CORRELATIONS BETWEEN CD31, CD68, MMP-2 AND MMP-9 EXPRESSION IN ALLOGRAFT CARDIAC REJECTION – IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Introduction. The cardiac allograft rejections from the post-transplant period are attributable to the acute cellular rejection monitored by multiple endomyocardial biopsies. Compared to this, humoral rejection remains a matter of debate, with multiple therapeutic strategies, poor prognosis, and persisting uncertainty about diagnostic criteria. Acute allograft rejection is associated with significant modifications of the extracellular matrix compartment mainly regulated by matrix metalloproteinases (MMPs). In this context, the aim of this study was to evaluate the expression of MMP-2 and -9 and CD31, CD68 (endothelial and histiocytic markers) and the correlations between them using immunohistochemistry, in patients with cardiac allografts. Materials and methods. Tissue fragments were obtained by endomyocardial biopsy from 5 patients with allograft heart transplant, 2 in the medium post-transplant phase and 2 in late phase. After identifying and characterizing the morphological context the probes were processed by standard immunohistochemical technique using anti-MMP-2 and anti-MMP-9 antibodies (Santa Cruz Biotechnology, Inc.) and anti-CD31, anti-CD68 antibodies (Sigma). The samples were examined using the Olympus BX40 microscope with an Olympus E330 camera attached. Results and discussions. Sample examination revealed in all 4 cases the lack of IR (-) for CD31 and weak IR (+) for CD68 compared to MMPs, where we found moderate IR (++) for MMP-9 and weak IR (+) for MMP-2. These aspects complete the histological lesional aspects of these cases, indicating the lack of acute rejection. In conclusion, CD31 and CD68 IR correlated with MMPs IR (especially MMP-9) appear to represent predictive markers for cardiac allograft rejection and require further studies.

Keywords: allograft rejection, biomarkers, immunohistochemistry

Introduction

Cardiac transplantation, performed for the first time in 1967 by Christian Bernard, is currently the only and most effective therapeutic method for patients with end-stage coronary or non-coronary heart diseases(1-3).

Currently, the survival rate is about 50%. The results of the published studies indicate that acute cellular rejection is the most common mechanism encountered in patients who underwent heart transplantation, compared with the much lower incidence of antibody-mediated (humoral) rejection. The „gold standard” for monitoring and diagnosing the heart

transplant patients is percutaneous transvenous endomyocardial biopsy developed by Caves et al. (4-8).

In order to improve long-term survival of heart transplant recipients, it is essential both to understand the cellular and molecular mechanisms involved in transplant rejection and to identify some specific biomarkers with prognostic value (9,10).

The results of recent literature reviews draw attention both to the involvement of CD31, CD68 in the pathogenesis of acute cellular rejection and to the interrelations with matrix metalloproteinases (MMPs) (11-13).

CD31 is an adhesion membrane

glycoprotein belonging to the immunoglobulin family, expressed at the level of the laterobasal pole of endothelial cells, apical pole of the bone marrow stem cells, platelets, and leukocytes, with an important role in the differential diagnosis of acute cell rejection. Its expression on the endothelial cell membrane confers them a certain immune privilege providing immune protection in the event of immune stress, blocking their entry into apoptosis by intrinsic pathways. It also facilitates transendothelial migration of neutrophils and monocytes, both in vitro and in vivo. However, the role of this molecule in protecting endothelial cells against the action of pro-inflammatory cytokines and apoptosis induced by extrinsic pathways is incompletely elucidated (14).

CD68 is a transmembrane glycoprotein expressed in macrophages, neutrophils, osteoclasts, lymphocytes as well as basophils, with a role in microvascular inflammation as well as in antigen (Ag) processing/presentation (12).

Fedriga et al. have associated CD68 immunohistochemical expression with acute humoral rejection, indicating the role of macrophages in the diagnosis of this rejection type both in symptomatic and asymptomatic patients. Thus, the role of this molecule in microvascular inflammation, representing the first sign of cardiac impairment, has been proposed as a potential therapeutic target (12,15).

Acute cardiac allograft rejection also involves a process of extracellular matrix (ECM) degradation, which is an important event in the process of tissue remodeling. This degradation is due to proteolytic enzymes including several types of MMPs and their inhibitors (TIMPs) (16).

It was found that some MMPs are overexpressed in atherosclerotic lesions, the possible therapeutic utility of some MMP inhibitors in this case being suggested (16-18).

It is also believed that a change in MMP and TIMP expression may indicate a phenotypic change of mesenchymal cells, even before the occurrence of morphological changes specific to acute cardiac allograft rejection (16,17).

