highly sensitive RT-QPCR method.

Vero/hSLAM and II-18 cells cocultured with stapes bones or primary cells derived from them did not show any CPE, and the N mRNA of MV was not detected in any samples (Fig. 5).
detected using total RNA obtained from cocultured cells, stapes bones, or primary cells.

In the 1950s, MV of genotype A was prevailing in acute cases in many countries (36). In Japan, Taniguchi et al. succeeded in isolating MV using the chorioallantoic membrane in 1954, and this isolate was of genotype A (43). Viruses of genotypes D1, E, and F were mainly reported during the 1960s and 1970s in some countries, but these genotypes have not been detected since the late 1980s. Nakayama et al. reviewed Japanese isolates and reported that the strains of MV isolated in Japan since 1984 were classified into three different genotypes, C1 (before 1985), D3 (1985 to 1990), and D5 (1990 to 2000), and that genotype C1 prevailed for a long period before 1985 (25, 32). Sporadic outbreaks of H1 in Tokyo and D9 in Yamagata were reported in 2000 and 2004, respectively (23, 45). In our study, the patient age ranged from 23 to 67 years (mean, 51.05 years). If these patients were persistently infected with MV, then genotype A, C1, D1, D3, or D5 would be able to be detected in otosclerotic samples. We confirmed that our method can amplified the N genes of genotypes A, D3, D4, D5, D8, D9, G3, and H1. We did not examine genotypes C1 and D1. However, given the sequence similarity among genotypes A, C1, D1, and D3 (Table 3), it is highly likely that our method can also amplify the N genes of genotypes C1 and D1.

It may be argued that the titer of MV in otosclerotic tissues is too low to be detected or that the virus may be mutated so that it cannot infect surrounding cells. In those cases, the coculture method might fail to isolate MV from otosclerotic tissues. However, these possibilities raise another question, i.e., whether such a virus could ever cause disease. Furthermore, the MV hypothesis cannot explain why otosclerosis is extremely rare among Africans (1, 42) despite the fact that MV is a major cause of child deaths in Africa. Additionally, the localization of filamentous structures observed in otosclerotic cells (22) is quite different from that in MV-infected cells, in which viral filamentous structures are observed in the cytoplasm, not in the ER.

MV infection may have the capacity to trigger a pathological process which can cause otosclerosis even after elimination of MV. In addition, autoimmunity and genetic factors have been suspected to play a role in triggering otosclerosis lesions. If so, it might be very difficult to demonstrate that MV plays a key role in triggering the pathogenic process, because there is no suitable animal model of otosclerosis.

Some studies have detected MV mRNA or protein in samples from patients with PD (7) like those with otosclerosis. However, others have never detected evidence of paramyxoviruses in sam-

FIG 4 Primary cultures and RT-QPCR for 11 samples in the second protocol. (A) Primary cell cultures were established from stapes bones. The cells migrated out of the bone samples and reached maximal growth after 3 to 4 weeks of culture. Two examples are shown. (B and C) Primary cells were cocultured with Vero/hSLAM (B) or II-18 (C) cells. No CPE was observed. (D and E) Total RNA was extracted from primary cells (D) and cultured stapes bones (E) in the second protocol. Patient numbers are indicated below the lanes (patients 2-1 to 2-11). The amplified RT-QPCR products were separated by electrophoresis on a 3% agarose gel.

FIG 5 RT-QPCR for 11 samples in the third protocol. Patient numbers are indicated below each lane (patients 3-1 to 3-11). Total RNA was extracted from stapes bone immediately after surgery. The amplified RT-QPCR products were separated by electrophoresis on a 3% agarose gel.
TABLE 3 Comparison of sequences for different measles virus genotypes

<table>
<thead>
<tr>
<th>Measles virus</th>
<th>Genotype</th>
<th>Sequence in comparison to primer:</th>
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<tbody>
<tr>
<td>Edmonston</td>
<td>A</td>
<td>N forward (5’GAACTCGGTATCACGTGCC) T3TCGTGAGTCTCATTCTC</td>
</tr>
<tr>
<td>Mvī/Tokyo, JPN/84/K</td>
<td>C1</td>
<td>GAACCTCGGTATCACGTGCC TCCGTGAGTCTCATTCTC</td>
</tr>
<tr>
<td>Mvī/Bristol, UNK/74</td>
<td>D1</td>
<td>GGACTCGGTATCACGTGCC TCCGTGAGTCTCATTCTC</td>
</tr>
<tr>
<td>Ichinose-B95a</td>
<td>D3</td>
<td>GAACCTCGGTATCACGTGCC TCCGTGAGTCTCATTCTC</td>
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* BASES different from the primer sequence are underlined.

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