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From Cell Biology to Tissue Engineering

Inflammatory cytokines and antimicrobial peptides in acquired heart diseases

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Summary. Introduction. One of the risk factors for cardiovascular disease is inflammation. The role that it plays in the pathogenesis of cardiovascular disease remains a topic of ongoing research.

The aim of this study was to identify the appearance and distribution of inflammatory markers, interleukins 1α (Il-1α) and 10 (Il-10) and β defensins 2 (βD2), 3 (βD3), and 4 (βD4), in the right atrial tissue from different acquired heart diseases.

Methods. During cardiac surgery, right atrial tissue fragments were taken from 23 patients with acquired heart diseases.

Tissue fragments were stained for immunohistochemical detection of Il-1α, Il-10, βD2, βD3 and βD4.

Results. Few to a moderate number of Il-1α-positive cells and a moderate to great number of Il-10-, βD2- and βD3-positive cells were detected in right atrial tissue. There was a positive correlation between the level of CRP and the number of βD3-positive cardiomyocytes (r_s 0.463; p 0.026). We found a negative correlation between the left ventricular ejection fraction and the number of βD2-positive cells in connective tissue (r_s -0.524; p 0.012).

Conclusions. The rich expression of antimicrobial peptides and its association with CRP support the idea that an inflammatory process is involved in the pathogenesis of acquired heart diseases.

The worst preoperative condition is associated with increased antimicrobial peptide expression in the right atrial cells.

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Introduction

Despite scientific progress and recent decreases in mortality rates in many countries, cardiovascular disease is still the leading cause of death in all of Europe (Nichols et al., 2014). There are many known risk factors for cardiovascular diseases, one of which is inflammation in response to injury, lipid peroxidation, and perhaps infection (Buja, 1996; Mehta et al., 1998; Ridker and Willerson 2004). The role of inflammation in the pathogenesis of cardiovascular disease remains a topic of ongoing research.

The β defensins are classical epithelial antimicrobial peptides and there is growing evidence about their role in the innate immunity. Different studies have screened for β defensin expression in a range of tissues from various species and described expression of βD1, βD2 and βD3 in whole heart homogenate (Linde et al., 2013). It is known that βD2 is also expressed in cardiac fibroblasts and is up-regulated in hypoxia (Karapetyan et al., 2013). In our study, we looked for proinflammatory cytokine Il-1α, antiinflammatory cytokine Il-10 and antimicrobial peptides human β defensins 2, 3 and 4 in the human right atrial tissues from different acquired heart diseases.

Il-1α is a proinflammatory cytokine that is induced and activated following tissue injury and plays an essential role in many inflammatory conditions (Dianarello, 1996). The primary source of Il-1α is fibroblasts, but it is also expressed in normal

keratinocytes of the skin, in the epithelial cells of mucosal membranes, in the liver, in the lung and kidney, in platelets, in the endothelium of the vasculature, and in fetal cardiac tissues (Turner et al., 2007; Westphal et al., 2007; Dinarello and van der Meer, 2013). Increased Il-1 production has been reported in patients with various viral, bacterial, fungal and parasitic infections; in patients with intravascular coagulation, solid tumors, leukemias, autoimmune disorders, trauma, and transplant rejection; and in healthy subjects after strenuous exercise (Dianarello, 1996). Il-1α is not secreted from human cells, but it accumulates intracellularly and at the plasma membrane and is released only when cells undergo necrotic cell death (Turner et al., 2009).

Il-10 is a cytokine in which the primary function appears to limit and control inflammatory responses by suppressing macrophages and inhibiting the production of a number of cytokines (Zdanov, 2006). The release of Il-10 during the acute or chronic inflammatory response regulates innate immune mechanisms following infection and specific immune responses to bacteria, fungi or protozoa (Fujii and Lotze, 2006). It has characteristics of being both an immunosuppressive and immunostimulatory cytokine (Fujii and Lotze, 2006). It is produced by activated T cells, B cells, monocytes/macrophages, mast cells and keratinocytes (Zdanov, 2006).

