REVIEW —Current Perspective—

Prospects for Gene-Based Immunopharmacology in Salivary Glands

Seiichi Yamano and Bruce J. Baum*

Gene Therapy and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892, USA

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ABSTRACT—The clinical potential of gene transfer is increasing. One likely major application of this emerging biotechnology will be for gene therapeutics, the use of a gene as a drug. Salivary glands provide an unusual but increasingly valuable target site for gene transfer. Studies in animal salivary glands from several laboratories, including our own, have provided proof of this concept. In this review, we provide a background and perspective on possible strategies for gene-based immunopharmacology in salivary glands. We use as a target disease model the autoimmune exocrinopathy Sjögren's syndrome.

Keywords: Gene therapeutics, Salivary gland, Autoimmune disease, Cytokine, Adeno-associated virus

Physiology of salivary glands

Salivary glands are classical exocrine organs composed almost entirely of well-differentiated epithelial cells of two general types (1, 2). The bulk of the gland consists of acinar cells, which are both capable of generating a trans-epithelial salt gradient and relatively water-permeable. They are considered to be the sole site of fluid secretion in these glands. Acinar cells also secrete approx. 85% of the exocrine proteins found in saliva. The second major portion of the gland is the ductal region. This is continuous with the acinar region and consists of epithelial cells that, in aggregate, are salt absorbing and relatively water-impermeable. Ductal cells secrete the remaining approx. 15% of salivary proteins but are considered incapable of fluid secretion. From a ductal orifice, which exits into the mouth, the lumen of the gland is lined with essentially a monolayer of ductal and acinar cells (2). This means that by cannulating a ductal orifice, such as is done clinically for obtaining contrast radiographs, a clinician or investigator has direct access to the luminal membranes of essentially every epithelial cell in a gland. Because of their easy access, ability to produce considerable quantities of protein and secretory nature, we have suggested that salivary glands provide an inviting, if unusual, target for *in vivo* gene transfer (3).

Sjögren's syndrome is the single most significant pathology affecting salivary glands

The most prominent disease affecting salivary gland function is Sjögren's syndrome (SS). SS is an autoimmune disease, characterized by a focal and diffuse lymphoid cell infiltration into the salivary and lacrimal glands (4). The consequence of chronic immune cell activation in these exocrine glands is diminished secretory function, due to the loss of acinar cells. This leads to symptoms of dry mouth (xerostomia) and dry eyes (xerophthalmia). SS occurs in two forms, primary and secondary. Primary SS exists in the absence of another autoimmune disease, while in secondary SS, an additional, co-existing autoimmune condition such as rheumatoid arthritis or systemic lupus erythematosus is present. In the U.S., there are an estimated 2 million persons with either primary or secondary SS. Although there is more restricted tissue involvement in the primary form, primary SS is a systemic disorder with extensive serologic immune reactivity and potential involvement of other organ sites, termed extraglandular manifestations. The cells comprising the infiltrates have been described in detail. They are predominantly mature T cells of the CD4⁺ subset, with a smaller percentage of CD8⁺ cells. These T cells account for about 80% of the total infiltrate, with the remaining 20% composed of B cells and plasma cells (5).

Studies in SS have especially focused on the infiltrating lymphocytes in the salivary glands, since T cells are a known and rich source of cytokines. Cytokines are an important group of secreted protein mediators which may

* Correspondence and reprint requests to: Dr. Bruce J. Baum, GTTB/NIDCR/NIH, Building 10, Room 1N113, 10 Center Drive, MSC 1190, Bethesda, MD 20892-1190, USA

regulate many aspects of cell growth, activation, differentiation and other effector functions. They are major modulators of inflammatory and immune responses. Studies of lymphocyte signaling in mice indicate a complex series of regulatory interactions between lymphocyte subsets. In mice, clones of CD4⁺ T cells show divergence into T helper 1 (Th1) and T helper 2 (Th2) subpopulations (6). The Th1 cells produce cytokines such as interleukin 2 (IL-2), interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) that are associated with cell-mediated immunity. Such activities are effective in combating viruses and extracellular organisms. The Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which stimulate humoral responses. Certain infections and atopic diseases show a predominance of Th1- or Th2-like activity (7). In many autoimmune conditions with localized manifestations, alterations in cytokine concentrations have been described at involved sites. Cytokine production and release at these specific tissue sites are believed to provide a possible mechanism for the destructive changes seen in these conditions.

