Immunofluorescent evaluation of renal biopsy: current point of view

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Immunomorphologic assessment of percutaneous renal biopsy became a standard procedure for establishing diagnosis in kidney disease in parallel with routine haematoxylin & eosin stained paraffin sections. Among various immunomorphologic techniques, direct immunofluorescence of cryosections with a panel of fluorochrome labelled polyclonal or monoclonal antibodies to various serum proteins turned out to be the most reliable and rapid diagnostic procedure. The panel of antibodies may be expanded to include those to microbial or tumour antigens, when needed. The authors specify major advantages of immunofluorescence for such task and potential pitfalls in the case of nonspecific staining. In the next step, various types of fluorescence within renal structures are confronted with particular kidney disorders. Special attention is paid to various types of glomerulonephritis. Lesions in transplanted kidney are also discussed and the role of deposition of C4d complement component along peritubular capillaries is underlined as the evidence of humoral anti-graft reaction. The article is supplemented with a detailed technical procedure for performing of immunofluorescent reaction and evaluation of kidney biopsy, including several control steps.

Key words: immunofluorescence, renal biopsy, glomerulonephritis.

Introduction

Immunopathologic assessment of the renal tissue should be a portion of every examination of the renal tissue. Correlation of light microscopy with immunofluorescence findings, electron microscopy evaluation and clinical history is needed to determine the nature and pathogenesis of the lesion in renal biopsy [1, 2]. It is noteworthy that in renal pathology many diagnostic lesions can be detected and classified only by immunohistochemistry [3, 4]. IgA nephropathy, C1q nephropathy, fibronectin glomerulopathy, and monoclonal immunoglobulin deposition disease belong to the glomerular diseases in which diagnosis is established exclusively relying on immunomorphological findings, thus these nephropathies are overlooked if immunofluorescence is not undertaken.

Direct immunofluorescence (IF) on frozen tissue remains the most widely used technique of immunohistochemistry for the detection of immune deposits in the kidney. However, when no glomeruli are available in frozen tissue, immunofluorescence, or immunohistochemistry can be performed on the formalin-fixed-paraffin-embedded tissue allocated for light microscopy after antigen retrieval with proteases [5-8].

Handling and preparation of renal biopsy specimens for IF

Renal tissue for immunofluorescence study should include a piece of cortex, approximately 20% of the biopsy containing > 5 glomeruli. The unfixed tissue sample should be sent on a wet swab on crushed ice to the pathology department, and processed within 20 minutes after taking the biopsy. Serial sections should be cut at 3-4 μm in cryostat. A routine antibody panel for immunofluorescence on native kidney biopsy includes the fluorescein-labelled antibodies directed against IgA, IgM, IgG, C3, C1q, fibrinogen,

λ light chains and κ light chains [7, 9, 10]. Additional immunohistochemical study with antibodies, such as collagen IV alpha chains can be performed in selected nephropathies. In all allograft renal biopsies immunostaining for C4d should be determined by an indirect immunofluorescence method.

Evaluation of IF findings

It is of value to decide if immunostaining is specific, because nonspecific deposits are due to plasma insudation, or leakage of serum protein: C3, IgM, and albumin into sclerosed or necrotic area. Non-specific deposition/absorption of immunoreactants may be seen in glomeruli, tubuli or vessels. Glomerular nonspecific staining is observed in sclerosis (global or segmental) – usually IgM/C3 – linear along GBM (diabetic nephropathy, tubular atrophy), or in the glomerular visceral epithelial cells (podocytes). Nonspecific tubular epithelial cells staining are due to proteinuric states. Staining of tubular casts (usually IgA, IgM, κ and λ) may be observed in various glomerulopathies. Vascular nonspecific staining in arteries/arterioles usually includes IgM or C3 [1, 2, 10].

The specific immunostaining should be evaluated quantitatively using computer image analysis, or semiquantitatively using an intensity score (0 to 4+). A report of the renal biopsy examination should consist of description concerning immunostaining in all renal compartments (glomeruli, vessels, tubuli and interstitium) [1, 3, 6, 10, 11, 12]. Glomerular immunostaining may be mesangial, along capillary walls, or both, as well as immune deposits may be observed within Bowman's capsules.