The β defensins are small cysteine-rich cationic peptides that, in multicellular organisms, are part of the innate local host response to microbial exposure or are inducible by various proinflammatory cytokines (Bals et al., 1998; Schneider et al., 2005; Shi et al., 2014). They are expressed predominantly in epithelial tissues, which provide the first line of defense between an organism and the environment (Schneider et al., 2005). Their remarkably broad antimicrobial spectrum includes fungi, gram-positive and gram-negative bacteria, and some parasites and enveloped viruses (Ganz et al., 1990; Dhople et al., 2006). In tissues with relatively high levels of defensin expression, bacterial infections are uncommon (García et al., 2001a,b; Dhople et al., 2006).

Human β defensin 2 was isolated for the first time from the skin of psoriatic patients (Harder et al., 1997). It can also be detected in gingival, esophageal, tracheal, and intestinal tissue (Mathews et al., 1999; Harder et al., 2001; Schneider et al., 2005; Shi et al., 2014). It is bactericidal to gram-negative bacteria and *Candida albicans* but is relatively ineffective against gram-positive *Staphylococcus aureus* (Huttner and Bevis, 1999). Some studies have demonstrated the association of hβD2 with pro-carcinogenic chronic inflammation (Shi et al., 2014).

Another antimicrobial peptide, human β defensin 3, was isolated from human psoriatic lesion scales (Harder et al., 1997). Similar to hβD2, hβD3 has microbicidal activity against gram-negative bacteria and yeasts, but it also works against gram-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus*, including multiresistant *S. aureus* strains, and even some viruses (Schneider et al., 2005; Jiang et al., 2015). Cellular sources for hβD3 have been found in epithelial cells of the respiratory, gastrointestinal and genitourinary tract, and significant expression has also been detected in nonepithelial tissues such as leukocytes, the mammary gland, cardiac and skeletal muscles (García et al., 2001a,b; Harder et al., 2001).

Human β defensin 4 was discovered in 2001 (García et al., 2001a,b). In contrast to hβD2 and hβD3, which are diffusely expressed throughout many organs, hβD4 expression is currently confined to certain epithelia in the testis, stomach, uterus, neutrophils, thyroid, lungs and kidneys (García et al., 2001a,b). Stimulation with inactivated gram-positive and gram-negative bacteria increases hβD4 expression in human respiratory epithelial cells (García et al., 2001a,b; Schneider et al., 2005).

The aim of this study was to identify the appearance and distribution of inflammatory markers such as interleukins 1α (Il-1α) and 10 (Il-10) and human β defensins 2 (hβD2), 3 (hβD3), and 4 (hβD4) in the right atrial tissue from different acquired heart diseases.

Materials and methods

The reported research activities are consistent with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects and the ethical requirements of Riga Stradins University (Ethics Committee meeting date 29.05.2014). All tissue specimens have been obtained with informed consent.

During elective cardiac surgery, right atrial tissue fragments were taken from 23 patients. All patients were operated on because of the following acquired heart diseases: degenerative aortic valve stenosis (7 patients), coronary heart disease (12 patients), rheumatic mitral and tricuspid valve disease (1 patient), non-rheumatic, non-ischemic mitral valve insufficiency (1 patient), tricuspid valve endocarditis (1 patient) and bicuspid aortic valve and ascending aortic aneurysm (1 patient).

Tissue fragments (~2 mm^2) were taken from the venous cannulae insertion site before cardiopulmonary bypass had begun and cardioplegic solution was administered. Tissue fixation was carried out immediately in the operating room, and for this purpose, previously prepared Eppendorf tubes with saturated picric acid solution (2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2)) were used. Tissue fragments were transported to a morphology laboratory at the Institute of Anatomy and Anthropology, Riga Stradins University. Tissue fragments were washed for 12 hours in 10% sucrose phosphate buffer, embedded in paraffin and cut into 8 μm thick slices.