Based on the results of these studies, it could be concluded that there are complex molecular interactions between immune response and induction of matrix protein expression in cardiac

transplantation. Practically, the interactions between the aforementioned molecules create the molecular picture of acute rejection (18).

Given these aspects, the roles of these molecular interactions and their involvement in a number of heart diseases such as acute myocardial infarction, ischemic or dilated cardiomyopathy, in order to identify biomarker molecules for a non-invasive, sensitive and specific diagnosis of acute cardiac allograft rejection with predictive and prognostic value, we thought necessary and useful to conduct a correlated study of the expression of MMP-2, MMP-9, CD31, CD68 by immunohistochemical technique, both in patients in which anatomopathological examination revealed the absence/presence of suggestive lesions for acute rejection.

Materials and methods

In the present study we used cardiac tissue fragments obtained by serial percutaneous transvenous endomyocardial biopsies performed at the Tg. Mureș "Emergency Institute for Cardiovascular Diseases and Transplant" from 5 male cardiac transplant patients, two of them in middle posttransplant period, with moderate acute rejection signs, with a history of a humoral rejection episode confirmed at that time immunohistochemically and histologically, and three patients of which 2 in middle and 1 in late posttransplant period without obvious signs of acute rejection. Informed consent was obtained from each patient prior to biopsy.

Some of the harvested tissue fragments were processed using paraffin embedding technique and classical hematoxylin-eosin staining in view of making a histopathological diagnosis, and other fragments were processed by standard immunohistochemical technique using anti-MMP-2 and anti-MMP-9 antibodies (dilution 1: 100, Santa Cruz Biotechnology, Inc.), anti-CD31 antibody (dilution 1:50, Sigma), and anti-CD68 antibody (dilution 1: 100) , Sigma). The preparations were examined with an Olympus BX40 microscope with an attached Olympus E330 camera at the Discipline of Cellular and Molecular Biology of the Iasi "Grigore T. Popa" University of Medicine and Pharmacy ".

Results

In order to assess the results obtained from the present immunohistochemical study, the presence of the target molecules was considered weakly positive (+) when immunoreactivity (IR) was detected in about 10% of the total cells (macrophages/endothelial cells, myocytes) and intensely positive (+++) when IR was present in > 50% of the total cells.

In the samples collected from the patients in whom the anatomopathological examination revealed the persistence of acute cellular rejection phenomena and regression of humoral rejection, the examination of the fragments processed by the immunohistochemical technique revealed in the small number of macrophages a moderately positive IR (++) for CD68 (IR <50% for macrophages), and for CD31, a diffuse, weakly positive IR (+) in endothelial cells. These findings suggest the presence of acute cellular rejection, confirming the results of the anatomopathological examination (Figure 1, 2).

Figure 1 Moderate acute cell rejection of myocardium – moderate (++) IR of CD68, X10

Figure 2 Moderate acute cell rejection lesions of myocardium – weak (+) IR of CD31 (detail, X100)

In the patients without obvious signs of rejection where the anatomopathological examination identified features of ischemia, reperfusion and perimyocytic fibrosis, IR for CD31 in endothelial cells was negative (-), and diffuse, weakly positive (+) for CD68 in macrophages (Figure 3, 4).

Figure 3 Myocardium without acute rejection - negative IR of CD31, X20

Figure 4 Myocardium without acute rejection - negative IR of CD68, X40

As to MMP expression in patients with acute cellular rejection, in myocytes IR for MMP-2 was moderately positive (++) at membrane, cytoplasmic and nuclear levels, while IR for MMP-9 was intensely positive (+++) only at membrane and peripheral cytoplasm levels (Figure 5, 6).

Figure 5 Moderate acute cell rejection of myocardium – moderate (++) IR at membrane, cytoplasmic and nuclear level of myocytes MMP-2, X40

Figure 8 Myocardium without acute rejection – strong (++++) IR of MMP-9 in the necrosis clusters and myocytes, at membrane and cytoplasmic level, X40

Figure 6 Moderate acute cell rejection of myocardium – strong (++++) IR at membrane and cytoplasmic periphery of myocytes MMP-9, X100

In the patients without rejection, in myocytes and some fibroblasts, membrane and cytoplasmic IR for MMP-2 was weakly positive (+) and for MMP-9 intensely positive (+++) in necrosis foci and myocytes, being accompanied by obvious hypertrophy of myocyte fibers (Figure 7, 8).

