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Immunohistochemistry of CFTR in native tissues and primary epithelial cell cultures

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Abstract

Studies on CFTR protein expression and localization in native tissues or in primary cultures of human epithelial cells are scarce due to the intrinsic instability of this protein, its low expression in most tissues and also to technical difficulties.

However, such data are of the highest importance to understand the pathophysiology of CF. The purpose of this article is to outline several assays for the characterization of primary epithelial cultures and to review different CFTR immunostaining protocols.
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1. Introduction

CFTR protein is expressed predominately in epithelia (for a review, see Ref. [1]). The original description of the *CFTR* gene was accompanied by analysis of the CFTR transcript by Northern blotting. Such results evidenced CFTR expression in the pancreas, sweat glands, intestine, lung, nasal polyps, and liver [2].

Most of the data on CFTR expression and function, however, result from studies performed on heterologous overexpressing cellular models or on immortalized cells endogenously expressing this protein [3]. Although polarized epithelial cell models are the best models to study expression and interactions of CFTR, they still exhibit deviations from native tissues brought about by the transformation process.

Very few groups have addressed the issue of CFTR protein expression in native human tissues or in primary cultures of human epithelial cells. Thus, the intracellular and histological distribution of CFTR in healthy controls and in patients is still poorly documented and data are often contradictory [4–10].

Direct studies of CFTR localization on human tissue are hampered by several difficulties, namely: (a) low abundance of CFTR protein in most tissues where it is endogenously expressed [11,12]; (b) lack of sensitivity of anti-CFTR antibodies (Abs) to detect this protein in native tissues (for a review on anti-CFTR Abs, see Ref. [13]); additionally, (c) epitopes recognized by some of these Abs frequently become inaccessible after routine tissue preservation

Abbreviations: Ab, antibody; BSA, bovine serum albumin; IHC, immunohistochemistry; PFA, paraformaldehyde; PBS, phosphate-buffered saline; RT, room temperature; TJ, tight junctions; TER, transepithelial resistance.

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[12,14], and therefore there are complex methodological challenges to be solved. An additional reason for the difficulty of these studies in native tissues from patients is that organs affected by CF such as the airways, pancreas, and intestine are massively destroyed by inflammation and remodeling processes.

Clearly, more studies on native tissues are mandatory as the knowledge obtained is critical to assess the impact of CFTR mutations on the cellular and subcellular localization of the protein.

For this purpose, a sensitive and efficient method of choice is often indirect immunostaining. However, the quality of the data largely depends on the specificity and sensitivity of the Abs and on the protocols applied, starting with the collection of patient cells/tissues and ending in the observation of the specimens at the microscope. It is thus crucial for CFTR research in general, and for studies of CFTR localization in native tissues, particularly the exchange of information among researchers on antibodies, tissue samples, and protocols [15].

The scope of the present article is to review methods for the localization of CFTR in native tissues and in primary cultures of epithelial cells. The isolation and primary culture of human airway epithelial cells are described elsewhere in this supplement [16–19] and in the literature [20]. Here, we provide an outline of the assays and tests necessary to confirm the differentiation and polarization status of these types of cultures. We also review the impact of the anti-CFTR immunostaining protocol used in terms of both preservation of tissue morphology and CFTR signal detected. The details of the protocols are given elsewhere [15].

2. Junctional and polarity markers in primary cultures of human epithelial cells

At cellular level, the degree of epithelial cell differentiation is of major importance.

In well-differentiated and polarized epithelial cell cultures, CFTR expression is comparable to that of freshly isolated cells [20]. When growing primary cultures of epithelial cells, optimal culture conditions have to be used, so that the cell culture preserves the ultrastructure, the ion transport properties, and the synthesis and secretion of molecules characteristic of the tissue from which the cells were originally derived. Thus, markers of polarity and differentiation should be analysed to confirm the differentiation status of cells, so that these are appropriate for CFTR localization and functional studies.

