A Novel Tool for Nasal Polyp Investigation: An Ex vivo Organ Culture System

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ABSTRACT: **Background:** Nasal polyps are three-dimensional structures arising from the mucosa of the upper airway. Due to their complexity, the reliability of single-layer cell cultures and animal systems as research models is limited.

Objectives: To evaluate the feasibility of an ex vivo organ culture of human polyps, preserving tissue structure and function.

Methods: Nasal polyps were excised during routine endoscopic sinus surgery for chronic rhinosinusitis and polyposis. Fresh tissue samples were used for pathological evaluation and for the preparation of 250–500 µm sections, which were incubated in culture media. Tissue viability was assessed by visualisation of cilia motility, measurement of glucose uptake, and an infectivity assay. Cytokine secretion was evaluated by enzyme-linked immunosorbent assay and real-time polymerase chain reaction before and after the introduction of steroids.

Results: Polyp tissue viability was retained for 2–3 days as demonstrated by cilia motility, glucose uptake and preserved cellular composition. Tissue samples maintained their capacity to respond to infection by herpes simplex virus 1 and adenovirus. Introduction of dexamethasone to cultured tissue samples led to suppression of interferon-g production.

Conclusions: The ex vivo nasal polyp organ culture reproduces the physiological, metabolic, and cellular features of nasal polyps. Furthermore, it shows a preserved capacity for viral infection and response to drugs. This system is a useful tool for the investigation nasal-polyps and for the development of novel therapies.

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KEY WORDS: chronic rhinosinusitis, explant, ex vivo organ culture, nasal polyps, polyposis

The nasal mucosa is central in upper airway defense against various pathogens through a multitude of mechanisms [1]. Dysregulation of these mechanisms leads to diseases, such as non-steroidal anti-inflammatory drug-exacerbated respiratory disease (NERD) and chronic rhinosinusitis (CRS). NERD is a chronic disease that may be challenging to manage. It is characterized by nasal polyposis and bronchial asthma, along with hypersensitivity to non-steroidal inflammatory drugs [2,3]. The pathogenesis of NERD involves impairment of arachidonic acid metabolism leading to the overproduction of cysteinyl leukotrienes and decreased prostaglandin E2 production.

CRS may appear with or without nasal polyps (NP) (CRSwNP or CRSsNP, respectively) [2]. The pathogenesis of CRS is multifactorial. Most studies report that CRSwNP, similar to allergic asthma, is the result of a Th2-type inflammation, with recruitment of eosinophils to the inflamed tissue via cytokines, such as interleukin (IL)-4, IL-5, and IL-13, and chemokines (specifically eotaxins) [4]. Regulatory T cell involvement in this Th2-driven immune process was also suggested [5]. The physical cell barrier, comprised of tight junctions, adherent junctions, and desmosomes, is damaged by the action of inflammatory mediators, causing abnormal ion transport, tissue permeability, and bacterial colonization [1,6-8]. While the pathogenesis of nasal polyps is not entirely understood, it seems that CRSwNP and asthma share common features in terms of inflammatory cell composition, suggesting that they represent different manifestations of the same disease [9,10].

Cell culture and animal model systems may serve as useful tools for the investigation of nasal polyps. Although cell cultures are relatively simple and reproducible, they fail to represent the complexity of human tissues and therefore may not be sufficiently reliable. Likewise, animal models may not represent the human immune system faithfully and, there is no reasonable animal model for CRSwNP. To address the need for an informative experimental system, we studied a novel ex vivo nasal polyp organ culture. This approach allows maintenance of the studied tissue under conditions designed to mimic the in vivo microenvironment, preserving organ viability and func-

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tion. Validation of such an experimental system requires proof of viability, normal metabolism, capacity to acquire infection and response to pharmacological intervention.

PATIENTS AND METHODS

EX VIVO NASAL POLYPS PREPARATION

NP samples were obtained from adult patients undergoing endoscopic sinus surgery for chronic sinusitis with nasal polyposis. Patients with underlying autoimmune diseases and those taking steroids were excluded from the study. Tissue processing began within 2 hours of surgery and warm ischemia time did not exceed 30 minutes. NP samples were washed twice in RPMI, and 250–500 µm-thick sections were prepared using a microtome. Sections were incubated in RPMI-1640 (Biological Industries, Beit Haemek, Israel) and supplemented by 5% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Glucose was added to a final concentration of 450 mg/dl. The tissue was incubated in 48-well plates (250 µl per well) at 37°C, 5% CO_2. Media was replaced every 24 hours. Tissue viability was assessed daily by microscopic recording of cilia movement.

