Essential role of the Notch ligand Delta-like 1 in coronary arteriogenesis and cardiac recovery after myocardial infarction

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ABSTRACT

The development, homeostasis, and regeneration after injury of the cardiovascular system comprise a huge array of factors and mechanisms which have to work in a temporal and spatial organized fashion. Myocardial infarction is a highly prevalent ischemic disease and multiple studies have demonstrated that only arteriogenesis has considerable ability to fully restore blood flow, which is absolutely critical for the regeneration of all ischemic organs. Notch signalling constitutes an evolutionary conserved pathway and – as activators of the pathway – the Notch ligands play a critical role. The ligand Delta-like 1 (Dll1) has been associated with the maintenance of arterial identity during development and peripheral limb arteriogenesis in the adult. Yet, its function in the coronary vasculature and in cardiac remodelling has not been analyzed to date. This study identifies the Notch ligand Dll1 as critical regulator of developmental/neonatal coronary arteriogenesis and provides evidence that Dll1 is involved in cardiac recovery after myocardial infarction.

To analyse expression and role of Dll1 in adult hearts, $Dll1^{+/lacZ}$ reporter mice were the focus of this study, serving as Dll1-lacZ reporter and Dll1 heterozygous strain. In the heart, Dll1 expression was specific for endothelium of coronary arteries >20µm. Coronary artery analysis revealed a reduced number of conductance vessels (>20 µm), but an increased number of arterioles <20 µm. Data suggest a model of the coronary artery phenotype, were reduced levels of Dll1 impairs developmental/neonatal coronary arteriogenesis, becoming evident in the adult by a reduced coronary vessel size in the heart basis and causing a reduced number of vessels reaching more distal heart areas. Altered development of the coronary vasculature caused reduced heart weight and size in Dll1 heterozygous animals, but body weight and size, as well as cardiac function were unchanged. Data demonstrated a relationship where the smaller heart size with concurrent normal body weight is compensated by an elevated ejection fraction, resulting in a normal stroke volume and cardiac output. This finding elucidated the normal 18 month survival of Dll1 heterozygous mice. In addition, monocyte subset analyses demonstrated reduced total monocyte numbers and reduced Ly-$6C^{lo}$ monocytes in Dll1 heterozygous spleen tissue.

To define the role of Dll1 in response to myocardial infarction, mice were subjected to permanent LAD occlusion. Whereas wildtype (WT) control animals demonstrated

functional remodelling and preservation of cardiac function, Dll1 heterozygous animals exhibited features of adverse remodelling: increased inflammation, infarct expansion, progressive dilation and hypertrophy, and complete lack of arteriogenesis, resulting in infarct size enlargement, ventricular dysfunction and progressive mortality. Adverse remodelling in Dll1 heterozygotes was rather based on altered infarct healing mechanisms, than on the extent of the initial ischemic incidence. There is evidence to suggest that impaired arteriogenesis and enhanced inflammation are direct effects of diminished Dll1 levels, whereas infarct expansion, and progressive dilation and hypertrophy are rather downstream effects. These data highlight the importance of Dll1 mediated Notch signalling for correct compensation and functional remodelling to preserve ventricular function after myocardial infarction.

Dll1 was selectively expressed in the heart in arterial endothelium of large coronary arteries and Dll1 expression was upregulated after infarction. However, at least in a setting of myocardial infarction by permanent LAD occlusion, this study provided first evidence that endothelial Dll1 is not the major determinant causing adverse remodelling effects upon absence. Data rather identified an extravascular role of Dll1 in infarct healing, adumbrating a role in the monocyte/macrophage system, but the exact site and mode of action remains an open question which will have to be addressed by future studies.

Keywords: Dll1, arteriogenesis, myocardial infarction

ZUSAMMENFASSUNG

Die Entwicklung, Homöostase und Regeneration des kardiovaskulären Systems umfassen eine enorme Anzahl an Faktoren und Mechanismen, die in zeitlich und räumlich geregelter Art und Weise zusammenwirken müssen. Der Herzinfarkt ist eine weit verbreitete ischämische Erkrankung und vielfache Studien haben belegt, dass Arteriogenese erheblich zur Wiederherstellung des Blutflusses beiträgt. Dies ist absolut kritisch für die Regeneration aller ischämischen Organe. Das Notch Signalsystem ist ein evolutionär erhaltener Signalweg, in dem die Notch Liganden eine kritische Rolle spielen, da sie den Signalweg aktivieren. Der Ligand Delta-like 1 (Dll1) wurde bis jetzt mit der Erhaltung der arteriellen Identität während der Gefäßentwicklung und mit peripherer Arteriogenese im Bein in Verbindung gebracht. Seine Funktion in koronaren Blutgefäßen und im kardialen Remodelling ist bislang noch nicht untersucht worden. Die vorliegende Studie identifiziert den Notch Liganden Dll1 als kritischen Regler in der entwicklungs/neonatalen koronaren Arteriogenese und erbringt Beweise, dass Dll1 an der kardialen Genesung nach einem Herzinfarkt beteiligt ist.