The plasma membrane of polarized epithelial cells is divided into two biochemically and functionally distinct domains, the apical and basolateral plasma membrane. This distinction generates *polarity*. Junctional protein complexes such as tight junctions (TJ), adhering junctions, and desmosomes, while playing a fundamental role in maintaining the polarized phenotype (TJs separate the apical and basolateral domains), also provide the *tightness* that is a characteristic of epithelia. Indeed, TJs selectively regulate the passage of molecules across the paracellular pathway (gate function) of epithelia and passively separate molecules into the apical and basolateral plasma domains (fence function). Epithelial cells thus asymmetrically distribute receptors, transporters, ion channels, and lipids between the apical and basolateral membranes to establish and maintain polarity and function (Fig. 1).

Thus, functional TJs are crucial to maintain the polarized phenotype of epithelial cells. Several tests can be used to monitor the existence of functional TJ. The experimental details and protocols for these tests are described elsewhere [15].

2.1. To control the presence of tight junctions

In epithelial cell monolayers grown on collagen-coated filters, two types of methods can be used to visualize the presence of TJs: transmission electron microscopy and immunolocalization of TJ proteins such as occludin (rat monoclonal Ab from Zymed Laboratories, San Francisco, CA, USA) and ZO-1 protein,[1] (mouse monoclonal Ab from Chemicon International, Temecula, CA, USA).

2.2. To monitor the gate function of TJ

Two assays can be utilized, namely: (1) transepithelial resistance (TER) measurements with the Ca^{2+} switch assay and (2) the lanthanum nitrate permeability test.

The measurement of TER allows the evaluation of the degree of tightness of the epithelial cells grown on collagen-coated filters. Commonly used for MDCK cells, the Ca^{2+}-switch assay monitors the formation of functional TJ. The protocol is described in detail elsewhere [15]. Briefly, cells incubated in Ca^{2+}-free medium are transferred to medium containing normal Ca^{2+} levels, which increases cell-to-cell contact and initiates assembly of junctional complexes. At selected times, the presence of TJ proteins is monitored by measuring the TER.

The lanthanum nitrate permeability test also allows the verification of the existence of functional TJ. When these are present, the lanthanum nitrate does not penetrate through the epithelial intercellular junctions and remains excluded at the surface of the epithelium. In contrast, if the TJs are disrupted or improperly formed, the lanthanum nitrate diffuses through the intercellular spaces and penetrates through the epithelial barrier.

[1] Claudins and occludins, present in TJs, associate with intracellular peripheral membrane proteins called ZO proteins (a tight junction is also known as a zonula occludens) which anchor the strands to the actin cytoskeleton (see Fig. 1).

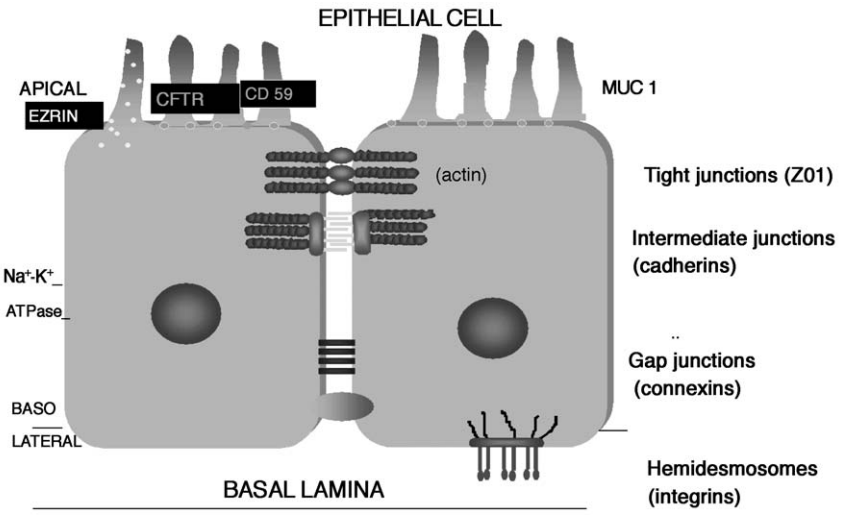

Fig. 1. Schematic representation of polarized epithelial cells. The junctional complexes and the apical or basolateral distribution of several polarity markers are shown (adapted from Ref. [15]).