Patterns of renal injury

Based on immunohistologic findings, glomerulopathies are classified into three categories that relate to the pathogenesis of inflammation [1, 3]. The pattern of immunostaining can be linear or granular. Linear glomerular staining of immunoglobulin indicates anti-GBM antibody-mediated injury. Granular staining of immunoglobulin and complement component is due to immune-complex-mediated injury. If no or scanty glomerular immunostaining of immunoglobulin is noted and light microscopy shows glomerular necrosis with crescents formation, pathologic lesions are related to the pauci-immune glomerulonephritis usually with circulating anti-neutrophil antibodies (ANCA).

An approach to the interpretation of IF findings in glomerular diseases

The precise diagnosis of glomerular diseases requires integration of immunopathological findings with light microscopic study and clinical data [1–3]. Patterns of immunostaining in glomerular diseases are shown in Table I and Table II. Linear GBM immunofluorescence staining caused by anti-GBM antibodies in a patient with lung haemorrhage points to the Goodpasture's syndrome. Granular glomerular IF staining due to immune-complex localization with domination of IgA, and no vasculitis referred to IgA nephropathy, whilst dominant IgA immunostaining and systemic vasculitis suggests Henoch-Schoenlein purpura. Full house pattern of immunostaining of three immunoglobulins and complement components (especially C1q), and clinically diagnosed SLE points to the lupus nephritis.

Mesangial localization of immunoglobulin is seen in mesangioproliferative glomerulonephritis. Peripheral capillary wall granular staining for C3 is seen in both types of membranoproliferative glomerulonephritis. The very coarsely granular staining of postinfectious glomerulonephritis is compared to the most finely granular staining along capillary loops in membranous glomerulopathy.

It must be also stressed that three subtypes of crescentic glomerulonephritis are categorized on the basis of immunohistochemical findings observed in the renal tissue: subtype I – with linear staining along glomerular capillaries, subtype II – with granular mesangial or capillary wall staining, or both, and subtype III – "pauci immune", which may occur as a component of microscopic polyangiitis, Wegener's granulomatosis, or Churg-Strauss syndrome.

Moreover, immunofluorescence may reveal abnormal accumulation of amyloid in glomeruli, tubuli, vessels, and in the interstitium. Immunohistochemical typing of amyloid proteins is of value in the patient's management and therapy. The majority of AL amyloidosis cases are λ chains-associated. However, it must be taken into consideration that negative stains for κ and light chains do not exclude the diagnosis of AL amyloidosis. Commercially available antibodies may not be able to detect abnormal or truncated monotypical proteins deposited in tissue [13]. Immunofluorescence on frozen sections for κ and λ light chains, as well as heavy chains allows diagnosing monoclonal immunoglobulin deposition disease, which includes light chain deposition disease (LCDD), heavy chain deposition disease (HCDD), or light and heavy chain deposition disease (LHCDD). In these cases immunofluorescence performed on frozen tissue is a better method than immoperoxidase on formalin-fixed paraffin-embedded sections, which often cause nonspecific background staining.

In minimal change disease immunofluorescence is usually negative. Diffuse mesangial hypercellularity (DMH) and IgM nephropathy are the variants of minimal change disease, which must be differentiat-

Table I. Patterns of immunostaining in acute diffuse intracapillary proliferative glomerulonephritis, membranoproliferative glomerulonephritis, IgA nephropathy, membranous glomerulopathy, crescentic glomerulonephritis, and lupus nephritis