Um die Expression und Rolle von Dll1 im erwachsenen Herzen zu analysieren. standen $Dll1^{+/lacZ}$ Reportermäuse im Mittelpunkt dieser Studie. Diese fungierten sowohl als Dll1-lacZ Reporter, als auch als Dll1 heterozygoter Mausstamm. Im Herzen wurde die Expression von Dll1 spezifisch im Endothel von Koronararterien nachgewiesen, die größer als 20 µm waren. Eine Analyse der Koronararterien ergab, dass die Anzahl der Konduktanzgefäße (>20 µm) signifikant verringert war, während die Anzahl der Widerstandsgefäße (<20 µm) erhöht war. Die Daten legen ein Modell des koronaren Phänotyps nahe, in dem verringerte Pegel von Dll1 die koronare Arteriogenese während der Entwicklung und postnatal beeinträchtigen. Dies wird im Erwachsenenalter ersichtlich durch eine reduzierte Größe der Koronararterien in der Herzbasis und hat zur Folge, dass eine verringerte Anzahl an Gefäßen distale Herzareale erreichen. Die beeinträchtigte Entwicklung der Koronargefäße bewirkte eine Reduktion des Herzgewichts und der Herzgröße in Dll1 heterozygoten Tieren, während Körpergröße und –gewicht, sowie Herzfunktion unverändert waren. Daten zeigen einen Zusammenhang in dem das geringere Herzgewicht bei gleichbleibendem Körpergewicht ausgeglichen wird durch eine Erhöhung

der Ejektionsfraktion. Dies führt zu normalem Schlagvolumen und Herzminutenvolumen und erklärt das normale Überleben der Tiere über 18 Monate. Zusätzlich zeigte die Analyse von Monozyten eine verringerte Gesamtzahl der Monozyten, sowie eine verringerte Anzahl von $Ly6C^{lo}$ Monozyten in der Milz von Dll1 heterozygoten Mäusen.

Um die Rolle von Dll1 während der Reaktion auf einen Herzinfarkt zu bestimmen, wurden Mäuse einer permanenten LAD Ligation unterzogen. Während Wildtyptiere (WT) funktionelles Remodelling und Erhaltung der kardialen Funktion aufwiesen, zeigten Dll1 heterozygote Mäuse Merkmale von adversem Remodelling: erhöhte Entzündung, Infarktexpansion, fortschreitende Dilatation und Hypertrophie und komplettes Fehlen von Arteriogenese, was im Ganzen eine Ausdehnung der Infarktgröße, ventrikuläre Dysfunktion und progressive Sterblichkeit zur Folge hatte. Adverses Remodelling in Dll1 heterozygoten Tieren war eher auf veränderte Infarktheilung zurückzuführen, als auf das Ausmaß des ursprünglichen, ischämischen Vorfalls. Vieles weist darauf hin, dass beeinträchtigte Arteriogenese und erhöhte Entzündung direkte Folgen verminderter Dll1 Pegel sind, während Infarktexpansion und progressive Dilatation und Hypertrophie eher nachgeordnete Ereignisse sind. Diese Daten zeigen die Wichtigkeit des Dll1 vermittelten Notch Signalweges in ordnungsgemäßer Kompensation und funktionellem Remodelling zur Erhaltung der ventrikulären Funktion nach einem Herzinfarkt.

Dll1 war spezifisch im Endothel großer Koronararterien nachzuweisen und die Expression von Dll1 war nach einem Herzinfarkt erhöht. Die vorliegende Studie konnte jedoch erste Nachweise erbringen (zumindest im Rahmen eines Herzinfarktes durch permanente LAD Ligation), dass nicht endotheliales Dll1 der bestimmende Faktor ist, der bei Fehlen adverses Remodelling bewirkt. Erste Ergebnisse zeigen eher eine extravaskuläre Rolle von Dll1 in der Infarktheilung und deuten auf eine Funktion im Monozyten/Makrophagensystem hin. Der genaue Wirkungsort und die Funktionsweise bleiben allerdings offene Fragen, die von zukünftigen Studien beleuchtet werden müssen.