2.3. To monitor the fence function of tight junctions

The characteristic distribution of certain proteins to the apical or basolateral domains of normal polarized epithelial tissues makes them useful as polarity markers. Their immunolocalization allows the analysis of the presence and functionality of TJ. As markers of the apical domain EBP50 (mouse monoclonal Ab from BD Transduction, San Diego, CA, USA), ezrin (goat polyclonal Ab from Santa Cruz Biotechnology, Santa Cruz, CA, USA) or CD59 (mouse monoclonal Ab from BD Pharmingen, San Diego, CA, USA) can be used. For the basolateral domain, two typical proteins are studied: β1 integrin (mouse monoclonal Ab from Beckman Coulter, Fullerton, CA, USA) and Na^+–K^+ ATPase.

3. Immunohistochemistry of CFTR

3.1. Optimization of the immunostaining protocol towards a good balance between tissue preservation and CFTR signal

A large part of the observed variability in CFTR immunostaining can be generally due to the protocol used. In fact, the differences in published results relative to CFTR localization may be explained, to a certain extent, by the lack of sensitivity of Abs and/or variability of protocols used among different laboratories.

A protocol of immunohistochemistry (IHC) of CFTR should result in a good compromise between morphology preservation (achieved through adequate fixation) and avoidance of epitope destruction (achieved only if mild fixation conditions are used). Before testing the specificity and sensitivity of the different anti-CFTR Abs (see Ref. [13]), optimization of critical steps (fixation, protease inhibition, dilution, and incubation time of primary Ab) should be performed.

Several studies have compared different fixation protocols and antibodies [7,10,21] using as starting point previously described IHC protocols for CFTR [12,4–6,22,23,8,9].

3.2. Collection of patient cells/tissues

Fresh specimens of human tissues (nasal polyps are obtained immediately after polypectomy or rectal/colon biopsies taken during colonoscopy) are immediately immersed in DMEM/F12 medium supplemented with 250 UI/ml of penicillin, 125 μg/ml of streptomycin, and 2.5 μg/ml amphotericin B and then rinsed with phosphate-buffered saline (PBS), coated with OCT compound (Tissue Tek, Miles, IN, USA), and the specimens quickly frozen in liquid nitrogen and stored at − 80 °C until use.

For colon tissue specimens, morphology is much better preserved, if samples are obtained by cutting off small pieces of tissue, rather than by a biopsy procedure [10]. This suggests that the additional cell and tissue disruption observed in intestinal samples when compared with airway tissue [9] may be associated with the biopsy (forceps) procedure.

Cryosections (6–8 μm thick) are obtained from frozen tissues in a cryomicrotome (Reichert-Jung, Bensheim, Germany) at − 20 °C, placed onto gelatin or silane-coated glass slides (Menzel-Glazer, Braunschweig, Germany), air-dried, and used immediately or stored at − 80 °C until use. IHC studies can be carried out either on fresh or thawed cryosections after air-drying and rehydration for 5 min in PBS.

3.3. Fixation of specimens

The fixation step is very critical because while preserving the tissue from autolysis, if too drastic, it may also cause a decreased signal due to concealment/disappearance of the antigenic motif (epitope).

The most common fixing agents used for CFTR IHC are methanol, acetone, or paraformaldehyde (PFA) solutions.

It was previously described that PFA-fixing solutions like the Zamboni's solution (composed of 85% of solution 1–2% v/v PFA/0.1 M Sorensen phosphate buffer and 15% of 51.5 mM picric acid [24]) are not suitable for IHC detection of CFTR [7,10]. Indeed, in Zamboni-fixed sections probed with anti-CFTR Ab, a CFTR-specific signal was described to be almost completely absent in intestinal samples and highly decreased in nasal samples [10]. Indeed, such drastic fixing conditions must render CFTR epitopes unavailable for detection.

Generally, methanol and acetone give better results on cryosections (as described above), while PFA works better for paraffin-embedded samples.

Without any fixation, labeling of intestinal tissue with anti-CFTR Abs is still evident but tissue morphology is not preserved, whereas in unfixed nasal samples, loss of morphology is not observed [10]. This also confirms that CFTR-IHC is considerably more difficult to perform in human intestinal tissue than in the airways.

Overall, methanol fixation is chosen for the general CFTR immunostaining protocol as the best compromise between CFTR signal and tissue morphology for both intestinal and nasal tissues.