The results of many studies with salivary tissue from SS patients indicate that CD4⁺ T cells produce predominantly the Th1-like cytokines IL-2, IFN- γ and TNF- α and that salivary epithelial cells also produce several cytokines including IL-1 β , TNF- α and IL-10 (8). These cytokines can also be detected in saliva of SS patients at increased levels. Thus, recent studies in both patients and animal models indicate that in SS, the glandular epithelial cells are not only victims of autoimmune attack but also active co-participants in the pathological process. While the roles that the individual cytokines play in the pathogenesis of the disease are still not established, the Th1 cytokines likely stimulate cytotoxic T cell processes within the gland.

The etiology of SS is still unknown, and consequently curative treatments are not available. The treatment options for managing the extraglandular manifestations of SS are also limited. For SS patients with some functional gland parenchyma remaining, it is possible to partially recover, or restore, some salivary function with the use of hydroxychloroquine, perhaps by effects on inflammatory processes (9), and treatment with parasympathomimetic agonists such as pilocarpine (10). Since some aspects of the immunopathogenesis of SS are appreciated, it seems reasonable to suggest that immune-regulating agents may be of therapeutic value. However, it is not yet possible to use an exact and evidence-based algorithm for such therapeutic decision making.

Gene-based protein therapeutics

The use gene transfer for clinical purposes was originally envisioned to correct untreatable inherited disorders of metabolism and malignancies refractory to conventional therapy. However, in recent years, it has been clear that

clinical gene transfer may be beneficial for numerous other circumstances including acquired conditions and for disorders affecting the quality of life. The key element underlying the development of all current clinically related applications of gene transfer is the absence of any effective conventional treatment for a given condition.

One major application for clinical gene transfer that has been the focus of considerable effort is the use of a gene as a drug, so called gene therapeutics (11, 12). There are many systemic single protein deficiency disorders, such as diabetes, hemophilia A, erythropoietin-responsive anemias, and growth hormone deficiency, that are currently managed by the repetitive injection of a recombinant protein. While this treatment method is useful and circumvents concerns about the possible co-administration of pathological contaminants in biochemically purified proteins from natural sources, it is still considered inconvenient and certainly not an ideal form of therapy (e.g., 13, 14).

In theory, the stable transfer of a gene encoding a deficient protein, and its appropriate regulation, could lead to the correction of such clinical defects. Thus far, no gene-based therapy has replaced the conventional use of repetitive protein injections. However, there have been several proofs of this concept demonstrated in animal model studies using gene transfer. Our laboratory has made a substantial effort to explore the potential of using salivary glands as a target site for gene therapeutics (e.g., 15–17).

Transferring genes to salivary glands

Salivary glands have proven to be very good targets for gene transfer using many different vectors. There are essentially two general ways to transfer genes: using non-viral methods or using viruses. The use of recombinant viruses takes advantage of natural, biologically evolved mechanisms. Viral-mediated gene transfer generally can be viewed as highly efficient but with some risk for safety. Conversely, non-viral methods currently are viewed as being much less efficient for gene transfer, but without significant safety risk. There are several non-viral methods used to transfer genes including cationic liposomes (18, 19); macromolecular conjugates of the DNA with, for example, a cell receptor ligand or a polycation (e.g., 20); and the direct injection of the DNA by itself (21). Both cationic liposome mediated gene transfer (22) and DNA conjugate mediated gene transfer (23) have been successfully accomplished in salivary glands.

The most common viral vectors utilized are derived from retroviruses and adenoviruses. Retroviral vectors are capable of integration into the host chromosome, thus affording the possibility of stable gene transfer. However, retroviral vectors cannot be grown to high titers. They also require cell division for transduction. Both of these characteristics

hinder the use of retroviral vectors *in vivo*. Consequently, retroviral vectors are typically employed for *ex vivo* gene transfer. With *ex vivo* gene transfer, genes are delivered to cells expanded *in vitro* and then the cells are returned to the host. *Ex vivo* gene transfer to salivary glands is not possible because the differentiated acinar cells cannot be grown and amplified *in vitro*. Consequently, while retroviral vectors are able to infect a small number of cells *in vivo*, and thus mediate gene transfer to salivary glands, they result in very low levels of cell transduction that seem unlikely to be of practical value (24).