Stichwörter: Dll1, Arteriogenese, Herzinfarkt

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1. INTRODUCTION

The development, homeostasis, and regeneration after injury of the cardiovascular system comprise a huge array of factors and mechanisms which have to work in a temporal and spatial organized fashion (Darland and D'Amore, 2001). Notch signalling constitutes an evolutionary conserved pathway which plays a pivotal role in the cardiovascular system; as activators of the pathway the Notch ligands play a critical role.

In order to provide the reader with all background information needed for an informed understanding of this dissertation, this chapter will give an overview of the cardiovascular system and healing mechanisms after myocardial infarction. Notch signalling and the ligand Dll1 will be reviewed with more detail to the currently available literature.

1.3 Notch signalling

1.3.1 The Notch signalling pathway

Role in adults – *myocardial infarction and heart stress response*

Although the engagement of Notch signalling in cardiovascular development has been the focus of a multitude of studies, its role in the postnatal heart and stressed conditions has attained less attention. Only in 2008 first studies attended to this topic.

Gude *et al*. were the first to analyse Notch signalling following myocardial infarction and showed the activation of protective mechanisms in the myocardium, mediated by Notch (Gude *et al*., 2008). In the mammalian heart endogenous Notch signalling is low (Gude *et al*., 2008, Collesi *et al*., 2008; Kratsios *et al*., 2010), but expression levels increase in response to acute infarction by permanent coronary occlusion (Gude *et al*., 2008). 4 days after infarction, NICD and Jag1 were detected in surviving cardiomyocytes of the border zone, whereas Dll4 was predominantly expressed in interstitial areas. Additional Hes1 detection indicated active Notch signalling (Gude *et al*., 2008). The group treated infarcted hearts with an adenoviral vector expressing NICD and could show improved hemodynamic function, compared to control animals after 4 weeks. Further analyses revealed the relationship of HGF (hepatocyte growth factor), binding to its receptor c-Met (increased in

hypertrophic and infarcted cardiac tissue) which in turn induces Delta expression, Notch activation and Hes1 expression. Among others, c-Met activates ERK and Akt/PKB survival signalling in the heart. As Notch1 signalling enhances Akt activity, results suggest Notch signalling as mediator of cell survival (Gude *et al*., 2008).

The most recent and second study focusing on Notch involvement after myocardial infarction was performed by Kratsios and co-workers. The group analyzed effects of sustained Notch1 activation after myocardial infarction, using an inducible cardiomyocyte-specific NICD1 mouse model or by intramyocardial delivery of a Notch1 pseudoligand. These approaches resulted in increased survival rates, improved functional performance, and decreased myocardial injury by promoting antiapoptotic mechanisms and cardiomyocyte survival. In addition, minimized fibrosis and enhanced neovascularisation could be observed (Kratsios *et al*., 2010).

1.3.3 Involvement of Notch signalling in immunity mediated wound repair

Already described in the chapter dealing with myocardial infarction, the inflammatory response involves the recruitment of two distinct and complementary monocyte subsets (Nahrendorf *et al*., 2007). Recruited monocytes differentiate to macrophages at the area of inflammation (Gordon and Taylor, 2005). Ly-6C^{hi} monocytes dominate early in the inflammatory phase, whereas Ly-6C^{lo} monocytes dominate rather in the proliferative phase and show enhanced healing properties (Nahrendorf *et al*., 2007). Polarized macrophages are classified as M1 (classical activated macrophages) with pro-inflammatory function and M2 (alternatively activated macrophages), associated with tissue repair and angiogenesis (Mosser and Edwards, 2008; Limbourg *et al*., *in review*). Just recently Limbourg and co-workers could provide evidence that Notch signalling is involved in macrophage differentiation. *In vitro* experiments revealed that cultures of PBMC (peripheral blood monocytes)-derived macrophages showed active Notch signalling and an M2 typical phenotype. Inhibition of Notch signalling resulted in macrophages of inflammatory M1 phenotype (Limbourg *et al*., *in review*). Next, the group tested the hypothesis that by regulating macrophage polarization, Notch signalling regulates the inflammatory and regeneration response after ischemia. In a M2 macrophage-deficient mouse model, effects of cultured M2 macrophages and cultured Notch-deficient M1 macrophages were studied. Cells were injected into ischemic muscles after hindlimb ischemia. Without treatment, mice showed impaired recovery from ischemia. Treatment with M2 macrophages resulted in decreased necrosis and increased angiogenesis, arteriogenesis and arterial branching in the

