Quantitation of indirect immunofluorescence on paraffin sections with confocal microscopy

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SUMMARY

Salivary and lacrimal gland secretions are reduced in primary Sjögren's syndrome (pSS). Aquaporins (AQP) are involved in transmembrane water transport and different isoforms show specific cellular and subcellular distributions in salivary and lacrimal glands. Aquaporin-5 is present in the apical membrane of acinar cells, and we have previously demonstrated that its expression is not altered in pSS. By contrast, we report here that AQP1, which is restricted to the myoepithelial cells surrounding acini, shows decreased expression in salivary glands from pSS patients compared with healthy controls.

In order to understand disease processes in human, characteristic alterations that occur need to be determined so that appropriate experimental models may be developed. One approach is to quantify alterations in antigen expression in human biopsy samples obtained from patients with disease and compare the data with that from healthy controls. Tissue antigens are commonly visualised with either indirect immunofluorescence or immunoperoxidase techniques. It has been demonstrated previously that indirect immunofluorescence methods are associated with both less background and non-specific staining in paraffin sectioned material (Beroukas et al, 2002, Lancet paper).

Quantitative immunohistochemistry methods have a chequered history. Three decades of application of such methods has not resulted in their acceptance in routine diagnostic pathology or in experimental research models due to many reasons outlined recently by Shin et al (2000). Lack of consistency of results across laboratories and the difficulties in standardization of the immunohistochemical procedures are cited as confounding factors. In addition, often there is requirement for manual experimenter intervention, high cost of

equipment and visual analysis of obvious differences in staining patterns and intensity continue to be reliable for diagnostic evaluation (Shin et al 2000). None the less, a valuable role for quantitative immuohistochemistry in establishing disease processes can be demonstrated (ref? Polkowski et al 1997).

In this paper, we demonstrate a method that combines a stereology based, systematic random sampling method and, evaluation of intensity of immunohistochemistry, for the analysis of the role of aquaporin and muscarinc receptor antigens in the disease processes of Sjogren's syndrome in human. Formalin fixed biopsies of human minor salivary glands were sectioned and prepared for indirect fluorescent immunohistochemistry using antigen retrieval techniques to restore antigens (Taylor in Shin et al 2000). Confocal microscopy was chosen to view and sample tissue for quantitation because i) antigens of interest have restricted subcellular localisation and ii) with confocal microscopy, it was possible to restrict the thickness of optical section from which measurements of emitted fluorescence were obtained. This allowed direct comparisons to be made in fluorescence intensity between control and disease tissue.

To quantitate alterations in level of expression of antigens in human biopsy tissue sections is problematic. Changes of large magnitude are readily distinguishable by visual analysis of sections from human control and disease tissue with immunohistochemical techniques, and there are a number of quantitative techniques available with low power light microscopy that can be utilised to demonstrate such alterations. However, some changes in the disease tissue may not be discernable at low power microscopy level because the substance of interest is

present in small amounts in restricted locations within cells. The fact that these components are present in small amounts may not reflect their importance. For example, receptor molecules for neurotransmitters are often arranged in restricted clusters at variable intervals along the plasma membrane (Southwall and Bennet paper?) and have obvious importance for post-synaptic cell function.

In this paper, we demonstrate a method to quantitate antigens visualised with indirect immunofluoresence and viewed with confocal microscopy. The indirect immunofluorescent method was chosen because of the demonstrated reduction in background and non-specific staining compared with immunoperoxidase methods (Beroukas et al 2002). Confocal microscopy was chosen for quantitation because i) antigens had restricted subcellular localisation and ii) it was possible to restrict the thickness of optical section from which measurements of emitted fluorescence were obtained. This allowed direct comparisons to be made in fluorescence intensity between control and disease tissue.

For this study, paraffin sections from human biopsy samples were taken from the labial salivary glands of healthy controls or patients with primary Sjögren's syndrome (pSS). PSS is an autoimmune disease characterised clinically by dry eyes and mouth, indicating a reduction in fluid secretion from lacrimal and salivary glands. Antigens that were analysed were two aquaporin molecules (AQP1 and AQP5) and the M3R muscarinic receptor. The M3R receptors are located on cells within the salivary gland (Beroukas et al Lab Invest 2002) to mediate responses following release of acetylcholine in the nearby parasympathetic nerves (ref ? who demonstrated cholinergic innervation?). Aquaporins are water channel proteins providing a pathway for osmotic water flow across cell membranes (Nielson et al 1997), including across salivary epithelial cells.