Type of glomerular disease	Pattern of immunostaining
Acute diffuse intracapillary proliferative (postinfectious) glomerulonephritis	Coarse granular staining of glomerular capillary loops with IgG and C3. Less frequently IgM, IgA and early complement components (C1q, C4). Three immunostaining patterns: "garland-type" – staining of peripheral capillary loops for IgG and C3, "starry sky" – appearance of IgG and C3 irregular deposits (along capillary walls and in mesangium), "mesangial" – IgG and C3, or predominantly C3.
Membranoproliferative glomerulonephritis type I	Intense fine to coarse staining for C3 along the periphery of glomerular capillary loops and in the mesangium. Similar staining patterns are often seen for IgG and IgM and for early complement components C1q and C4.
IgA nephropathy	Mesangial granular dominant or co-dominant staining for IgA. Co-deposition of C3, IgG and IgM. Positive staining for C1q is infrequent (secondary IgAN). Granular staining of IgA in peripheral capillary loops is associated with more proliferative forms of IgA nephropathy.
Membranous nephropathy	Strong fine granular staining for IgG and less intense granular staining for C3 along capillary loops.
Crescentic glomerulonephritis	1. Subtype I (anti-GBM glomerulonephritis): linear IgG and C3 staining of GBM. Staining for fibrin is positive in areas of necrosis, crescents, periglomerular interstitium, and in Bowman's capsules. 2. Subtype II (immune complex disease): granular immune deposits of variable immunoglobulins and complement components (mesangial, along capillary loops, or both). 3. Subtype III ("pauci-immune"): negative or very weak staining. Fibrinogen positivity in glomerular necrosis, cellular crescents and in fibrinoid vascular necrosis. Nonspecific staining for immunoglobulins and complement components in areas of glomerular necrosis.
Lupus nephritis	1. Mesangial lupus nephritis: granular mesangial positivity of all three immunoglobulins and both complements (C1q and C3) – ("full house" pattern) 2. Proliferative lupus nephritis: granular mesangial and along capillary loops positivity for all three immunoglobulins and both C3 and C1q ("full house" staining). Immune deposits are also identified in tubular basement membranes, interstitial capillary walls, interstitial collagen, arterial intima, and media. Fibrinogen positivity in necrotizing lesions.

ed from the other forms of mesangial glomerulopathies. The differential diagnosis may be done using immunofluorescence method. Despite glomerular hypercellularity confined to mesangium, immunofluorescence is usually negative in a variant of minimal change disease called DMH. In IgM nephropathy mesangial deposits of IgM exhibiting strong intensity of staining are observed. These mesangial deposits probably are not true immune complex deposits, and are thought to result from nonspecific entrapment of circulated plasma protein due to impaired mesangial clearance. It is noteworthy that, minimal change disease activation of complement components on tubular cells, due to increased protein trafficking, may result in tubular deposits of C3.

In focal segmental glomerulosclerosis (FSGS) granular depositions of IgM and C3 in the area of the glomerular sclerosis and hyalinosis are frequently noted. Intracytoplasmic deposits of albumin, immunoglobulins and complement components corresponding to the protein resorption droplets may be seen within podocytes and proximal tubules in renal biopsies in patients with FSGS.

As noticed earlier, a renal biopsy report must include immunofluorescence findings not only observed within glomeruli, but in all renal compartments. Vascular immunostaining may be seen in lupus vasculitis, cryoglobulinemic vasculitis or "pauci-immune" lesions. Fibrin and fibrinogen in thrombi within glomerular capillaries are seen in thrombotic microangiopathies and necrotizing glomerulopathies. In malignant hypertension immunostaining of arteriolar or artery walls in renal biopsy samples are noted. About a quarter of IgA nephropathy specimens have focal segmental capil-

Table II. Patterns of immunostaining in minimal change disease, focal segmental glomerulosclerosis, C1q nephropathy, IgM nephropathy, diabetic nephropathy, dense deposit disease, fibrillary glomerulonephritis, immunotactoid glomerulopathy, fibronectin glomerulopathy, nephropathy in Alport syndrome, thin-basement-membrane disease, and monoclonal immunoglobulin deposition disease