Tissues for routine light-microscopical examination were stained with hematoxylin and eosin.

All tissue fragments were stained for immuno-histochemical detection of the following:

- Interleukin 1α (sc-9983, working dilution 1:50,

Santa Cruz Biotechnology, Inc., USA)
- Interleukin 10 (ab34843, working dilution 1:400, Abcam, England)
- β defensin 2 (AF2758, working dilution 1:100, R&D Systems, Germany)
- β defensin 3 (orb1832298, working dilution 1:100, Biorbyt, UK)
- β defensin 4 (sc-59496, working dilution 1:50, Santa Cruz Biotechnology, Inc., USA)

CD34 staining was used to verify endothelial cells.

For negative controls the primary antibody was replaced by a diluent. Positive controls (in tissues which always have positive reaction) were prepared for each preparation series as well.

All specimens were observed using a Leica VM 6000B microscope.

For the quantification of structures, a semiquantitative counting method was used. The designations were as follows: 0, negative reaction; 0/+, occasional positive structures in the view field; +, a few positive structures in the view field; +/++, few to a moderate number of positive structures in the view field; ++, moderate count of positive structures in the view field; ++/+++, moderate to great number of positive structures in the view field; +++, numerous positive structures in the view field; ++++, abundance of positive structures in the view field (Pilmane et al., 1998).

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics 22. To determine the differences of distribution of different variables between groups, we used the Mann-Whitney U test. A Spearman's rank-order correlation was used to determine the relationship between variables. Statistical significance was considered at the level of $p<0.05$. Data are presented as the mean± standard deviation (SD).

Patients baseline characteristics

The mean age was (mean±SD) 67.4±14.7 years (range 19-83 years), and there were 11 male patients. All patients were examined as usual prior to heart surgery to exclude focal infection. Six patients had a history of diabetes mellitus. One patient was operated on because of endocarditis, and one patient had rheumatic disease. Four patients (17%) had increased CRP plasma levels and 15 patients received statin therapy before surgery. 74% of patients had good left ventricular systolic function with an ejection fraction of greater than 55%. The left ventricular systolic function of five patients was slightly impaired and one patient had a moderately reduced left ventricular ejection fraction. The right atrial size of all patients was less than 18 cm^2. Ten patients had no signs of pulmonary hypertension, but 12 patients had increased right ventricular systolic pressure; of these 12 patients, eight and four patients had mild and severe pulmonary hypertension, respectively. The right ventricular systolic pressure of one patient was not measured by echo because of severe tricuspid regurgitation. The primary preoperative data are shown in Table 1.

Results

In all specimens, especially in the perinuclear region, myocardial degeneration with diffuse vacuolation of cardiomyocytes was detected. Cardiomyocytes and their nuclei in all specimens varied in size and shape. In all tissue fragments, pyknotic nuclei of cardiomyocytes were detected. In three patients, we found significant vascular sclerosis.

A few to moderate number of interleukin 1α-positive cells were found in all specimens. These cells were most commonly found among connective tissue cells (Fig. 1) and epi-/endocardial cells, but interleukin 1α -positive cardiomyocytes were not found in any specimens aside from one specimen which showed an atypical reaction - all structures were positive for Il-1α.

In all tissue fragments, a moderate to great number of interleukin 10-, β defensin 2-, and β defensin 3-positive cells were detected. Factor positive cells were found among cardiomyocytes (Fig. 2A-C), connective tissue (Fig. 2D-F), endothelial cells, and endocardial and epicardial cells.

In almost all tissue fragments, regions with cube shaped endocardial endothelial cells (Fig. 3A-C) were detected. These cells were positive for βD2 (Fig. 3A), βD3 (Fig. 3B) and Il-10 (Fig. 3C).