GLUCOSE UPTAKE ASSAY

Media from each well were analyzed every 24 hours before replacement with fresh media. Glucose concentrations in the media were measured by a glucometer. Glucose uptake is expressed as the difference in glucose concentration between a well containing a tissue to a well without tissue.

HISTOPATHOLOGICAL ANALYSIS

Tissue samples were analyzed every 24 hours to assess viability and structure integrity. The samples were fixed in 3.7% formalin for 24 hours. Formalin-fixed paraffin-embedded tissues were cut into 5 µm sections, fixed on slides, de-paraffinised in xylene, and dehydrated. Sections were stained with hematoxylin and eosin (H&E) and analyzed under a microscope for variation in structure and cellular composition.

INFECTIVITY ASSAY

Viral vectors that specifically infect human respiratory epithelium were used to infect the NP tissue samples. Tissues were incubated for 1 hour in minimum essential media (150 µl) for viral absorption, after which an additional 100 µl of media was added to maintain tissue viability. Two types of vectors were used for these experiments. The human herpes simplex virus 1 (HSV-1) 17+/pR20 5/5 containing the β-galactosidase (β-Gal) gene under the control of the Rous sarcoma virus (RSV) promoter and the green fluorescent protein gene under the control of the constitutively expressed cytomegalovirus (CMV) promoter [11]. Type 5 adenovirus (AD5) viral vector, an E1A-deleted, replication-defective adenovirus, encoding β-Gal and driven by the constitutively expressed CMV promoter.

IN SITU β-GAL DETECTION

Organ cultures were fixed for 10 minutes in 0.2% glutaraldehyde, 2% formaldehyde, and 2 mM $MgCl_2$ in phosphate buffer saline (PBS) (pH 7.4), washed in PBS, and incubated for 1 h at 37°C with X-Gal substrate 20 mg/ml (Sigma, Rehovot, Israel) followed by fixation with 4% formaldehyde.

QUANTIFICATION OF CYTOKINE AND CHEMOKINE SECRETION

Tissue samples were placed in a 48-well plate with 250 µl of media per well. The tissue was incubated overnight for recovery and the media was then replaced. Media was collected after 24 and 48 hours for measurement of cytokine levels. Quantification was measured by a custom-made Q-Plex™ Human Cytokine array for 9 cytokines in the Q-View Imager system (Quansys Biosciences). Nine cytokines and chemokines were detected in the media: eotaxin, interferon (IFN)γ, IL-1b, IL-2, IL-5, IL-6, IL-10, IL-13, and tumor necrosis factor (TNF) α, using a multiplex ELISA assay.

QUANTIFICATION OF CYTOKINE AND CHEMOKINE EXPRESSION BY REAL-TIME POLYMERASE CHAIN REACTION

Tissue samples were placed in a 48-well plate with 250 µl of media. The tissue was incubated overnight for recovery and media was replaced with organ culture media supplemented with dexamethasone 0.2 and 20 mg/ml. After 24 and 48 hours, the tissues were collected for quantification of cytokine-mRNA. Total RNA extraction was performed according to the protocol described in the Nucleospin RNA II kit (Macherey Nagel's Duren, Germany). cDNA was generated with random primers using the reverse transcriptase reaction (Promega, Madison, Wisconsin, USA). Cytokine mRNA levels were quantified by real-time polymerase chain reaction (PCR) using cyber green (Quantabio Beverly, Ma., USA) specific primers for the different genes (Trailer end: 5'-GTCTCATTCACCAAATC-3',

