

HeartJnl-2011-300960 revision

Supplementary data: histological and immunohistochemical methods

Kuusisto J et al.: Low -grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy

Histology

The most representative EMB biopsy sample was designated for the traditional histological staining. Endomyocardial biopsy and myocardial autopsy specimens were formalin fixed (pH 7.0), paraffin embedded, sectioned at 5µm and stained with haematoxylin and eosin, and Weigert van Gieson staining. The evaluation of histopathology was performed by an experienced pathologist (V.K.). The following histological characteristics of HCM were determined: heterogeneity of myocyte size, myocyte hypertrophy, myofiber disarray, myocardial fibrosis, inflammatory cell infiltration (mononuclear inflammatory cells, eosinophilic granulocytes and macrophages) and narrowing of intramyocardial small arteries. We used the semiquantitative evaluation of histological and immunohistochemical findings, which we have previously used to show an inflammatory response in aortic stenosis and aortic valves of necropsy subjects (1,2). The extent of these variables was graded as 0= none, 1= mild, 2= moderate, and 3=severe changes. All samples were blinded and examined twice by the same pathologist, and if a different grade was obtained, the samples were re-examined to obtain intraobserver consistency.

Immunohistochemistry

The second best EMB sample, which was not always sufficient for all analyses, was designated for immunohistochemical stainings. Each staining was performed from a different EMB microscopic slide in the patients with HCM.

To study CD3 positivity of mononuclear inflammatory cells, indicating presence of T-lymphocytes in endomyocardial samples and control cadaver samples, immunostaining was performed by an experienced pathologist (I.K.) with rabbit anti-human antibody (Sigma, 1:50 dilution) using trypsin pretreatment and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed).

The slides of EMB and control cadaver samples designated for antibody M755, MO814 and NCL-CD3-PS1 studies were pretreated in microwave oven in ChemMate Target Retrieval Solution (dilution 1:10, DAKO, Glostrup, Denmark) at 700W for two cycles of five minutes. Antibody M755 (DAKO, dilution 1:400) was used to label B-lymphocytes, and MO814 (DAKO, dilution 1:2000) to label macrophages. NCL-CD3-PS1 (Novocastra, Newcastle upon Tyne, UK, dilution 1:50) was used to label T-lymphocytes. The slides were stained in a Techmate 500 Plus automat (DAKO) using the labelled streptavidin biotin method. Peroxidase was the marker enzyme and it was visualized by hydrogen peroxide as a substrate and diaminobenzidine as a chromogen. Human tonsil was used as positive control in each staining batch and samples from the same series without primary antibody served as negative controls.

Myocardial fibrosis was verified in Masson's Trichome and PicroSirius Red stained sections in endomyocardial biopsy and myocardial autopsy specimens (I.K.).

To evaluate NF- κ B activity, immunostaining was performed in endomyocardial samples of 15 patients with HCM and in 20 cadaver myocardial samples after pretreatment in citrate buffer in microwave oven and avidin-biotin-HRP system (Vector Laboratories) with DAB as chromogen (Zymed). Mouse anti-NF- κ B, p65 subunit monoclonal antibody (Chemicon, 1:100 dilution), which

recognizes an epitope overlapping the nuclear location signal of the p65 subunit of the NF- κ B heterodimer and thus selectively binds to activated form of NF- κ B, was used.

References

1. **Otto CM**, Kuusisto J, Reichenbach DD, *et al.* Characterization of the early lesion of degenerative valvular aortic stenosis – histological and immunohistochemical studies. *Circulation* 1994;**90**:844-53.
2. **Kuusisto J**, Räsänen K, Alarakkola E, *et al.* Atherosclerosis-like lesions of the aortic valve are common in adults of all ages: a necropsy study. *Heart* 2005;**91**:576-82.