There were 3 patients – 2 patients with coronary

Table 1. Baseline characteristics.

n=23		n(%)
Male		11 (48%)
Diabetes mellitus		6 (26%)
Rheumatic disease		1 (4%)
Endocarditis		1 (4%)
CRP, mg/l		
	<5,0	19 (83%)
	>5,0	4 (17%)
Use of Statins		15 (65%)
LVEF, %		
	>55	17 (74%)
	39-55	5 (22%)
	29-38	1 (4%)
RAA, cm^2		
	>18	23 (100%)
	<18	0
RVSP, mmHg (n=22)		
No PH	0-24	10 (45%)
Mild PH	25-40	8 (36%)
Moderate PH	41-55	0
Severe PH	>55	4 (18%)

CRP, C reactive protein; LVEF, left ventricular ejection fraction; RAA, right atrial area; RVSP, right ventricular systolic pressure; PH, pulmonary hypertension.

heart disease and 1 patient with non-rheumatic, non-ischemic mitral valve insufficiency – in which the cube shaped endocardial endothelial cells in the right atrial tissue were not found; however, their squamous endothelial epithelium showed positive responses to Il-10, βD2 and βD3.

There was one specimen in which we found groups of newly performed microvessels (Fig. 3D-G) with cube shaped endothelial cells. These cube-shaped endothelial cells were positive for Il-1 (Fig. 3D), Il-10 (Fig. 3E), β defensin 2 (Fig. 3F), and β defensin 3 (Fig. 3G).

β defensin 4-positive cells were not found in any of the specimens.

Statistical analysis

The distribution of Il-1-positive cells in the connective tissue of patients with coronary heart disease was significantly higher than in the right atrial tissue of patients with degenerative aortic valve stenosis (p 0.011). However, the distribution of Il-10-positive cardiomyocytes was significantly higher in patients with aortic valve stenosis compared to patients with coronary heart disease (p 0.020). There were no statistically significant differences of distribution of βD2-, βD3- and βD4-positive cells in patients with coronary heart disease and aortic valve stenosis.

There was a statistically significant moderate positive correlation between the plasma level of CRP and the number of βD3-positive cardiomyocytes (r_s 0.463; p 0.026). Using Spearman's rank correlation, we also found a statistically significant moderate positive correlation between the plasma level of CRP and the number of βD2-positive cells in connective tissue (r_s 0.414; p 0.050). We found a negative moderate correlation between the left ventricular ejection fraction and the number of βD2-positive cells in the right atrial epi/endocardium (r_s -0.489; p 0.021) and in connective tissue (r_s -0.524; p 0.012). However, the number of Il-1-positive cells in the connective tissue showed a statistically significant strong positive correlation with the number of Il-10-positive right atrial vascular endothelial cells (r_s 0.655; p 0.001), a moderate positive correlation with the number of βD2-positive connective tissue cells (r_s 0.418; p 0.047), but a moderate negative correlation with the number of IL-10-positive cardiomyocytes (r_s -0.494; p 0.016). There was a statistically significant moderate positive correlation between the number of Il-10-positive cardiomyocytes and the number of βD3-positive cardiomyocytes (r_s 0.571; p 0.004). A statistically significant moderate negative correlation was found between Il-10 expression in the epi/endocardium and βD2 expression in the vascular endothelial cells (r_s -0.434; p 0.038), IL-10 expression in connective tissue and βD3 expression in epi/endocardium (r_s -0.586; p 0.003). The number of βD2-positive cardiomyocytes showed a statistically significant moderate positive correlation with the number of βD3-positive connective tissue cells (r_s 0.530;