Materials and Methods.

In order to quantitate the immunofluorescence, it is necessary to sample the tissue appropriately. The samples should be unbiased and representative and sufficient samples need to be taken.

Tissue preparation

Salivary gland tissue was obtained by labial gland biopsy from patients who fulfilled the European consensus criteria for the diagnosis of pSS, including seropositivity for anti-Ro/La antibodies, unstimulated salivary flow <1.5ml per 15 min and labial salivary gland biopsy focus scores or >1 per 4 mm^2. Disease duration ranged from 1-22 years. Biopsy tissues were obtained from healthy controls; one group from Adelaide, Australia and another group from Sweeden. Unstimulated salivary flow was >4ml per 15 min and control labial salivary gland tissues were histologically normal, with no foci of lymphocytes.

Indirest immunohistochemical localisation on paraffin-embedded tissue

A four-micron thick section was cut from each paraffin block containing the salivary gland biopsies. Sections were dried onto histogrip-coated slides overnight at 57° C, deparaffinised, and microwaved for 10 mins using the antigen-retrieval method protocol of Shi et al (1991) in citrate buffer (pH 6.0)(Toshiba microwave at 1000W). Non-specific protein binding was blocked by incubation with undiluted Protein Block Serum-Free (PBS-F, Dako Corporation, Carpinteria, CA) and sections were then incubated with the appropriate previously characterised primary antibody (Table I), washed in 0.1% Tween 20 in Tris buffered Saline pH 7.4 and then incubated with the appropriate second antibody conjugated to indocarbocyanin fluorphore (CY3) (Table I). Sections were washed and mounted in

phosphate buffered glycerol containing 1,4 Diazabicyclo-(222)-octane anti-fade chemical. Consecutive sections from each tissue block were stained with Mayer's haematoxylin and eosin for histological examination.

Controls

The specificity of the primary antibodies were determined by 1) omission of primary antibody from incubation protocols, 2) pre-absorption with immunising peptide (and in the case of AQP5, pre-absorption with peptide corresponding to the human C terminus) and 3) pre-absorption with unrelated peptide, calreticulin at 100 µg/ml. In all cases, specific immunofluorescent labelling was abolished in the control sections (Beroukas et al 2002, 2001 and in press).

Unbiased sampling.

Single random tissue sections from each patient, healthy control or with pSS, were chosen for analysis. An image was then taken from separate salivary gland acini. To avoid bias, sampling on the sections was systematic. Images were taken from acini at 100um intervals arranged in a grid pattern across the tissue. This was achieved with the aid of a motorised microscope stage. A beginning point was chosen at random (x10 objective lens), this position was set as zero, then the next position was obtained by moving 100um from this. This distance was chosen as acini are approximately 70um in diameter, and at 100um intervals between samples ensured that a new acini was sampled with each new image. (diagram – photograph (H&E with drawing over it with grid with points marked; then zoom into the membrane for image)

Data was collected with operator blinded to sample details.

Slides with tissue sections adhered were coded 1, 2, 3, etc. Images were thus collected without the microscopist knowledge of the samples disease state.

Minimisation of variation

Sections to be quantitated were prepared for immunohistochemistry on the same day and at the same time. Tissue from control and diseased patients were subject to all washes and antibody incubations together.

Data collection was standardised to collect from 0-256 pixel range.

Images for quantitation were collected on the same day. The sections from each experiment were initially observed and the sample with the brightest fluorescence was found. This sample was then viewed in the confocal microscope and the settings to collect the full range of brightness from 0-256 pixels of intensity, were established. Using these settings, the images for quantitation were then collected for all other samples for that experiment. Of critical importance is that confocal microscopy with the iris setting the same for all samples allows collection of emitted fluorescence from the same optical section thickness.

Images were collected from sections viewed with an Olympus AX70 microscope attached to a Biorad 1024 scanning confocal system equipped with a Helium/Argon laser. The 100x objective was used (NA 1.4) and a software zoom of 3.5 to obtain images 31.2µm x 31.2 µm in size. Confocal settings were laser power 1%, iris 2.0 and gain around 950 (variable between experiments but constant for each experimental group).