Type of glomerular disease	Pattern of immunostaining
Minimal change disease	Usually negative in IF.
Focal/segmental glomerulosclerosis	IgM and C3 positivity in sclerotic area.
C1q nephropathy	Prominent glomerular mesangial C1q deposits with lesser deposits of C3 and immunoglobulins.
IgM nephropathy	Prominent glomerular mesangial IgM staining.
Diabetic nephropathy	Nonspecific linear staining for IgG, IgM, albumins, and C3 along the glomerular capillary walls and tubular basement membranes.
Dense deposit disease	Granular staining of capillary walls and mesangium for C3. Early complement components (C1q and C4) are usually absent. Staining of C3 may be seen in Bowman's capsules and tubular basement membranes.
Fibrillary glomerulonephritis	Granular IgG immunopositivity (mesangial and along capillary loops). Ribbon-like, linear or pseudolinear staining for IgG, C3, κ and λ light chains. κ staining is noted more often.
Immunotactoid glomerulopathy	Coarsely granular IgG and C3 immunopositivity (mesangial or mesangial and along peripheral capillary loops). IgM and IgA positivity is often. Light chain restriction with κ predominance may be seen.
Fibronectin glomerulopathy	Immunoreactivity for fibronectin in mesangial area.
Nephropathy in Alport syndrome	Nonspecific, many biopsies negative for immunoglobulin and complement components. Distribution of type IV collagens 1 (IV), 3 (IV), and 5 (IV) in glomeruli and tubular basement membranes is of value.
Thin-basement-membrane nephropathy	Usually negative for immunoglobulins and complement components. In differential diagnostics with nephropathy in Alport syndrome immunofluorescence for 1 (IV), 3 (IV), and 5 (IV) may be performed.
Monoclonal immunoglobulin deposition disease	Deposition of monotypical light (κ or λ), or heavy chains along the glomerular basement membranes, mesangium, along tubular basement membranes, in the interstitium, and in vasculature.

lary wall staining that has the same composition as the mesangial deposits [15]. Linear tubular immunostaining resembles anti-tubular basement membrane (anti-TBM) disease. Granular, discrete immune complex deposits along TBM most often are associated with lupus nephritis. Immunopositivity of κ light chains or immunopositivity of λ light chains in tubuli is noted in light chain deposition disease.

IF in transplant renal biopsy

In transplant renal biopsy deposition of C4d along peritubular capillaries (PTC) has been found to be the sensitive marker for the antibody-dependent humoral reaction [16]. C4d is a degradation product of the activated complement factor C4 and can be covalently bound to the endothelium. Positive C4d immunostaining in peritubular capillaries of renal transplant biopsies has been incorporated into the criteria for the diagnosis of humoral rejection. C4d-positive cases are connected with a high prevalence of transplant glomerulitis/glomerulopathy, transplant endarteritis and high risk of graft dysfunction. According to the Banff update, focal peritubular capillary staining for C4d is defined as 10% to 50% C4d positive PTC [17]. C4d is not only positive in the cortical PTC, but also in the vasa recta, therefore biopsy tissue which includes only renal medulla is adequate for immunofluorescence evaluation. Literature data show that detection of C4d by immunofluorescence in frozen renal allograft biopsies with a monoclonal antibody is a highly sensitive and highly reproducible method [18, 19].

Moreover, in renal transplant biopsies the absence of immune deposits by immunofluorescence helps to differentiate transplant glomerulopathy from recurrent primary membranoproliferative glomerulonephritis. Routine histology of renal transplant biopsy should be supplemented by immunofluorescence in cases where recurrent glomerular disease or de novo glomerular disease is suspected on the basis of clinical presentation and light microscopic findings.

In conclusion, it must be stressed that the immunomorphological evaluation of the renal biopsy specimen should be done in all cases to avoid diagnostic mistakes. Immunofluorescence findings provide information about pathogenesis of the renal injury and allow us to establish a precise diagnosis on the type of the glomerular disease.

Appendix

Detailed procedure of performance and evaluation of immunofluorescence reaction of kidney biopsy.

The aim: to show immunopathologic background of clinical symptoms in renal disease and thus, to facilitate diagnosis.

The principle

Direct immunofluorescence (IMF) reaction on frozen (cryostat) sections of percutaneous kidney biopsy specimen, using polyclonal fluorochrome labelled antibodies (most commonly used fluorochromes: FITC – fluorescein isothiocyanate – green fluorescence, TRITC – rhodamine isothiocyanate – red fluorescence).

