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COMPARISON OF C4D IMMUNOSTAINING BY IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Pathology

Pallavi Arvind Patil Assistant Professor, Dept of Pathology ,Smt.Kashibai Navale Medical College and general hospital.

ABSTRACT

Renal allograft biopsy is the gold standard for diagnosis of rejection. Incorporation of C4d as a marker for humoral rejection is a major addition for Banff Schema, 2005. We evaluated the pattern of C4d staining in indicated renal allograft biopsies from January 2009 to March 2012 in Ruby hall Clinic, Pune. There were total 108 renal allograft biopsies received in the department; out of which 59 biopsies had features consistent with rejection.

C4d is the degradation product of the activated complement factor C4, a component of the classical complement cascade which is typically initiated by binding of antibodies to specific target molecules.

C4d staining was compared by two methods- Immunofluorescence and IHC. The intensity of expression of C4d in formalin-fixed paraffin-embedded tissues byIHC (P-IHC) was significantly reduced in comparison with frozen sections analysed by immunofluorescence (F-IF).

KEYWORDS

C4d, Allograft, Rejection, Immunohistochemistry

INTRODUCTION

Transplant rejection is a complex process in which both cell-mediated immunity and circulating antibodies play a role. Renal biopsy is the gold standard for diagnosis of acute rejection in renal transplant recipients. For the diagnosis of cellular rejection, well-defined histological criteria were laid down under theBanff system in 1993 and were further revised in 1997. Acute antibody mediated rejection is associated with the appearanceof donor-specific antibodies that can be detected using various methods, e.g. panel reactive antibody (PRA) levels, flow cytometrycross match or flow PRA bead assays. In view of these observations, theBanff (1997) classification was revised in 2003 incorporating morphological criteria for acute antibody mediated rejection, supported by immunopathological criteria, and serological evidence for acute humoral rejection. The diagnostic criteria of antibody mediated rejection in renal allograft biopsy include:

Type 1: ATN like- C4d positivity in peritubular capillaries
Type 2: Capillary glomerulitis, polymorphonuclear and/or mononuclear leucocytes in peritubular capillaries (with C4d positivity)
Type 3: Arterial-transmural inflammation/ fibrinoid change with C4d positivity [1]

Acute antibody mediated rejection is mediated by antibodies to the donor HLA that activate the classical complement pathway. This leads to a number of split products of complement (C_{3a},C_{3b}, C_{3d}, C_{4b}). C4d is a fragment of C_4 released during activation of the classic complement pathway by the antigen–antibody complex. Because C4d contains an internal thioester bond, itbinds covalently to tissue elements at the site of activation and is therefore a durable marker of antibody-mediated antidonor humoral response. Detection of C4d is regarded as an indirectsign, a 'footprint' of an antibody response.

C4d is the degradation product **of** the activated complement factorC4, a component **of** the classical complement cascade which is typically **in**itiated by b**indin**g **of** antibodies to specific target molecules. Follow**in**g activation and degradation **of** the C4 molecule, thio-ester groups are exposed which allow transient, covalent b**indin**g **of** the degradation product **C4d** to endothelial cell surfaces and extracellular matrix components **of** vascular basement membranes near the sites **of** C4 activation. **C4d** is also found **in in**tracytoplasmic vacuoles **of** endothelial cells [2]. Covalent b**indin**g renders **C4d** a stable molecule that can easily be detected by immunohistochemistry[3-15].

AIMS AND OBJECTIVES

To study concordance of C4d positivity by immunohistochemistry and direct immunoflorescence.

MATERIAL AND METHODS

Tissue processing of the renal biopsies was carried out for 22 hours in LEICA automated tissue processor. After tissue processing, the tissue was embedded in molten paraffin wax (60-65^{0}C). Sections were taken and stained by hematoxylin and eosin (H & E) stain. C4d immunostaining were done by standard Immunohistochemistry and Immonofluorescence technique in all cases to substantiate the diagnosis.

OBSERVATIONS

Immunofluorescence C4d staining was done in 13 cases since most of the nephrologists submitted only one formalin fixed renal biopsy core for evaluation.

The mean serum creatinine at the time of biopsy was 4.4 mg/dl.

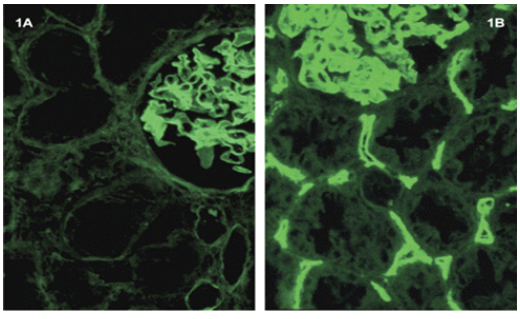

Figure 17:- Immunofluorescence microsopy demonstrating C4d positivity in Glomerulus (1A) and in Peritubular capillaries (1B)

Table 11 – Grading of C4d staining by Immunohistochemistry.

BANFF Grade	Interpretation	% Biopsy area (cortex &/or medulla
C4d0	Negative	0%
C4d1	Minimal	1-10%
C4d2	Focal	10-50%
C4d3	Diffuse	>50%

Table 14 – Staining characteristics of C4d Positive cases by Immunofluorescence and Immunohistochemistry.