Number of Data points

Ten images were collected from each tissue section from control or diseased patients. The images were taken from two fields in each section and were generally away from the

lymphocytic infiltrate. This resulted in data from 10 acini per sample, selected in an unbiased manner (see above). The position for image was obtained using the motorised stage at low power (10 or 20x objective) and then the nearest piece of labelled acinar membrane was scanned at 100x objective with zoom of 3 (see Figure 1).

Quantitation of Images

Measurements of pixel intensity were made according to previous descriptions (ref – us). In each image, labelling of the acinar membrane was drawn around with the mouse and the average image pixel intensity was obtained using the Biorad 1024 Lasersharp software. Each image had 8-10 regions sampled (either the entire length of labelled membrane present for AQP1 and AQP5, or M3R clusters at 1cm intervals along the acinar membrane). Background pixel intensity for each image was measured in three locations without fluorescence and these were averaged, and subtracted from each measurement of labelled membrane in that image.

Results

? Should we do some experimentation with

- larger distance between acini so that more acini over larger area of section are sampled?
- Larger number of samples? – would need to do – control and diseased

Discussion

Is the data representative?

Demonstration of changes in tissue function either during development of normal animals (counts of cells?), resulting from gene manipulations in genetically engineered animals (Nurcombe et al 2001) or resulting from disease processes in human (Clark & Bertram, 2000 or something similar) or animal models of disease, depend upon appropriate quantitative methods.

Sampling

For quantitative analysis of data, the sampling procedure is very important to ensure that the sample chosen for analysis is representative of the tissue and thus able to be compared statistically with other representative data (Nurcombe et al 2001). The preferred method of sampling is the systematic uniform random approach (Bertram, 1995). At each stage of the sampling process there needs to be a systematic and a random component. For example, to choose sections through a tissue, the first section is chosen at random, and then systematically there after through the thickness of the tissue (Bertram 1995). Secondly, within the section, the first sample is chosen at random and then subsequently systematically for the fields within the section (Bertram 1995). Thus, at each stage of the sampling of a tissue, a random start is chosen and subsequent sampling is systematic (Bertram, 1995).

Quantitation of Immunofluorescence – requirements – reference Pawley J?

. Localisation of antigen –ie. Location of antigen in specific cell location

. Measurement of “amount”

Blinded measures - reference

In human salivary glands, AQP5 is restricted to the apical membrane of acinar cells, where it allows fluid outflow; we have recently demonstrated however that expression of this protein is unchanged in pSS [2]. Similarly AQP3, which is present in the basal membrane of the acinar cells [3] and presumably allows water inflow, is not altered in expression in Sjögren’s syndrome (Beroukas et al., unpublished results). Myoepithelial cells surrounding the secretory acini of salivary glands are believed to play an important role in maintaining intraluminal pressure during salivary secretion. Since AQP1 has been reported in

myoepithelial cells [3], we studied the distribution of this AQP isoform in control and pSS glands.

To assess expression and subcellular localisation of aquaporin-1, we used indirect immunofluorescence to stain paraffin sections of labial salivary gland (LSG) biopsies from 11 healthy controls and 12 pSS patients who had been used in our previous studies [2,4]. The patients all met European consensus criteria, had labial salivary gland focus scores greater than 1 per 4 mm^2 and were anti-Ro/La autoantibody-positive. Disease duration ranged from 1 – 22 years. Tissue sections were probed with an affinity-purified rabbit antibody (Alpha Diagnostic International, San Antonio, TX) that had been raised against the 19 amino acid sequence (aa 251 – 269) from the carboxy terminus of human AQP1. Immunoreactive cells were visualised using fluorescent-labelled secondary antibody. We used a microwave antigen-retrieval technique (citrate buffer: pH 6.0) to unmask epitopes and increase the detection sensitivity.