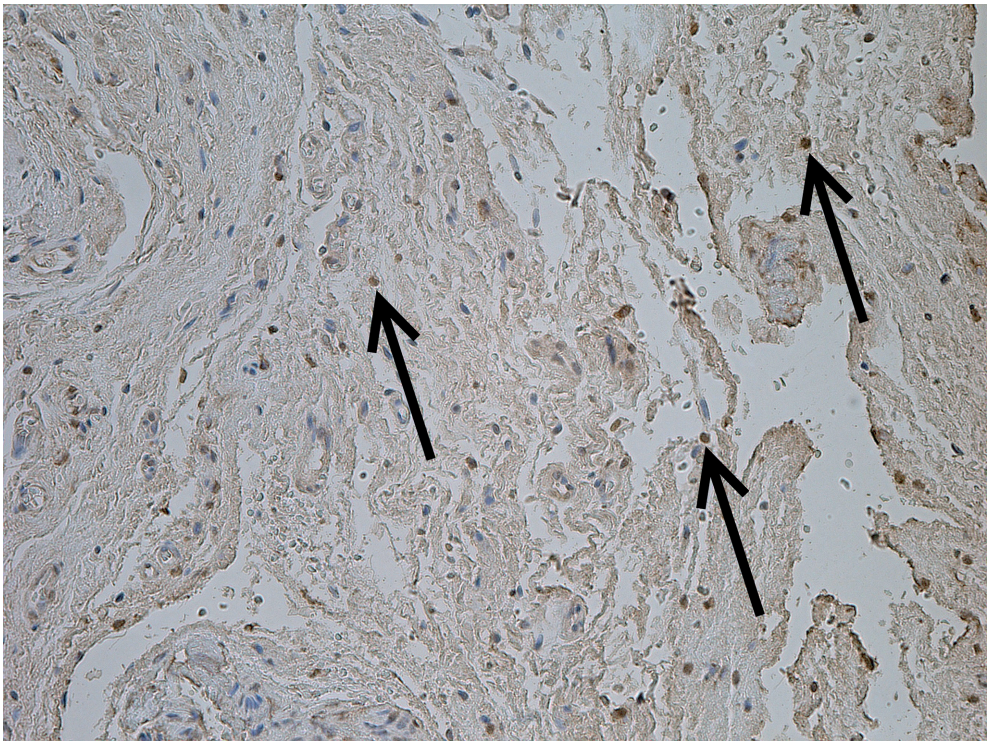

Fig. 1. Few interleukin 1α-positive cells among right atrial connective tissue cells (arrows, IMH). x 200.

