Peer-reviewed version available at *Medicina* **2019**, *55*, 766; doi:10.3390/medicina55120766

Short communication

Indexes of Angiogenic Activation in Myocardial Samples of Patients with Advanced Chronic Heart Failure

Klara Komici K[1*], Isabella Gnemmi I[2], Claudia Sangiorgi[2], Fabio Luigi Massimo Ricciardolo [3], Mauro Rinaldi[4], Antonino Di Stefano[2#], Eleuteri E[5#].

[1] Department of Medicine and Health Sciences, University of Molise, Italy. klara.komici@unimol.it
[2] Pulmonary Rehabilitation Unit and Laboratory of Cytoimmunopathology of the Heart and Lung, Istituti Clinici Scientifici Maugeri, Veruno, Italy isabella.gnemmi@icsmaugeri.it; san.85@hotmail.it antonino.distefano@icsmaugeri.it
[3] Department of Clinical and Biological Sciences, University of Torino, San Luigi Hospital , Turin , Italy. fabioluigimassimo.ricciardolo@unito.it
[4] Department of Cardiovascular and Thoracic Surgery, University of Turin, Italy. mauro.rinaldi@unito.it
[5] Division of Cardiology, Istituti Clinici Scientifici Maugeri, Veruno, Italy. ermanno.eleuteri@icsmauger.it
# These authors contributed equally to the work.
* Correspondence:
Klara Komici, MD
Department of Medicine and Health Sciences, University of Molise
Via De Sanctis, 86100 Campobasso
Tel. +390874404771 Fax +390874404778 e-mail: klara.komici@unimol.it

Abstract: Background and Objectives: Ischemic and idiopathic heart failure are two different etiologies, however reactive cardiac fibrosis together with impaired vasculogenesis has been described in both of them. Implication of main proangiogenic factors as: angiogenin, agiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) has been described mainly in experimental models of heart failure. However, differences in molecular pathways between these cardiomyopathies are still under investigation. In this short communication we aimed to evaluate and compare the expression of pro-angiogenic molecules in the heart tissue of patients with advanced chronic heart failure (CHF) of ischemic and idiopathic etiology. Methods and Results: Heart tissue from left ventricular walls was obtained at transplantation from ischemic heart disease (IHD), idiopathic cardiomyopathy (ICM) patients. Tissue samples were examined using immunohistochemistry for angiogenic molecules. Immunopositivity (I-pos) for angiopoietin-1 was mainly observed in the cardiomyocytes, while I-pos for Ang-2 and Tie-2 receptor mainly in endothelial cells. Procollagen-I (PICP), angiogenin, Ang-1, Tie-2 receptor, were similarly expressed in IHD and ICM patients. In contrast, endothelial immunopositivity for Ang-2 was higher in IHD samples compared to ICM (p=0.03). Conclusions: Ang-2 expression is different in heart tissue of ICM and ICM patients and distribution of Ang-1 and angiogenin is higher in cardiomyocytes, whereas Ang-2 higher in endothelial cells, suggesting a different pattern of angiogenic stimulation, or at least of altered endothelial integrity. This data may serve for further studies investigating angiogenesis signaling pathways and in HF of different etiology.

Keywords: heart failure, angiogenesis, angiopoietin-1, angiopoietin-2, cardiac fibrosis.

1. Introduction

Ischemic and idiopathic heart failure (HF) are different etiologies, however reactive cardiac fibrosis together with impaired vasculogenesis has been described in both of them [1]. Angiogenesis process, is essential in a number of pathophysiological responses to injury such as gastrointestinal ulcer, stroke, myocardial infarction and left ventricular hypertrophy [2-4] and is regulated by Vascular Endothelial Growth Factor (VEGF) and of angiopoietins-Tie (Ang-Tie) signaling pathway [5-6]. Ang-1 and Ang-2 are vascular growth factors expressed mainly on endothelial cells. Ang-1 induces Tie-2 receptor activation and facilitates endothelial cell sprouting and vascular network maturation. Ang-2 also binds to Tie-2 receptor but inhibits Ang-1-Tie signaling by blocking Ang-1-induced phosphorylation of Tie-2, resulting in vascular destabilization and remodeling [7-8]. Angiogenin, a member of the ribonuclease (RNAse) superfamily, is a potent inducer of neovascularization in vivo, and its circulating levels reflect various angiogenic activities that include increased vessel permeability, endothelial proliferation and vascular maturation [9]. While idiopathic HF may have different triggers as immunological, metabolic or genetic, the current pathophysiological model of ischemic HF is focused on atherosclerosis process and impairment of angiogenesis. Data from experimental models and evaluation of circulating levels of angiogenesis factors in patients with idiopathic dilative cardiomyopathy report also that there is abnormal angiogenesis associated to cardiac remodeling and HF progression [10-11]. From the other side Ang-2 role in atherosclerosis report conflicting results. If Ang-2 antibodies administration leads to inhibition of atherosclerotic plaque progression, also it has been described that Ang-2 has a protective role against LDL oxidation [12-13]. Anyway most of the mentioned studies are performed on experimental models and data that describe vasculogenesis pattern in end stage HF are lacking. Furthermore, the differences in molecular pathways between idiopathic and ischemic cardiomyopathy are still under investigation. Therefore, in this brief report we aimed to evaluate and compare the expression of pro-angiogenic molecules in the heart tissue in patients with advanced CHF of ischemic and idiopathic etiology.