Figure 7 Myocardium without acute rejection – weakly (+) IR of MMP-2 on myocytes and few fibroblast, at membrane and cytoplasmic level, X40

Discussions

The results of the present study regarding the expression of membranous and cytoplasmic MMP-2 in fibroblasts and myocytes in cases without signs of acute rejection confirm the hypothesis of V. Polakova et al. who suggest that in these cases fibroblasts and myocytes are sources of MMPs (5). Weakly positive IR for MMP-2 indicates decreased collagen deposition and remodeling of degraded components by MMP-2, according to data from studies by Campbell et al (19).

MMP-9 overexpression in samples collected from patients in middle posttransplant period and without signs of acute rejection suggest according to literature data on the roles of this MMP type, degradation of ECM components, collagen IV, V, XI, elastin, fibronectin as well as the presence of a chronic inflammatory status. According to the results of the experimental research by Campbell et al. MMP-9 overexpression correlates with inhibition of mechanisms that usually lead to cardiac allograft rejection (19).

Also, according to the results of the studies conducted by Polyakova et al., the intensely positive IR for MMP-9 can also be correlated with the degree of myocardial fibrosis and hypertrophy (5).

In patients without signs suggestive of acute rejection, there is also a weakly positive CD68 expression and the lack of IR for CD31. The negative IR for CD31 reveals the lack of cytoprotective mechanisms, of that immune privilege, indicating at the same time the presence of extensive vascular lesions and necrosis, aspects

confirmed otherwise by the anatomopathological examination. CD31 expression usually indicates the existence of a molecular mechanism that prevents the entry into apoptosis of endothelial cells, by intrinsic, immune-mediated pathways suggesting according to literature data the possibility of preserving vascular integrity, delayed onset of phenomena of chronic rejection and even atherosclerosis (14).

Negative IR for CD31 correlated with MMP-9 overexpression and weakly positive MMP-2 expression suggest the severity of inflammatory lesions and a significant degradation of some ECM molecules.

In the cases without acute rejection, the weakly positive IR for CD68 correlated with the absence of CD31 expression, MMP-2 and 9 expression and presence of ischemia and reperfusion phenomena found at the anatomopathological examination indicate the possibility of chronic rejection.

Comparatively, in the patients in middle posttransplant period but showing signs of acute rejection, there was a positive IR for CD31 and CD68 overexpression and an intensely positive IR for MMP-9 and moderately positive for MMP-2.

MMP-9 overexpression indicates an intense process of remodeling and destruction at EMC level, being, according to the studies by Vanhuetten et al., the only molecule whose level correlates significantly with the level of TNF- α , IL-6 and TGF- β expression and even with the influx of T lymphocytes and monocytes into the cardiac allografts (18).

Also, the moderately positive IR for CD68 correlates functionally with the increased expression of MMP-9, which in its turn indicates extensive inflammatory phenomena and the presence of the extracellular proteolytic processes.

The weakly positive IR for CD31 and CD68 overexpression suggest an increased expression of inflammatory cytokines and a diminished antiapoptotic protection for endothelial cells.

On the other hand, the studies by Lacey et al. shows that cardiac allograft rejection is inhibited in cases where IR for MMP-2 is absent compared to those in which MMP-2 is expressed (17). The correlation of these literature data

with the results of the present study indicating a weakly positive IR for MMP-2 could suggest an extension of cardiac allograft acceptance period.

On the other hand, the same of Lacey et al. found that the presence of MMP-9 decreases the rejection risk although it indicates an intense inflammatory process. The results of their studies show that rejection can occur after a shorter period of time in patients in which MMP-9 IR is negative compared with cases in which MMP-9 is expressed (17).

Thus, in our study MMP-9 expression correlated with MMP-2 expression could be a predictive marker suggesting a low risk of acute cardiac allograft rejection and extension of its acceptance period.

For better accuracy and definition of the predictive value of these MMPs, it is necessary to investigate the expression of other molecules having in view their interrelationships with the expression of proinflammatory cytokines, and not only, such as: IL-6, TGF- β and synd-1, testican-1 etc.

Conclusions

Despite the limitations and small sample size, the results of this study correlated with the literature data, highlight the existence of a correlation between the expression of MMP-2 and MMP-9, the degree of inflammation in the case of cardiac allografts and CD68 expression suggesting the possibility of their use as predictive markers of the risk of acute rejection and / or the occurrence of chronic rejection.

It also confirms the hypothesis of the molecular interactions between the immune response and the induction of matrix protein expression in the case of heart transplantation.

Further studies are needed to elucidate these molecular interrelationships in order to complete the diagnostic modalities of acute cellular rejection and to develop targeted therapies to prevent or limit it.

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