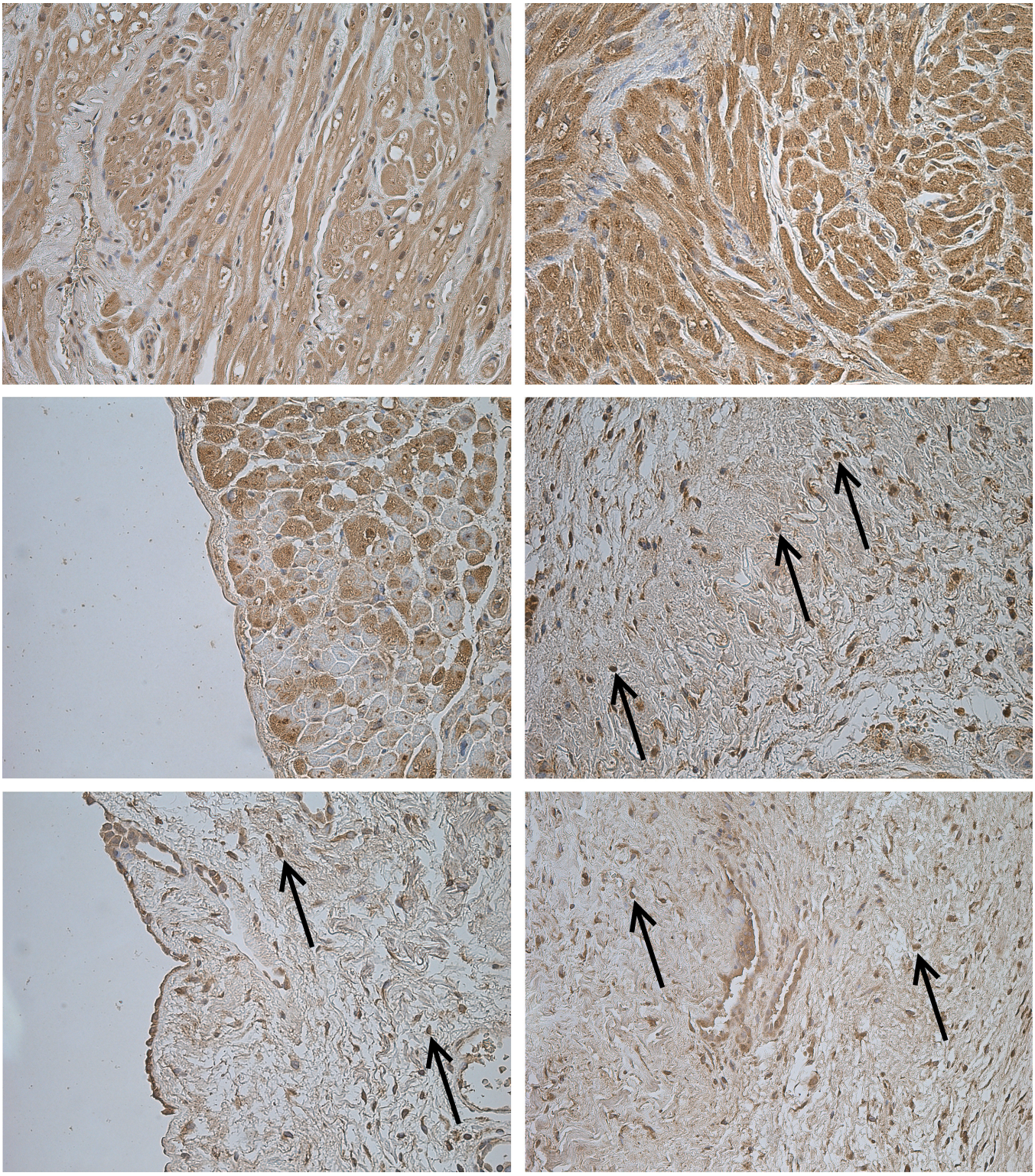

Fig. 2. A. Numerous β defensin 2-positive cardiomyocytes. **B.** Numerous β defensin 3-positive cardiomyocytes. **C.** Moderate interleukin 10-positive cardiomyocytes. **D.** β defensin 2-positive connective tissue cells. **E.** β defensin 3-positive connective tissue cells (arrows). **F.** Interleukin 10-positive connective tissue cells (arrows). (IMH) A-E, x 250; F, x 200.