Conversely, adenoviral vectors can be grown to very high titers, allowing their ready use *in vivo*. Recombinant adenoviruses are also able to infect a wide variety of dividing and non-dividing cells. However, adenoviral vectors are essentially incapable of genomic integration (adenoviral DNA exists in the nucleus in an extra-chromosomal location), and they elicit a potent immune response that may cause tissue damage (see below). Both of these traits are drawbacks that render adenoviral-mediated gene transfer unstable or transient. Nonetheless, adenoviral vectors are extremely useful for proof of concept studies, and accordingly, they have been widely employed. There are many studies reporting adenoviral vectors for gene transfer to the salivary gland. A variety of genes have been transferred including genes encoding hormones (human and mouse growth hormone: 17, 25, respectively), an antimicrobial agent (histatin 3: 15), membrane proteins (aquaporins 1 and 5: 26, 27, respectively), a transcription factor (E2F-1: 28), a protease inhibitor (α 1-antitrypsin: 16), and several bacterial or insect reporter proteins (β -galactosidase, chloramphenicol transferase, luciferase: 3, 29, 30, respectively). Quantitative immunochemical detection studies in rat submandibular glands have shown that about 25–30% of the glandular parenchymal cells expresses the transferred gene following recombinant adenoviral infection.

Because of the above problems arising with these two viruses in salivary and other tissues, many laboratories are exploring the use of different viral vectors for gene transfer, e.g. adeno-associated virus (AAV), lentivirus and herpes virus. Of these vectors only the use of AAV in salivary glands has been reported (31). AAV is a small (<5 kb), single strand DNA, dependent parvovirus. Wild type AAV has the unique ability to integrate into human chromosome 19 at a specific site. As yet, this is not achievable with recombinant AAV vectors, but the theoretical possibility means that long term, stable transgene expression may be achievable with AAV. Braddon et al. (31) have transferred the aquaporin-1 cDNA to murine salivary glands using a recombinant AAV. Ductal cells were the primary, but not only, cell type transduced in their experiments.

Several studies demonstrating proof of “clinical” con-

cept have been reported using recombinant adenoviral vectors in rat salivary glands. For example, Delporte et al. (26) showed that adenoviral-mediated transfer of the aquaporin-1 cDNA could achieve a type of repair of irradiation-damaged salivary glands. O’Connell et al. (15) provided an example of localized gene therapeutics with salivary glands. In these studies adenoviruses were employed to transfer the gene for an exocrine secretory protein, histatin 3. The goal was to use histatin 3 to augment the protein content of saliva in order to treat azole-resistant mucosal candidiasis, an upper gastrointestinal tract disorder. Finally, a number of studies have shown that gene transfer to salivary glands may be useful for systemic gene therapeutics; i.e., the treatment of single protein deficiency disorders, as noted earlier. Although salivary glands are considered classic exocrine glands, they may also secrete in an endocrine manner (e.g., 32, 33). Several groups including our own have used adenoviral and other gene transfer vectors to demonstrate that biologically active proteins can be secreted directly into the bloodstream from rodent salivary glands (e.g., 16, 17, 22, 34–36).

Some problems associated with salivary gland gene transfer

The major problem presently limiting clinical progress in gene transfer to salivary glands and other tissues is the lack of adequate vectors. All currently used vectors, both viral and non-viral, have problems that render them non-ideal. Many vectors do not lead to adequate expression of the transgene, such as mentioned above for retroviral vectors and cationic liposomes. Additionally, most vectors in use today are non-regulated, i.e., the transgene expression is either present or absent; and the level of expression is determined mainly by the choice of promoter for the construct. While several laboratories are working to develop convenient elements for regulating transgene expression in vectors, there is as yet no satisfactory general method.