Indirect immunofluorescence detection showed that the distribution of AQP1 labelling was confined to the basal aspect of acini and intercalated ducts as shown previously [3]. AQP1 labelling patterns were similar in both control and pSS glands (Figure 1); however, the labelling was less intense in glands from each of the pSS patients. AQP1 labelling was also present in endothelial cells of non-fenestrated capillaries; however the intensity of this labelling did not alter in pSS (Figure 1). In the apical or lateral membranes of acinar cells (Figure 1), in striated ducts and in infiltrating lymphocytes, AQP1 labelling was absent. All labelling was abrogated by pre-absorption with 100 μg/ml of the human AQP1 peptide (Figure 1) but not by unrelated peptide (AQP5; not shown). Using high resolution confocal microscopy, we confirmed the basal labelling of AQP1 in acini and intercalated ducts.

To determine precisely the subcellular localisation of AQP1, double labelling immunofluorescence was performed. Labelling myoepithelial cells with an α-smooth muscle

actin antibody (MAB-003, Maxim Biotech; Figure 2) demonstrated that the AQP1 labelling is present principally luminal to the myoepithelial cell cytoplasm, in the luminal membrane of myoepithelial cells, and to a lesser extent in the abluminal membrane of the myoepithelial cells. Double-labelling for the M3-muscarinic receptor, which we have previously demonstrated is present on myoepithelial cells [4] , confirmed that AQP1 is on the luminal membrane of myoepithelial cells (data not shown). This suggests that acetylcholine released from parasympathetic nerve terminals acts on M3-muscarinic receptors on myoepithelial cells and that these in turn trigger water inflow through AQP1 channels in these cells; this mechanism is likely to be important in the rapid control of myoepithelial cell volume which is required to enable these cells to provide structural support during salivary secretion [5].

High resolution confocal microscopy was used to quantitate differences in AQP1 labelling of myoepithelial cells in pSS and control LSG biopsies. Quantification of fluorescence intensity of basal labelling of 10 acini per 5 control and 6 pSS glands on confocal microscopic images showed a highly significant decrease in pixel intensity in the same pSS patients who had previously shown no difference in AQP5 labelling [2] and increased M3-muscarinic receptor expression [4] (controls, mean 57.6 pixels [SE 2.6], pSS, mean 35.6 pixels [SE 2.4] Mann-Whitney *U* test, $p < 0.0001$). Investigators who viewed sections were masked to group, and all images were taken at the same settings on a BioRad 1024 laser scanning confocal system with 100x objective lens and a zoom of three. For each individual, we took 60 intensity measurements, corrected for background fluorescence, from 10 acini that had been selected by systematic sampling.

Our studies suggest that water flow into and out of myoepithelial cells may be altered in pSS, since AQP1 expression in these cells is decreased. Translocation of AQP5 molecules to the apical membrane of rat parotid acinar cells is dependent on M3-muscarinic receptor stimulation [6]. If the normal expression of AQP1 in myoepithelial cells is similarly dependent

on intact M3-muscarinic receptor-mediated neurotransmission, then the decreased expression of AQP1 may be a secondary consequence of the partial block of M3R-mediated neurotransmission in pSS glands [7]. These findings suggest that bypassing the block in neurotransmission and restoring AQP1 levels may represent a novel approach to treatment of the dry eyes and dry mouth in pSS. Furthermore, the myoepithelial cell may play a more important role in salivary secretion than previously recognised.

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Figure legend:

Figure 1. Immunolocalisation of AQP1 in labial salivary gland biopsies.

A, C, D=control. B=patient with pSS. Sections were labelled with an affinity purified, polyclonal antibody to human AQP1 and visualised using fluorescent labelled secondary antibody with conventional fluorescence microscopy (A, B) or confocal microscopy (C, D). AQP1 was detected in the basal aspect of acini in both control and pSS glands, but the intensity of labelling was greatly decreased in pSS glands. AQP1 labelling also occurs in blood vessels (bv) (B, C) and intercalated ducts (C). Labelling was blocked by pre-absorption with the human AQP1 peptide (D). Scale bars = 100 μm (A, B) and 50 μm (C, D).

Figure 2. Co-localisation of AQP1 with α-smooth muscle actin in labial salivary gland biopsies.

Actin (green) is used to label myoepithelial cells surrounding the secretory acini and intercalated ducts. AQP1 labelling (red) appears predominantly as continuous membrane labelling luminal to the actin in the myoepithelial cell (arrows). AQP1 also occur on the abluminal membrane of the myoepithelial cell (arrowheads).

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