1. Reagents used:
   a) facilities for rapid freezing of tissue (liquid nitrogen, dry ice – acetone slurry etc.);
   b) medium for tissue embedding during freezing (Tissue-tek);
   c) microscope slides of high adhesiveness, cover slips;
   d) PBS pH 7, 4-7, 6 – 5 l;
   e) labelled antibodies: rabbit (goat) anti-human IgG FITC (or TRITC), IgA, IgM, C3, C1q, C4, fibrinogen, κ light chain, λ light chain, normal rabbit (goat) γ-globulins (control); this is an essential panel of antibodies. It may be extended to include other, such as those to albumin, microbial antigens, lymphocyte differentiation etc.;
   f) glycerogel (glycerol: PBS 80 : 20%, v/v) mixture);
   g) Meyer's haematoxylin & eosin (H+E) stain;
   h) alcohol row, xylol, Canada balsam (for H+E stained section).
2. Equipment needed:
   a) cryostat with rotary microtome;
   b) fluorescence microscope of high quality with a set of filters for green and red fluorescence and tungsten lamp for visible light;
   c) automatic pipettes;
   d) humid chamber (usually plastic box with cover, coated with wet blotting paper).
3. Tissue preparation:
   a) kidney tissue fragment collected by means of a needle biopsy should be immediately shipped to the laboratory in a sealed pre-cooled container, preferably on crushed ice in a wet swab;
   b) human lymph node or tonsil (hypertrophic) previously snap frozen and stored in –70°C (control tissue);
   c) kidney biopsy tiny fragment (usually 5-8 mm long) is snap frozen in a drop of Tissue–Tek embedding compound;
   d) just before sectioning both tissue specimens are left in a cryostat chamber for 30–60 min in order to obtain the same temperature as that of cryostat interior (ca –25°C);
   e) tissues are cut in a cryostat for sections 4-6 μm thick. It is preferable to deposit control tissue (lymph node or tonsil) on the same slide as kidney section.
4. Description of analytic procedures:
   a) tissue sections are dried, subsequently fixed in pre-cooled (4°C) fixative (acetone or acetone-absolute alcohol v/w) for 10 min and thereafter dried again for 20 min;
   b) sections rehydrated for 5 min in chilled PBS, excess of fluid is removed with blotting paper and tissue surface is covered with suitably diluted fluorescent reagent (ca 10-15 μl). The procedure is repeated with next slides, taking care to avoid drying of tissue;
   c) sections are incubated in a humid chamber in the dark at room temperature for 30 min;
   d) following incubation, sections are washed in 3 changes of PBS for 5 min each, thereafter embedded in a drop of glycerogel and covered with cover slip;
   e) sections are stored in a refrigerator (4°C) up to the time of microscopic evaluation.
5. Quality control:
   a) negative control:

Sections are incubated with normal (rabbit or goat) γ-globulins fluorochrome labelled, instead of antibodies: there should be no positive staining.

   b) positive control:

Apart from kidney, sections of a lymph node or tonsil are incubated with labelled antibodies: plasma cells, fibrin or complement components if present, should provide positive staining.

6. Microscopic evaluation of reaction.

Prior to assessment using fluorescence microscope H+E stained section is checked in visible light for histologic quality, number of glomeruli, inflammatory infiltrates etc.

7. While examining sections by fluorescence microscopy, the following parameters are taken into account:
   a) glomeruli:
   – their number,
   – the involvement (diffuse > 80%, focal < 80%),
   – the type of alteration (linear, granular, pseudogranular deposits),
   – the extent of alterations (global or segmental),
   – localization of deposits (peripheral, vascular, mesangial, mixed, Bowman capsule),
   – intensity of deposits (coarse, microgranular, subtle, punctual);
   b) renal canaliculi (positive or negative: +/–):
   – deposits (linear or granular),
   – their localization (in the lumen, epithelium or basal membrane),
   – their content (Ig, complement components etc.);
   c) extraglomerular vessels (+ or –):
   – the site of deposits (linear, granular),
   – their content;
   d) kidney stroma:
   – content of deposits.

Immunofluorescence evaluation of kidney biopsy should end with the conclusion suggesting the type of lesion and possible pathologic entity. The results should be verified with routine histological evaluation of paraffin embedded sections and, if necessary, with ultrastructural kidney preparations.