Trailer start**:** 5'- GTACTTGACTCGTGCTCAAC -3'; β-actin forward: 5'-CCAACCGTGAAAAGATGACC-3', β-actin reverse:5'-GCTGTGGTGGTGAAGCTGTA-3'; Gapdh forward: 5'-ATGGGGAAGGTGAAGGTCGG-3', Gapdh reverse: 5'-TGACGGTGCCATGGAATTTG-3'; IL10 forward: 5'-GACTTTAAGGGTTACCTGGGTTG-3', IL10 reverse: 5'-GAAGAAATCGATGACAGCGCCGT-3'; IFNγ forward: 5'- GCAACAAAAAGAAACGAGATGACTTCG-3', IFNγ reverse: 5' TGAGTTCATGTATTGCTTTGCGTTG-3'; IL1β forward: 5' TGGAGCAACAAGTGGTGTT-3', IL1β reverse: 5'-TTGGGATCTACACTCTCCAGCT-3'; MX1 forward: 5'-ACACATATCTGTAAATCTCTGCCCCT-3', MX1 reverse: 5'-GGCATTAACTTTATCTATCAGGAAGAACA-3'). Analysis was performed by the SDS 2.3 software (ABI) and normalized to the GADPH gene.

This study was approved by the Hadassah institutional review board.

RESULTS

HISTOLOGY AND MOTILITY OF CILIA IN CULTURED HUMAN NASAL POLYPS

NP histology and motility of the cilia were evaluated in tissue sections that were kept in media. H&E staining showed no significant change in the respiratory epithelium, blood vessels, and cellular infiltrate on the day of tissue collection as well as 24 and 48 hours later [Figure 1]. Furthermore, cilia motility was also intact after 48 hours of incubation as observed under an inverted microscope (data not shown).

GLUCOSE UPTAKE BY TISSUE SECTIONS

Glucose uptake by the NP tissue was measured as an indication for viability and preserved metabolism. Glucose concentration was measured every 24 hours, prior to each daily change of culture media, and the concentration decrease served as an index for glucose consumption. A slight decline in glucose consumption was noted during the first 72 hours of incubation, followed by a considerable decline on the fourth day [Figure 2].

INFECTIVITY ASSAYS

Lac Z transfected viruses expressing the β-galactosidase enzyme were used for tissue infection, as an additional proof of tissue viability. NP tissue in culture was infected with the AD5 and HSV-1 viruses. The resulting blue-colored reaction demonstrated viral β-galactosidase expression within the tissue [Figure 3]. Viral replication was restricted to the respiratory epithelium on the surface of the polyp.

CYTOKINE EXPRESSION AND RESPONSE TO CORTICOSTEROIDS

To confirm the applicability of our model to the evaluation of inflammatory processes, we examined cytokines and chemokines secreted by cultured NP to the media. Nine cytokines and chemokines were evaluated: eotaxin, IFNγ, IL-1b, IL-2, IL-5, IL-6, IL-10, IL-13, and TNFα, using a multiplex ELISA assay. All cytokine levels, most notably IL-10, IL-5, and IL-6, were within the detection range of the assay [Figure 4A]. Cytokine levels were generally stable for the first 48 hours in culture media.

Figure 1. Histology of nasal polyps
Hematoxylin and eosin-stained sections of nasal polyps, immediately after excision (0) and following incubation of 205 µm sections for 24 hours and 48 hours, as indicated. Arrows point to an intact respiratory mucosa. The tissue samples demonstrate a noticeable inflammatory infiltrate

Figure 2. Glucose uptake by nasal polyps tissue during incubation
Media was replaced daily and glucose content was measured by a glucometer prior to media exchange. Consumption was calculated as the reduction in glucose concentration, expressed in mg/dl, compared to media without tissue. Data was collected from three independent experiments

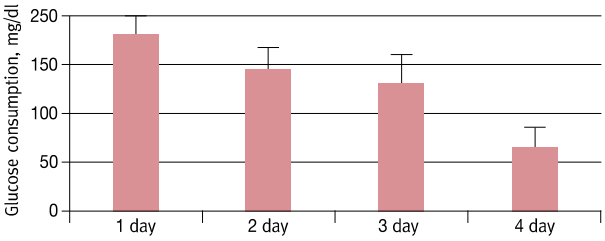
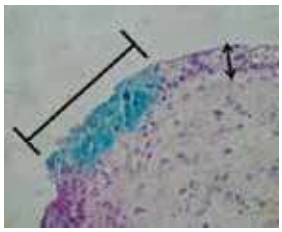