2. Materials and Methods

This study was carried out in accordance with the recommendations of the ethical committee of the Fondazione Salvatore Maugeri, IRCCS, Pavia, Italy. The protocol was approved by the Central Ethics Committee (CEC) of Fondazione Salvatore Maugeri, Pavia, Italy (archivial n.382 CEC). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

2.1 Heart tissue specimens from IHD and ICM patients

Heart tissue from anterior or basolateral left ventricular walls was obtained at transplantation from nine CHF patients (mean±SE: age 60.8±1.9, all male) with ischemic heart disease (IHD), and from seven CHF patients (age 59.6±2.4, four male) with idiopathic cardiomyopathy (ICM).

2.2 Immunohistochemistry in the heart tissue

Heart tissue samples were snap frozen within less than four hours after transplantation. Frozen sample was then oriented and 6 microns thick cryostat sections were cut and immunostained with a panel of primary antibodies applied in TRIS-buffered saline and revealed with the use of appropriate secondary antibodies and fast-red substrate. The following panel of primary antibodies was used: goat anti angiogenin, Santa Cruz, sc-1408 (1:150); goat anti Ang-1, Santa Cruz, sc-6319 (1:200); goat anti Ang-2, Santa Cruz, sc-7016 (1:200); rabbit anti Tie-2, Santa Cruz, sc-9026 (1:100); goat anti procollagen-I, Santa Cruz, sc-8782 (1:50). Human tonsil or nasal polyps were used as a positive control. For the negative control slides, normal goat or rabbit non-specific immunoglobulins (Santa Cruz Biotechnology) were used.

2.3 Scoring system for immunohistochemistry

Light microscopic analysis was performed at a magnification of 630X. The immunostaining was scored in each cell compartment (range: - = absence of immunostaining, + = 1-33% of immunostained cells; ++ = 34-66% of immunostained cells; +++ = 67-100% of immunostained cells) in the heart tissue.

2.4 Statistical analysis

Data were expressed as mean±standard deviation for functional data and median (range) for morphologic data. Differences between groups were analyzed using analysis of variance (ANOVA) for functional data. The ANOVA test was followed by the unpaired t-test for comparison between groups. The Mann-Whitney U test was applied for comparison between groups of morphologic data. Probability values of $p<0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA, USA).

3. Results

3.1. Immunohistochemistry in the heart tissue

High levels of immunopositivity for angiogenin, Ang-1 and Tie-2 has been found both in IHD and ICM patients. Immunopositivity (I-pos) for angiogenin was frequently observed in the cardiomyocyte perinuclear space, in the subendothelial layer of endocardium and occasionally in endothelial cells and in inflammatory cells infiltrating the heart tissue, while I-pos for angiopoietin-1 was mainly observed in the cardiomyocytes, and only occasionally in endothelial cells and in infiltrating inflammatory cells; I-pos for Tie-2 was widely expressed in endocardial endothelial cells, cardiomyocytes and occasionally in the inflammatory cells . I-pos for Ang-2 showed a different distribution, being mainly observed in the endothelial cells and occasionally in inflammatory cells infiltrating the heart tissue (Table 1). Though less represented in the heart tissue in comparison to other angiogenic molecules, I-pos for Ang-2 was significantly higher in IHD vs ICM patients (Mann-Whitney: $p=0.03$). Scored I-pos for procollagen I was similar in IHD and ICM patients (Table 2) and (Figure 1, panels A and B).