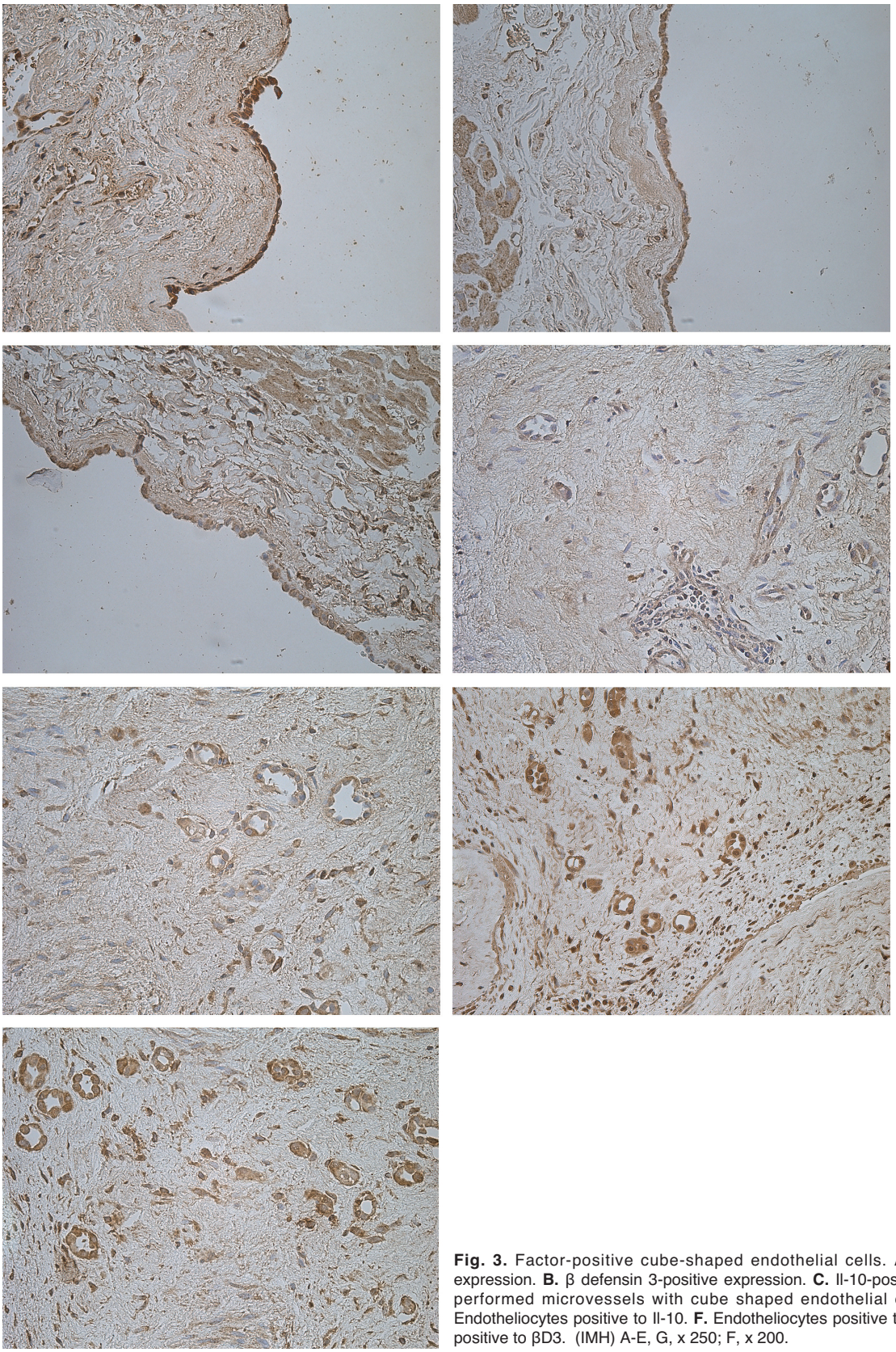

Fig. 3. Factor-positive cube-shaped endothelial cells. **A.** β defensin 2-positive expression. **B.** β defensin 3-positive expression. **C.** Il-10-positive expression. **D.** Newly performed microvessels with cube shaped endothelial cells positive for Il-1. **E.** Endotheliocytes positive to Il-10. **F.** Endotheliocytes positive to βD2. **G.** Endotheliocytes positive to βD3. (IMH) A-E, G, x 250; F, x 200.

p 0.009) and epi/endocardial cells (r_s 0.522; p 0.011), but the number of βD2-positive cells in connective tissue showed a positive moderate correlation with the number of βD3-positive cells in the same tissue (r_s 0.447; p 0.032) and an even stronger correlation with the number of βD3-positive epi/endocardial cells (r_s 0.641; p 0.001). There was a moderate correlation between βD2 expression and βD3 expression in epi/endocardium (r_s 0.565; p 0.005). The right ventricular systolic pressure showed a statistically significant correlation with the number of βD2 (r_s 0.661; p 0.004) and βD3 (r_s 0.487; p 0.047) positive epi/endocardial cells.

Discussion

Our study demonstrates a slight to moderate distribution of inflammatory markers such as interleukins 1α and 10 and a moderate to rich distribution of antimicrobial peptides such as human β defensins 2, 3 and 4 in different structures of three diseased right atrial tissues – cardiomyocytes, connective tissue, and endothelium. These findings support the possibility of "hidden" inflammation and even infection in the pathogenesis of acquired heart diseases.