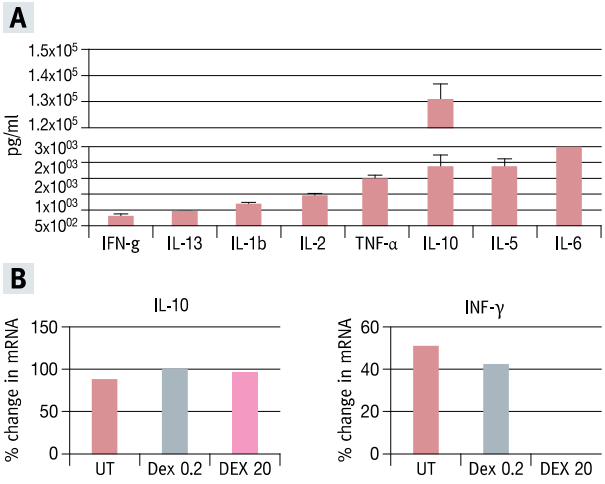
Figure 3. Viral infectivity assay with HSV-1 infection



H&E stain, 400-fold magnification. The bar indicates the blue-colored reaction demonstrating β-galactosidase expression in a virus-infected viable tissue. The arrow shows the respiratory mucosa. A similar result was obtained with AD5 infection (data not shown). This is a representative result of 3 independent experiments

H&E = hematoxylin and eosin

Figure 4. Cytokine production and response to dexamethasone
[A] Media was collected after 24 hours of nasal polyps-incubation for measurement of cytokine levels by a cytokine array. The results show the average and standard error for 9 independent experiments; **[B]** Real-time PCR for interleukin (IL)-10 and interferon (IFN)-γ in NP following introduction of a corticosteroid to the media



DEX 0.2 = dexamethasone 0.2 mg/ml, DEX 20 = dexamethasone 20 mg/ml, PCR = polymerase chain reaction, UT = untreated control

The validity of our model to pharmacological studies was demonstrated by the introduction of dexamethasone for 2 days in tissue cultures. For this purpose, we examined IL-10 as a cytokine that was not expected to decrease by dexamethasone and compared it to the steroid-sensitive IFN-g. Changes in cytokine expression were determined by real-time PCR. IL-10 expression remained unchanged after 24 and 48 hours of incubation with dexamethasone while IFNγ levels decreased considerably. Incubation with high-dose dexamethasone completely suppressed IFNγ expression after 24 hours [Figure 4B].

DISCUSSION

Cell cultures have traditionally been regarded as a model for studying the airway epithelium [12]. However, primary cultures are limited by a short lifespan, thereby requiring transformation, or immortalization for sustained survival, resulting in loss of certain differentiated functions, such as ciliary activity [13]. The nasal epithelium has often been used a source for cell cultures due to its availability and to the common features that it shares with the lower airways [12]. NP-derived cell cultures have been used for the study of topical drug delivery [14], mechanisms of wound repair [15], cytokine gene expression [16], and attachment of microorganisms [17]. It is accepted that cell functions depend on the coexistence of different cell types [18]. However, the benefit of cultured cells is compromised by the fact that they do not fully retain the primary phenotype and do not reproduce the complex interactions among the various cell layers that constitute the original tissue.

Animal models of nasal polyps have also been investigated. Models that are based on the rat middle ear [19] and the rabbit sinus [20] have shown that polyps may be induced by local trauma (epithelial disruption) and bacterial inoculation. Nevertheless, the histological differences between human airways and those of animal species have questioned the reliability of these models [21]. This concern has been addressed by the human xenograft system, involving the transplantation of human epithelial cells in immune-tolerant (or severe combined immunodeficient mice). The chimeric model reconstitutes a human adult airway and can be used for the study of inflammatory responses to different types of injuries, the effect of pharmacological molecules, and stimulation of epithelial regeneration by viral vectors [21].

Contrary to cell cultures and animal models, organ cultures have the potential to provide experimental conditions that retain the human physiology. Explants from various human tissues (e.g., bronchus, pancreas, and esophagus) can be obtained during surgery or autopsy and grown in culture media. These specimens maintain the cellular composition and architecture of the original organ [22]. For example, cultured adenoid tissue represents the respiratory mucosa and is used to investigate cellular responses to various agents [23]. Organ cultures of human nasal turbinates have been instrumental in the study of the carcinogenic effects of xenobiotics in vitro [22]. Furthermore, organ cultures demonstrate a metabolic capacity that is absent in monolayer cells [22]. Proteolytic enzymes that are used for dissociation of cells, may modify their surface properties [18] thereby inhibiting their ability for uptake of drugs and other molecules.