Table 1. Immunopositivity scored distribution of angiogenic molecules in heart tissue samples coming from advanced CHF patients.

	ANGIOGENIN	**ANGIOPOIETIN-1**	**ANGIOPOIETIN-2**	**TIE-2**	**PICP**
CPS	+++	+++	-	+++	+++
EC	+--	+--	+++	+++	---
IIC	+--	+--	+--	+--	---

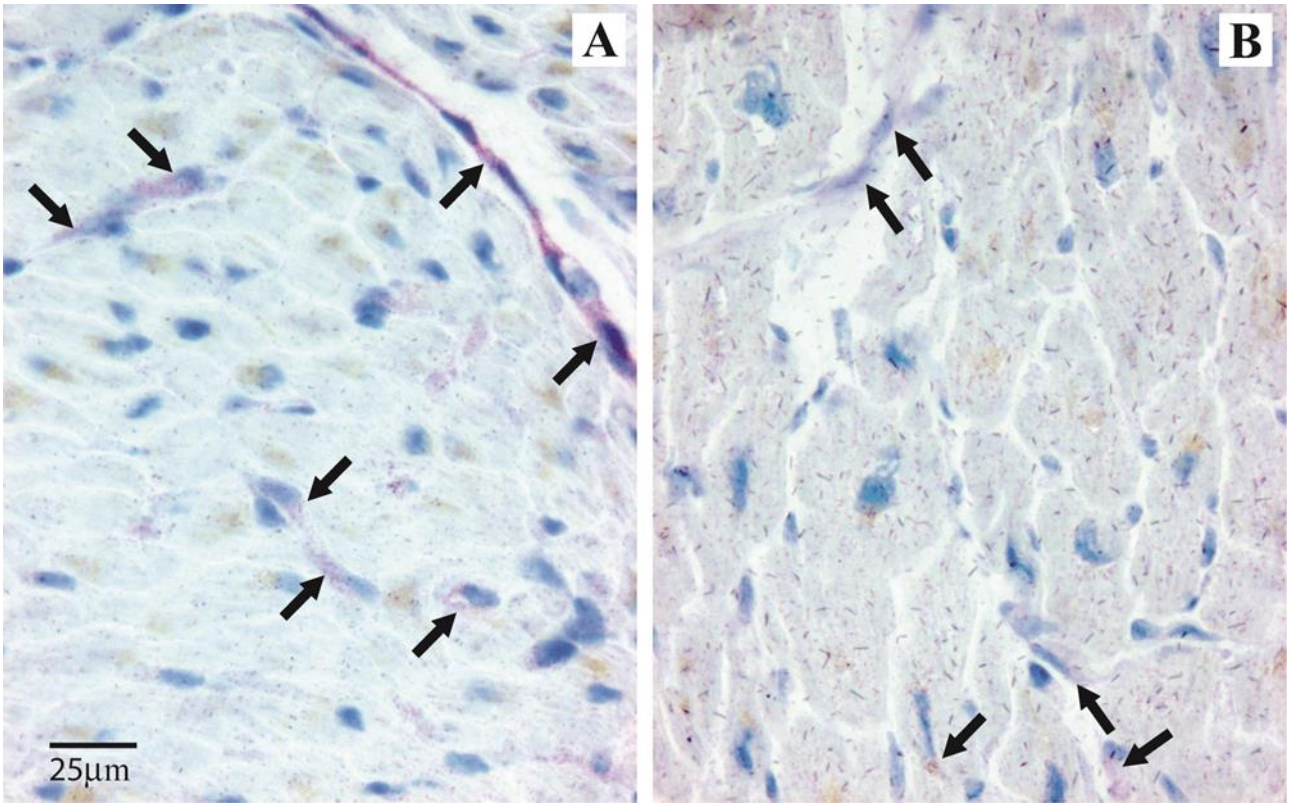
CPS: cardiomyocyte perinuclear space; EC: endothelial cells; IIC: infiltrating inflammatory cells.

Table 2. Immunohistochemical analysis of angiogenic proteins in the heart tissue obtained at transplantation from ischemic and idiopathic CHF patients.

