Evidence for Oncolytic Viral Eradication of Cholesteatoma In Vitro

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Abstract
Cholesteatomas (CHSTs) are congenital or acquired lesions of the temporal bone that are associated with significant morbidity. We hypothesized that an oncolytic herpes simplex virus (oHSV) could preferentially eradicate primary human CHST cells in vitro and that this virus will replicate selectively and efficiently in CHST cells when compared with control cells. In this work, primary human CHST cells were cultured from surgically collected tissue. Cholesteatomas and control cells were grown and infected by oncolytic oHSV. More than 80% CHST cells versus <5% control cells were killed by oHSV. The oHSV showed a significant enhanced cytotoxic effect against CHST cells in a time- and dose-dependent manner. Therefore, this novel therapy has promise as a future treatment to minimize the spread and recurrence of CHST.

Keywords
cholesteatoma, oncolytic, virus

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Cholesteatomas (CHSTs) are lesions of the temporal bone that can lead to significant complications. The standard of care is surgery to eradicate the disease. However, patients require lifelong follow-up after surgery because recurrence rates can be >50%.1 Given the lack of optimal outcomes achieved by surgery alone, there is a need for new approaches for CHST treatment.

One possible alternative is oncolytic virotherapy. Although this concept originated in the early 20th century, only in the past 2 decades did it truly emerge as a potentially viable therapeutic approach for the treatment of malignancies (eg, melanoma, head and neck squamous cell carcinoma, glioblastoma multiforme, pancreatic adenocarcinoma).2 Utilized viruses have included parvovirus, polio virus, reovirus, and herpes simplex virus (HSV).3

Oncolytic HSV (oHSV) is the first and most studied virus for oncolytic virotherapy. It is the only virus approved by the Food and Drug Administration for clinical use. The rationale for treating CHST with oHSV stems from the fact that wild-type HSV is a lytic virus that can infect epithelial cells, as in the CHST matrix. oHSV is attenuated and can be mutated to preferentially grow in dividing cells (as in CHST).3 Additionally, CHST is associated with bacterial superinfection and biofilm formation, which can prime a low-grade immune response. We postulate that acute oHSV infection might facilitate this immune response and lead to CHST eradication.

For this in vitro experiment, we hypothesized that oHSV could selectively and efficiently replicate in CHST cells as compared with control cells. This work represents the first step toward the development of a novel oncolytic virus treatment for management of CHST.

Materials and Methods
After an Institutional Review Board exemption (Federalwide Assurance 00003152) was obtained for the collection of de-identified CHST tissue from 4 patients, primary human CHST cells were cultured from surgically collected tissue. CHSTs were isolated and subsequently cultured via a keratinocyte culture protocol. CHST tissue was trypsinized briefly with agitation (30-60 minutes) in 0.25% trypsin-EDTA at 37°C. The tissue was then neutralized by adding fetal bovine serum to the cell suspension, followed by pipetting the suspension to release the CHST cells. The isolated primary keratinocytes from disaggregated tissue were collected...
and plated in keratinocyte growth medium: F-12 and Dulbecco’s Modified Eagle’s Medium media (3:1 mixture) containing fetal bovine serum (5%), insulin (5 μg/mL), adenine (24.2 μg/mL), hydrocortisone (0.4 μg/mL), cholecalciferol (8.3 ng/mL), epidermal growth factor (10 ng/mL), penicillin-streptomycin (50 IU/mL), and Fungizone (0.5 μg/mL) in a 5% CO₂ and humidified incubator. The CHST cells were cultured with replication-inactivated murine fibroblast feeder layers to provide optimal stromal conditions to maximize the growth of CHST cells. Within 1 to 2 weeks, keratinocyte colonies had formed. After gaining confluence, primary fibroblasts were removed by trypsinization, allowing CHST cells to be transferred into a new plate. Samples from each patient were kept and tested separately.

The primary cultured human CHST cells were validated and cryopreserved at low passage (1 or 2) for oHSV experiments. CHST cells were grown and infected with oHSV in 96-well plates. Cell viability was assessed by the colorimetric MTS Assay Kit (Promega, Madison, Wisconsin) after viral infection. To demonstrate the specificity of the oHSV, cytotoxic effects in CHST cultures infected with 2 types of oHSV (parent and progeny oHSV) were compared with 2 types of control cells (primary fibroblasts and normal human foreskin keratinocytes).

**Results**

CHST cells were successfully grown in vitro (Figure 1), and the progeny version of oHSV showed a significant cytotoxic effect against CHST cells in a time- and dose-dependent manner (Figure 2). The parent version of oHSV also had a cytotoxic effect against CHST, but the effect was not as potent as that of the progeny oHSV (results not shown). Multiplicity of infection (MOI), the ratio of infectious particles to target cells, was used to quantify response. More than 50% of CHST cells versus <5% of control were killed by oHSV at an MOI of 0.001, suggesting that the oHSV selectively targets and kills the CHST cells. Repeated measures analysis of variance indicated significant main effects of cell type and MOI level and a significant interaction between those factors (P < .001). Pairwise multiple comparisons (Holm-Sidak method) confirmed highly significant differences (<.001) between mean CHST cell viability and that of the control cell types at all MOI levels other than 0 and 1.0.
Discussion
In our preliminary in vitro results, oHSV showed a significant cytotoxic effect against human CHST cells in a time- and dose-dependent manner. Oncolytic virotherapy is emerging as a promising new therapeutic approach for human cancer treatment. Attenuated oHSV mutants engineered with deletions of normally critical genetic functions that are dispensable in cancer cells are being actively pursued as novel therapeutic agents. Clinical trials have confirmed the safety of administering attenuated herpes viruses in humans, and the Food and Drug Administration recently approved the HSV-1 virus T-VEC (Imlygic, Amgen) for advanced melanoma.

The mechanism of action of oHSV relies on cytotoxicity and immunomodulation, inducing direct cellular lysis, cytokine-induced apoptosis, innate immune cell cytotoxicity, and adaptive antigen-specific T-cell response. The specific mechanism by which oHSV induced CHST cell death is still unknown and requires further evaluation.

To the best of our knowledge, this is the first study to show the efficacy of oncolytic virotherapy for a benign disorder such as CHST. Future research will include validation of these results in animal models and safety assessment of oHSV treatment in vitro and in vivo.

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Ravi N. Samy, design of the work; acquisition, analysis, interpretation of data, drafting the work; Noga Lipschitz, analysis and interpretation of data, drafting the work; Brian R. Earl, analysis and interpretation of data, drafting the work; Timothy P. Cripe, design of the work; acquisition, analysis, interpretation of data, drafting the work.

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