In our study, we found that Il-1α in the right atrial tissue from diseased hearts was mostly expressed by connective tissue cells and endocardial endothelial cells and that its expression did not differ among different acquired diseases. It has already been reported that Il-1α protein levels are increased in infarcted myocardium following myocyte necrosis (Timmers et al., 2008; Turner et al., 2009). Cardiac myofibroblasts cultured from different patients consistently respond to Il-1α through increased expression of other specific proinflammatory cytokines – interleukin 1β, tumor necrosis factor α, interleukin 6 (Turner et al., 2009). Il-1α, together with other proinflammatory cytokines, play a key role in the myocardial inflammatory response and, in the long term, the sustained expression of proinflammatory cytokines is detrimental and can lead to progressive heart failure (Turner et al., 2007). Slight expression of Il-1α in all specimens might indicate myocardial remodeling. It should be noted that 65% of our patients received statin therapy, which could reduce myocardial expression of proinflammatory cytokines in human hearts, but we did not find any correlation between the use of statins and the expression of inflammatory markers (Turner et al., 2007).

Interleukin 10 is a potent antiinflammatory cytokine (Downing et al., 1998). The detection of a moderate to great number of Il-10-positive cells in all tissue fragments with a mild, statistically nonsignificant tendency to be expressed more in the right atrial tissue from patients with chronic coronary heart disease compared to those with aortic valve stenosis suggests that Il-10 secretion is related to a common pathogenic factor. Both coronary heart disease and aortic valve stenosis are polyetiologic diseases, and include etiologic factors such as chronic inflammation and atherogenesis (O'Brien, 2006; Zakynthinos and Pappa 2009; Parisi et al., 2015). Atherosclerosis is an inflammatory disease; furthermore, several reports have shown a correlation between the incidence of atherosclerosis and the presence of infectious microorganisms (Ross, 1999). It has already been shown that Il-10 is expressed in human atherosclerotic plaques and in thrombosed vein walls and that it is associated with decreased signs of inflammation (Downing et al., 1998; Mallat et al., 1999a,b). Additionally, there are several lines of evidence suggesting that Il-10 is important in the remodeling of the myocardium after myocardial infarction, but it has little or no effect on proinflammatory cytokine expression (Turner et al., 2009).

The most striking discovery in our study was the rich expression of antimicrobial peptides, such as human β defensins 2 and 3, in the right atrial tissues from all specimens. Beta defensins are antimicrobial peptides that form a part of the first line of host defense against bacterial infections. Furthermore, βD2 concentration depends on the stage of infection; high concentrations occur in the active stage, and normalization occurs during the recovery period (Yanagi et al., 2007) There are no doubts about the presence of inflammation during the pathogenesis of acquired heart diseases such as coronary heart disease and degenerative aortic valve stenosis, but our findings may support the notion of the importance of infection during the pathogenesis of these diseases (Leopold, 2012; Yayan, 2013). We also found a correlation between the expressions of βD3 in cardiomyocytes and in the right atrial connective tissue and the plasma level of C-reactive protein, which is an acute-phase protein whose levels rise in response to inflammation (Pepys and Hirschfield 2003). It should also be noted that the worst preoperative conditions (e.g., reduced left ventricular systolic function, pulmonary hypertension and history of atrial fibrillation) showed moderate and strong correlations with the number of βD2 and βD3-positive epi/endocardial and connective tissue cells.

Conclusions

The rich expression of human βD2 and βD3 in the right atrial tissue of diseased hearts and their association with CRP support the notion of an inflammatory process in the pathogenesis of these acquired heart diseases.

The worst condition (e.g., reduced left ventricular systolic function, pulmonary hypertension and history of atrial fibrillation) is associated with increased levels of antimicrobial peptide - βD2 and βD3 - expression in the right atrial cells.

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