SUBJECTS (n)	ANGIOGENIN	ANGIOPOIETIN-1	ANGIOPOIETIN-2	TIE-2	PICP
IHD (9)	1.25 (0.75-1.75)	2.625 (1.25-3)	0.625 (0.25-1.25)*	1.375 (1.125-1.75)	2.25 (1.5-2.5)
ICM (7)	0.5 (0.375-2)	2.5 (0.25-2.75)	0.25 (0-0.75)	1.75 (0-2.125)	2.25 (1.5-2.5)

Heart tissue immunopositivity for angiogenic proteins was scored from 0=absence of immunopositivity to 3=extensive immunopositivity involving all endothelial cells and cardiomyocytes). Ang=angiopoietin; IHD= Ischemic Heart Disease; ICM= Idiopathic

Figure 1. Angipoitein-2 expression in heart tissue samples from ischemic and idiopathic cardiomyopathy



4.1 Discussion

In our series of heart tissue samples coming from CHF patients we demonstrated detectable signs of angiogenic molecules presence, as shown by intense immunopositivity for angiogenin, Ang-1 and Tie-2, both in IHD and ICM patients. In the present literature, is reported that ischemic heart disease is characterized by a compensatory increase in angiogenic factors [14], and previous studies [15-16], describe an increase of angiogenic factors in chronic HF patients compared to healthy controls. Importantly, these reports are based on serum evaluation of angiogenic biomarkers while we report the presence of angiogenic pattern in human cardiac samples. In IHD samples, compared to ICM a statistically significant higher expression of Ang-2 was evident. Different studies have reported an important role of Ang-2 in the prediction of negative outcome in ischemic heart disease patients and a study performed on adults with congenital heart disease as well

confirmed this result [15,17]. In our study Ang-2 was less pronounced in heart tissue of ICM patients suggesting a different pattern of angiogenic stimulation, or at least of altered endothelial integrity. Based on the immunohistological analysis we found a greater distribution of Ang-1 and angiogenin in cardiomyocytes, whereas Ang-2 was higher in endothelial cells.

Deletion of specific Ang-1 in cardiomyocytes has demonstrated to contribute to defective formation of coronary vessels during embryonic development [18]. Furthermore overexpression of Ang-1 has shown a protective effect in cardiomyocytes against doxorubicine induced hypoxia [19]. These data have suggested a protective effect of Ang-1 in cardiomycocytes. Indeed we found a greater distribution of Ang-1 in cardomyocyte cells compared to endothelial cells in both HF. Similar with experimental data in the literature, reporting that Ang-2 is stored at endothelial cells [20], at heart tissue level we found a distribution pattern of angiopoietins and its receptor Tie-2 that suggest that Ang-2 acts selectively through activation of endocardial endothelial cells. This may suggest an activation state of vascular bed in this subgroup of patients, and the up-regulation of Ang-2 that we found in IHD patients may suggest a more peculiar attempt at cardiac revascularization taking place in this subgroup of patients with CHF. Unfortunately, the low levels of immunopositivity for Ang-2 in the heart tissue can only lead us to speculate on the role of this molecule in terms of induction of tissue repair and/or remodeling. Furthermore, the similar scored immunopositivity for procollagen I in IHD and ICM , independently from the initial cause of cardiomyopathy, may suggest at least in part, different intermediate molecular mechanisms involved in the fibrotic process developing in IHD and ICM patients.

4.2 Study limitation

Our data are based on a relative small group of patients and we did not include heart samples from healthy controls. Further experiments would be necessary for deepening of angiogenesis signaling. However these are short communication data and we hope that this will serve for future studies.

5. Conclusions

Ang-2 expression is different in heart tissue of ICM and ICM patients suggesting a different pattern of angiogenic stimulation, or at least of altered endothelial integrity. The heart tissue distribution of Ang-1 and angiogenin is higher in cardiomyocytes, whereas Ang-2 higher in endothelial cells. This data may serve as a platform for further studies investigating the possible angiogenesis signaling pathways and mechanism in HF of different etiology.

Author Contributions: conceptualization, E.E,K.K. and A.DS.; methodology, A.DS, C.S and I.G. formal analysis, K.K.; data curation, A.DS.; writing—original draft preparation, K.K, M.R, FLM.R.;

Funding: This research received no external funding

.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Roura, S., Bayes-Genis, A. Vascular dysfunction in idiopathic dilated cardiomyopathy. Nat Rev Cardiol. 2009 Sep;6(9):590-8. doi: 10.1038/nrcardio.2009.130. Review.
2. Moryja, J., Minamino, T. Angiogenesis, Cancer, and Vascular Aging. Front. Cardiovasc. Med., 24 October 2017
3. Eklund, L., Kangas, J., Saharinen, P. Angiopoietin-Tie signalling in the cardiovascular and lymphatic systems. Clin Sci (Lond). 2017 Jan 1;131(1):87-103. Review. DOI: 10.1042/CS20160129.
4. Felmeden, D.C., Blann, A.D., Lip, G.Y. Angiogenesis: basic pathophysiology and implications for disease. Eur Heart J. 2003;24:586-603.