3.4. Antibodies

For CFTR immunostaining, several Abs have been evaluated for their sensitivity and specificity towards CFTR. The results are described elsewhere in this supplement [13].

Anti-cytokeratins 18/19 Abs (mouse monoclonal Ab AE1–AE3 from Dako, Glostrup, Denmark), staining cytoskeletal elements of differentiated epithelial cells, besides confirming epithelial nature of cells observed in sections, can be used to assess the preservation of tissue morphology.

Specificity of immunoreactive signals can be confirmed by the generally accepted negative controls: omission of CFTR (primary) antibody and/or its replacement by the pre-immune serum (or isotype control) or by peptide competition. Furthermore, tissues from CF patients carrying two nonsense mutations, e.g., R553X, G542X, and W1282X, can represent the golden-standard negative control, as no full-length CFTR protein is produced in these cells. However, these samples are not easy to obtain.

3.5. Optimized protocols

As mentioned above, several studies were performed to optimize the immunodetection protocol concerning the effect of fixation conditions, the presence of protease inhibitors, and the use of different incubation times of the primary Ab [2 h at room temperature (RT) versus overnight at 4 °C], both on preservation of tissue morphology and on the CFTR signal detected. These protocols are described in detail at the European Working Group on CFTR Expression Web site [15].

Overnight incubation of the primary Ab at 4 °C leads to a significant loss of tissue morphology compared to 2 h incubation at RT without significant gain in CFTR labeling [10]. Therefore, the shorter incubation time with primary Ab should be used because sample tissue morphology is better preserved without diminishing the signal.

Proteolytic activity, particularly intense in intestinal tissue samples due to the presence of pancreatic and intestinal proteases and bacteria [25], may also cause loss of tissue integrity. Adding a protease inhibitor cocktail (mg/ml): leupeptin 1; aprotinin 2; benzamidine 121; pefabloc 50; and E64 3.5, during primary Ab incubation does not significantly improve the preservation of colon tissue morphology [9].

Briefly, the general protocol consists of fixing the fresh or thawed cryosections (see above) in methanol for 10 min at −20 °C or acetone at 4 °C. Alternatively, fixation in 4% (w/v) PFA for 16 h is used prior to paraffin embedding. Nonspecific binding sites are blocked with 1–3% (w/v) BSA solution in PBS (optional) before labeling with anti-CFTR Ab. Incubation time with primary Abs is usually 1–2 h at RT in a humid chamber. Similar incubation with secondary Abs lasts for 45 min. Sections are washed three times for 5 min in PBS between each step and finally mounted in Vectashield antifading medium (Vector Labs, Burlingame, CA, USA) containing DAPI (Sigma, St Louis, MO, USA) for nuclei labeling. Samples can be counterstained with Harris' hematoxylin (Sigma).

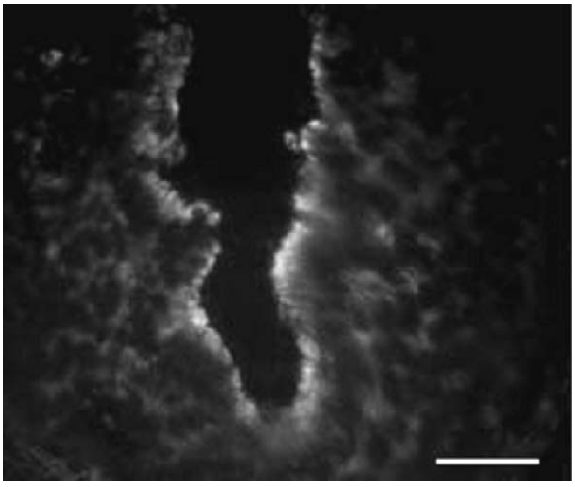

Fig. 2. Immunodetection of CFTR in human nasal epithelium. Cryofixed section from nasal polyp showing apical CFTR immunolabeling with MATG 1061 (Transgène, Strasbourg, France).

Immunofluorescence staining is observed using a fluorescence microscope (e.g., Axioskop, Carl Zeiss Optische Systeme, Göttingen, Germany). Specific staining and negative controls should always be photographed under identical conditions (filters, microscope magnification, and fluorescence exposure time). Fig. 2 provides typical results obtained with this protocol.

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