In salivary glands, most published studies have used recombinant adenoviruses, and they provide an excellent example of some important drawbacks with existing vectors. As noted earlier, adenoviral-mediated gene transfer does not result in genomic integration, rather the foreign DNA remains in an extra-chromosomal location in the nucleus. Thus, by definition, with each cell division, the number of cells containing the transgene is reduced exponentially. This trait certainly limits use of adenoviral vectors for purposes requiring long term, stable transgene expression, such as for the correction of an inborn metabolic error. However, for other purposes, a transient expression of the transgene may be either desirable or acceptable. For example, a time limited pharmacotherapeutic use, analogous to using a conventional antimicrobial regimen, may be ideal for our envisioned use of histatin 3 gene transfer to treat azole-resistant candidiasis, as described

Fig. 1. Schematic depiction of strategy for using recombinant adeno-associated virus (rAAV)-mediated immunopharmacological regulation in Sjögren's syndrome. Interleukin 10 (IL-10), is a Th2 lymphocyte-derived cytokine, which has the ability to inhibit cytokine production (IL-2, IFN- γ and TNF- α) by Th1 lymphocytes. rAAV encoding IL-10 (rAAVIL10) is delivered into the affected salivary glands, where IL-10 is locally secreted. Major salivary glands (parotid and submandibular) are shown in white. We hypothesize that the increased local concentration of IL-10 will lead to a down regulation of Th1 cytokines and, consequently, the glandular inflammatory infiltrate.

above.

However, the greatest complication of adenoviral vector use is their induction of a potent, often fulminant, host immune response, involving the innate, humoral and cellular arms of the immune system (e.g., 29, 37). Worgall et al. (38) showed that up to 90% of viral DNA delivered to the liver is lost within 24 h due to dendritic cell activity. Similarly, neutrophils and tissue macrophages appear to greatly limit early transgene expression in salivary glands (30, 37). Thereafter, the cellular immune response destroys transgene expressing target cells, resulting in damage to salivary gland tissue, while neutralizing antibodies will effectively prevent successful re-administration of the vectors (29).

Possible immunopharmacological therapy for Sjögren's syndrome

Recently, gene therapy strategies have begun to be applied to autoimmune diseases (39–41). For example, cells targeted for autoimmune attack may be genetically modified to express immunoregulatory cytokines that protect them from autoimmune-mediated destruction. Such target cells include pancreatic β cells in type I insulin-dependent diabetes mellitus (42), myelin-producing oligodendrocytes in multiple sclerosis (43) and synoviocytes in rheumatoid arthritis (44). In a complementary strategy, T cells that are tissue-specific and autoreactive can be transduced with anti-inflammatory cytokine genes and delivered to autoimmune lesions (45). Furthermore, autoimmune target cells and autoreactive T cells may be genetically engineered to

produce therapeutic transgenes. These include genes encoding growth factors for mediating the repair of tissue damaged during autoimmune disease (46).

In our laboratory, we are examining gene-based strategies potentially useful for SS. For this purpose, we have chosen a vector, AAV, which may be appropriate for long term transgene expression. Our initial emphasis has been quite simple: we used a recombinant AAV (rAAVIL10) to direct the expression of a single transgene, IL-10. We hypothesize that the encoded IL-10 will modulate the diseased cytokine profile in SS salivary tissues (Fig. 1; see ref. 47). IL-10 is a Th2-derived cytokine with a variety of biological effects *in vitro*. For example, IL-10 can inhibit macrophage-dependent antigen presentation, antigen-specific T cell proliferation, as well as cytokine production (IL-2, IFN- γ and TNF- α) by Th1 cells. These findings suggested to us that local delivery of IL-10 to salivary glands might be able to inhibit SS disease progression.

Studies from several laboratories have suggested that NOD mice provide a reasonable animal model for SS (47–50). We have begun to use NOD mice to test our hypothesis; i.e., that rAAVIL10 administration to mouse salivary glands will lead to both a reduction in the glandular inflammatory infiltrate and an increase in salivary secretion when compared with findings from control mice. We expect that these outcomes will occur as a result of a shift in the local Th1/Th2 cytokine balance. However, this initial simple approach may be inadequate, and it may be necessary to employ alternative strategies.

Conclusion

Many animal studies have shown that gene transfer to salivary glands is readily achieved and reasonably efficient. Vector deficiencies clearly limit its routine clinical application at present. We have chosen to use this technology in order to manage the salivary gland pathology associated with the autoimmune exocrinopathy SS. At present, there is no conventional therapeutic approach successful in treating patients with this disease. We propose a novel gene transfer-based strategy for treating SS, which aims at using a recombinant AAV encoding IL-10. We hypothesize that the recombinant IL-10 produced will be capable of modulating the localized cytokine profile in diseased salivary tissue, thus limiting SS disease progression. Regardless of the outcome of this initial approach, we believe that the prospects for gene-based protein therapeutics in salivary glands are excellent.

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