5. de Lucia, C., Eguchi, A., Koch, W.J. New Insights in Cardiac β-Adrenergic Signaling During Heart Failure and Aging. Front Pharmacol. 2018 Aug 10;9:904. doi: 10.3389/fphar.2018.00904. eCollection 2018.
6. Rengo, G., Cannavo, A., Liccardo, D., et al. Vascular endothelial growth factor blockade prevents the beneficial effects of β-blocker therapy on cardiac function, angiogenesis, and remodeling in heart failure. Circ Heart Fail. 2013 Nov;6(6):1259-67.
7. La Rocca, G., Di Stefano, A., Eleuteri, E., et al. Oxidative stress induces myeloperoxidase expression in endocardial endothelial cells from patients with chronic heart failure. Basic Res Cardiol. 2009;104:307-320.
8. Chin, B.S., Chung, N.A., Gibbs, C.R., Blann, A.D., Lip, G.Y. Vascular endothelial growth factor and soluble P-selectin in acute and chronic congestive heart failure. Am J Cardiol. 2002;90:1258-1260.
9. Chong, A.Y., Caine, G.J., Freestone, B., Blann, A.D., Lip, G.Y,. Plasma angiopoietin-1, angiopoietin-2, and angiopoietin receptor tie-2 levels in congestive heart failure. J Am Coll Cardiol. 2004;43:423-428.
10. Patel, J.V., Sosin, M., Gunarathne, A., et al. Elevated angiogenin levels in chronic heart failure. Ann Med. 2008;40:474-479.
11. Ohtsuka, T., Inoue, K., Hara, Y., et al. Serum markers of angiogenesis and myocardial ultrasonic tissue characterization in patients with dilated cardiomyopathy. Eur J Heart Fail. 2005 Jun;7(4):689-95.
12. Ahmed, A., Fujisawa, T., Niu, X.L., et al. Angiopoietin-2 confers Atheroprotection in apoE-/- mice by inhibiting LDL oxidation via nitric oxide. Circ Res. 2009 Jun 19;104(12):1333-6. doi: 10.1161/CIRCRESAHA.109.196154. Epub 2009 May 21.
13. Theelen, T.L., Lappalainen, J.P., Sluimer, J.C., et al. (2015) Angiopoietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice. Atherosclerosis 241, 297–304.
14. Eleuteri, E., Di Stefano, A., Tarro Genta, F., et al. Stepwise increase of angiopoietin-2 serum levels is related to haemodynamic and functional impairment in stable chronic heart failure. Eur J Cardiovasc Prev Rehabil. 2011 Aug;18(4):607-14. doi: 10.1177/1741826710389410. Epub 2011 Feb 11.
15. Tello-Montoliu, A., Marín, F., Patel, J., et al. Plasma angiogenin levels in acute coronary syndromes: implications for prognosis. Eur Heart J. 2007 Dec;28(24):3006-11. Epub 2007 Nov 2. DOI: 10.1093/eurheartj/ehm488.
16. Eleuteri, E., Di Stefano, A., Giordano, A., et al. Prognostic value of angiopoietin-2 in patients with chronic heart failure. Int J Cardiol. 2016 Jun 1;212:364-8. doi: 10.1016/j.ijcard.2016.03.005.
17. Lukasz, A., Beutel, G., Kümpers, P., et al. Angiopoietin-2 in adults with congenital heart disease and heart failure. PLoS One. 2013 Jun 24;8(6):e66861. doi: 10.1371/journal.pone.0066861. Print 2013.
18. Arita, Y., Nakaoka, Y., Matsunaga, T., et al. (2014) Myocardium-derived angiopoietin-1 is essential for coronary vein formation in the developing heart. Nat. Commun. 5, 4552.
19. Ren, D., Zhu, Q., Li, J., et al. Overexpression of angiopoietin-1 reduces doxorubicin-induced apoptosis in cardiomyocytes. J Biomed Res. 2012 Nov;26(6):432-8. doi: 10.7555/JBR.26.20120006. Epub 2012 Jun 6. DOI: 10.7555/JBR.26.20120006.
20. Kim, I., Kim, H.G., Moon, S.O., et al. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. Circ Res. 2000;86:952-959.