In the current study, we presented a novel ex vivo model for NP. Our results show that the tissue samples survive for a minimum of 3 days. This conclusion is supported by tissue histology, glucose uptake, cilia function and infectivity assays. Analysis of cytokine and chemokine secretion by the tissue demonstrated considerably high levels of IL-6 and IL-5. These findings correspond with previous reports that IL-6 and other pro-inflammatory cytokines and growth factors, and is increased in nasal polyp tissue [24]. We have also demonstrated that incubation of excised NP tissue with high-dose steroids resulted in suppression of IFN-γ. This result is in accordance with other studies showing inhibition of cytokine release with dexamethasone [14,16].

Although NP are considered benign lesions, nasal polyposis is associated with significant morbidity and an economic burden for the healthcare system. Furthermore, due to the resemblance of NP-related inflammatory processes to certain asthma endotypes, ex vivo models will become useful not only for the investigation of nasal polyposis and chronic rhinosinusitis but also for novel aspects of asthma pathophysiology and therapeutic approaches. Our ex vivo model presents several advantages. It allows characterization of cellular dynamics within the tissue and delineation of differences between allergic and non-allergic NP. Moreover, the fact that sections can be infected with pathogens can be useful in elucidating the mechanisms of infection and their pathogenic role. In addition, this model allows quantification of cytokine levels and their response to various therapeutic modalities. Monoclonal antibodies with proven efficacy in asthma can be readily tested in the NP ex vivo system, including omalizumab (anti-IgE), mepolizumab (anti-IL-5), and dupilumab (anti-IL-4α and IL-13) [6,7]. The model we presented can be used for the studying the modification of the nasal microbiome, restoration the epithelial barrier, and T cell responses [1].

CONCLUSIONS

The pathogenesis of chronic rhinosinusitis, with and without nasal polyps, has not been fully elucidated thus far. The existing experimental tools are limited, raising the need for new model systems. We presented a novel ex vivo nasal polyp culture, which is simple and readily accessible. We propose that this model will allow better understanding of the structural, mechanical, toxic, and immunological factors underlying this disease, leading to the development of novel therapeutic approaches.

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Capsule

Bile acid metabolites control T_H17 and T_{reg} cell differentiation

Bile acids are abundant in the mammalian gut, where they undergo bacteria-mediated transformation to generate a large pool of bioactive molecules. Although bile acids are known to affect host metabolism, cancer progression, and innate immunity, it is unknown whether they affect adaptive immune cells such as T helper cells that express IL-17a (T_H17 cells) or regulatory T cells (T_{reg} cells). **Hang** et al. screened a library of bile acid metabolites and to identify two distinct derivatives of lithocholic acid (LCA), 3-oxoLCA, and isoalloLCA, as T cell regulators in mice. 3-OxoLCA inhibited the differentiation of T_H17 cells by directly binding to the key transcription factor retinoid-related orphan receptor-γt (RORγt) and isoalloLCA increased the differentiation of T_{reg} cells through the production of mitochondrial reactive oxygen species (mitoROS), which led to increased expression of FOXP3. The isoalloLCA-mediated enhancement of T_{reg} cell differentiation required an intronic *Foxp3* enhancer, the conserved noncoding sequence (CNS) 3, which represents a mode of action distinct from that of previously identified metabolites that increase T_{reg} cell differentiation and require CNS1. The administration of 3-oxoLCA and isoalloLCA to mice reduced T_H17 cell differentiation and increased T_{reg} cell differentiation, respectively, in the intestinal lamina propria. The data suggest mechanisms through which bile acid metabolites control host immune responses, by directly modulating the balance of T_H17 and T_{reg} cells.

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Eitan Israeli

"Guard within yourself that treasure, kindness. Know how to give without hesitation, how to lose without regret, how to acquire without meanness"

George Sand [pen name of Amantine-Aurore-Lucile Dupin] (1804–1876), novelist