Case Reg.no	C4d Staining by IF (BANFF Grade)	C4d Staining by IHC (BANFF Grade)	PTC		
			Dilatation	Cellular infiltration	Cell type
605/09	C4d2	C4d1	-	+	N,M
630/09	C4d1	C4d1	-	+	N
1165/09	C4d3	C4d2	-	++	N,M,P
2208/09	C4d1	C4d1	-	+	N,M
2744/09	C4d2	C4d2	-	++	N,M
3042/09	C4d2	C4d2	-	++	N
4250/09	C4d2	C4d2	-	++	N,M
4500/10	C4d3	C4d3	+++	++	N,M
561/11	C4d2	C4d2	++	++	N,M,P
683/11	C4d3	C4d3	+++	+++	N,M

581/11	**C4d3**	**C4d2**	-	++	**N,M**
2969/11	**C4d3**	**C4d2**	-	++	**N,M**
4428/11	**C4d3**	**C4d2**	++	++	**N,M**

M, mononuclear cells (lymphocytes, monocytes); N, neutrophil; P, plasma cells; PTC, peritubular capillary; −, negative; +, present, scant; ++, moderate; +++, abundant.

DISCUSSION

The intensity of expression of C4d in formalin-fixed paraffin-embedded tissues by P-IHC was significantly reduced in comparison with frozen sections analysed by F-IF. For instance, of the 10 diffuse C4d expressing cases by F-IF (dilution 1:10), 7 were scored as focal or focal minimal expression by P-IHC. The estimated percentage area of C4d-positive PTC was lower in paraffin-embedded biopsies. This result is of great importance for the adequate interpretation and comparison of staining results from different studies. On average, the degree of staining in paraffin was lower by about one degree. As a rule of a thumb, diffusely staining cases in frozen sections (F-IF) turned to be focally positive in paraffin (P-IHC), and even more pronounced were the differences between F-IF and P-IHC for the focally positive cases. This finding was in line with previous studies (comparing frozen and paraffin-embedded materials) revealing a reduced sensitivity to stain immunoglobulins and complement factors after paraffin embedding[16].

The P-IHC investigation of C4d revealed two major problems: (i) reduced sensitivity and (ii) difficulties in interpretation. Therefore, we propose to utilize frozen unfixed material for the detection of the C4d antigen in renal allograft specimens. If paraffin-embedded sections only are available, the interpretation of the result should be performed with caution and the knowledge of a decreased sensitivity of this method. Equivocal diffuse expression of C4d by P-IHC in paraffin may occur. Negative findings for C4d in paraffin do not exclude positive findings in frozen material.

In our study, the higher frequency of C4d detection by the Quidel antibody is not explained by an additional unspecific staining of C4, C4b or C4c by this antibody, because, in parallel sections stained with a specific anti-C4 antibody (DakoCytomation), no co-localization of the F-IF signals of both antibodies was seen.

Previous studies comparing frozen *vs.* paraffin sections were based on a smaller number of cases. Regele and co-workers[17] used 25 normal native kidneys without any staining in the PTC and only 12 kidney allograft biopsies, of which five stained positive in the PTC both in paraffin and frozen sections. In endomyocardial biopsies, Chantranuwat *et al*[18.] compared F-IF and P-IHC detection of C4d implying a slightly reduced sensitivity for the P-IHC group (n = 35 C4d-positive cases).

Most recently, Nadasdy *et al*[19] published a comparative study for the detection of C4d in PTC of 20 renal allograft biopsies. Similar to our study, the authors compared F-IF using the Quidel antibody with P-IHC utilizing the polyclonal Biogenex anti-C4d antibody and an immunoperoxidase technique.

Intriguingly and seemingly in contrast to our results, Nadasdy and co-workers concluded from their data that none of the applied methods appeared to be clearly superior to the others. However, analysing their presented raw data, we find striking differences between F-IF (Quidel) and P-IHC (BI-RC4D). Out of 15 cases with a diffuse C4d expression in PTC analysed by F-IF (Quidel), only 12 expressed C4d by P-IHC. This is fully in line with the results of the current study indicating a loss of sensitivity for IHC in paraffin of 61.53 % *(8/13)* in the diffuse C4d-expressing group.

In a series of studies, the C4d status in PTC was correlated with morphological lesions and clinical course. Groups from Basel [20,21], and Oxford[22], worked with 66 materials and applied monoclonal antibodies, in the majority the Quidel antibody. Even if the results of most studies (independent of the material and antibodies used) point in the same direction, i.e. higher prevalence of transplant glomerulitis/glomerulopathy, transplant endarteritis and higher risk of graft dysfunction in C4d-positive cases, the results are strictly comparable only for the diffuse C4d-positive cases in both frozen and paraffin sections.

Our study shows concordance of intensity scoring in 8/13 cases using IHC and IF techniques for C4d demonstration.

The results from our study clearly demonstrate that all the investigations in paraffin harbour the risk of being less sensitive than studies being performed in frozen material, and must be interpreted with great caution, since they might not have unravelled all potential associations that could be recognized in frozen material.

This could well explain the striking differences in the prevalence of C4d positivity in different studies.

The results of our study warrant also some comments to the grading of C4d in PTC in frozen and paraffin sections. In different studies, the grading of C4d expression varied substantially.

CONCLUSION

C4d staining was compared by two methods- Immunofluorescence and IHC. The intensity of expression of C4d in formalin-fixed paraffin-embedded tissues by P-IHC was significantly reduced in comparison with frozen sections analysed by Immunofluorescence F-IF.

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