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References

1. Jennette JC, Olson JL, Schwartz MM, et al. Primer on the pathologic diagnosis of renal disease. In: Heptinstall's Pathology of the Kidney. Jennette JC, Olson JL, Schwartz MM, Silva FG (eds) 6th ed. Lippincott Williams & Wilkins, Philadelphia 2007; 97-123.
2. Lajoie G, Silva FG. Approach to the interpretation of renal biopsy. In: Renal Biopsy Interpretation. Silva FG, D'Agati VD, Nadasdy T (eds). Churchill Livingstone New York: 1996; 31-70.
3. Zhou XJ, Laszik Z, Silva FG. Algorithmic approach to the interpretation of renal biopsy. In: Silva's Diagnostic renal Pathology. Zhou XJ, Laszik Z, Nadasdy T, D'Agati VD, Silva FG (eds). Cambridge University Press, New York 2009; 55-78.
4. D'Agati VD, Jennette CJ, Silva FG. An approach to the pathologic diagnosis of glomerulonephritis. In: Atlas of Nontumor Pathology. Non-neoplastic Kidney Diseases. D'Agati VD, Jennette CJ, Silva FG (eds). ARP PRESS Silver Spring, Maryland 2005; 189-198.
5. Nasr SH, Galgano DJ, Markowitz GS, et al. Immunofluorescence on pronase-digested paraffin sections: a valuable salvage technique for renal biopsies. Kidney Int 2006; 70: 2148-2151.
6. Furness PN: Best Practice No 160. Renal biopsy specimens. J Clin Pathol 2000; 53: 433-438.
7. Molne J, Breimer ME, Svalander CT. Immunoperoxidase versus immunofluorescence in the assessment of human renal biopsies. Am J Kidney Dis 2005; 45: 674-683.
8. Chowdhury AKMRH, Ehara T, Higuchi M, et al. Immunohistochemical detection of immunoglobulins and complements in formaldehyde-fixed and paraffin-embedded renal biopsy tissues; an adjunct for diagnosis of glomerulonephritis. Nephrology 2005; 10: 298-304.
9. Lajoie G, Silva FG. Technical aspects of renal biopsy processing. In: Renal Biopsy Interpretation. Silva FG, D'Agati VD, Nadasdy T (eds). Churchill Livingstone, New York 1996; 423-35.
10. Fogo AB. Approach to renal biopsy. Am J Kidney Dis, 2003; 42: 826-836.
11. Walker PD, Cavallo T, Bonsib SM. Practice guidelines for the renal biopsy. Modern Pathology 2004; 17: 1555-63.
12. Regele H, Mougenot B, Brown P, et al. Report from Pathology Consensus Meeting on Renal Biopsy Handling and Processing, Vienna, February 25, 2000, (http://www kidney-euract.org/Rbpathologyconsensus.htm).
13. Picken MM, Herrera GA. The burden of "sticky" amyloid. Typing challenges. Arch Pathol Lab Med 2007, 131: 850-851.
14. D'Agati VD, Jeannette CJ, Silva FG: Focal segmental glomerulosclerosis. In: Atlas of Nontumor Pathology. Non-neoplastic Kidney Diseases. D'Agati VD, Jeannette CJ, Silva FG (eds). ARP PRESS, Silver Spring, Maryland, 2005; 125-161.
15. D'Agati VD, Jeannette CJ, Silva FG. IgA nephropathy and Henoch-Schoenlein purpura. In: Atlas of Nontumor Pathology. Non-neoplastic Kidney Diseases. D'Agati VD, Jeannette CJ, Silva FG (eds). ARP PRESS, Silver Spring, Maryland, 2005; 297-316.
16. Böhmig GA, Exner M, Habicht A, et al. Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. J Am Soc Nephrol 2002; 13: 1091–1099.
17. Solez K, Colvin RB, Racusen LC, et al. Banff 2007 classification of renal allograft pathology: updates and future directions. Am J Transplant 2008; 8: 753-60.
18. Seemayer CA, Gaspert A, Nickleit V, et al. C4d staining of renal allograft biopsies: a comparative analysis of different staining techniques. Nephrol Dial Transplant 2007; 22: 568-576.
19. Nadasdy GM, Bott C, Cowden D, et al. Comparative study for the detection of peritubular capillary C4d deposition in human renal allografts using different methodologies. Human Pathol 2005; 36: 1178-1185.

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