ischemic muscle. Recruitment of CD45+ leukocytes to the ischemic muscle was reduced and tissue necrosis suppressed. In contrast, injection of Notch-deficient M1 macrophages resulted in significantly reduced angiogenesis and arteriogenesis; tissue necrosis was increased. CD45+ leukocyte recruitment, corresponding inflammatory response and fibrosis was enhanced and prolonged, consistent with the development of chronic inflammation. These results were consistent with the role of macrophage subsets in inflammation and tissue recovery, and proved an according involvement of Notch signalling (Limbourg *et al*., *in review*).

1.4 The Notch ligand Delta-like 1 (Dll1)

1.4.2 Dll1 in the cardiovasculature

Dll1 in vasculature development

A complete Dll1 knockout is lethal around embryonic day 12 (E12) due to severe bleeding. However, lethality is based rather on defects in surrounding tissues, than on vascular functions (Hrabé de Angelis *et al*., 1997; Gessler, 2009). Embryos heterozygous for Dll1 are phenotypically normal and survive until adulthood, despite significantly reduced Notch activity (Hrabé de Angelis *et al*., 1997; Beckers *et al*., 1999; Schuster-Gossler *et al*., 2007).

Dll1 is expressed in vascular endothelium of late embryonic stages (Hrabé de Angelis *et al*., 1997; Beckers *et al*., 1999; Limbourg *et al*., 2007). Sörensen et al. showed specific expression of Dll1 in fetal arterial endothelial cells beginning at E13.5 (Sörensen *et al*., 2009). The group analysed embryos with reduced levels of Dll1 or endothelial-specific Dll1 ablation and found lost Notch1 activation, reduced VEGFR2, Nrp1 and ephrinB2 expression, and upregulation of COUP-TFII after E13.5. As Dll4 expression was unchanged in these animals, Dll4 appears to be required for arterial specification, whereas Dll1 mediated Notch activity is required in large arteries to maintain arterial identity in embryonic development (Sörensen *et al*., 2009). This finding suggests that there is a switch which renders Dll4 incapable to sustain Notch activity, arterial marker expression and vein marker suppression (Gessler, 2009). In normal conditions, COUP-TFII suppresses *Nrp1* expression and arterial differentiation in venous endothelium (You *et al*., 2005; Sörensen *et al*., 2009). Sörensen et al. revealed that VEGFR2 and Nrp1 (together forming the VEGF-A receptor complex) downregulation in mutant arteries preceded COUP-TFII expression. This indicates that Dll1-mediated Notch1 activation upregulates VEGFR2 and Nrp1, thereby enhancing VEGF-A responsiveness of arterial endothelial cells (Sörensen *et al*., 2009; Kume, 2009).

Thus, the group showed that Dll1 positively regulates VEGF signalling, which constitutes a contrasting function to Dll4 in growing capillaries where Dll4-mediated Notch signalling occurs downstream of VEGF (Kume, 2009). Summarizing, the study showed that there are functions of Dll1/Notch signalling which cannot be compensated by other ligands, and revealed a novel connection were in arteries Dll1/Notch1 facilitate VEGF sensitivity (Gessler, 2009).

Furthermore, Dll1 has been implicated in smooth muscle differentiation and maturation. Together with Jag1, Dll1 are the primary ligands on arterial endothelial cells, inducing the expression of Notch3 and Jag1 in the neighbouring mural cells. This subsequently promotes and maintains the differentiated mural cell phenotype (High *et al*., 2008; Liu *et al*., 2009; Kume, 2009)

Dll1 in adult vasculature

In the adult cardiovascular system Dll1 is selectively expressed in arterial endothelial cells. It is not found in capillaries, venous endothelial cells or other cell types present in the heart (Limbourg *et al*., 2007). The basal role of Dll1 in the adult vasculature has not been studied so far, but Limbourg and co-workers discovered a critical role of Dll1 in postnatal arteriogenesis (Limbourg *et al*., 2007). In the mouse model of hindlimb ischemia the expression of Dll1 was induced, Notch signalling activated, ephrin-B2 upregulated, and perivascular cells expressed VEGF and EphB4 while arteries grew. Analogous analyses in ischemia-induced $Dll^{+/-}$ animals revealed the absence of endothelial Notch activation and ephrin-B2 induction. Perivascular VEGF expression and microvascular angiogenesis were not altered, but arteriogenesis and blood flow recovery were severely impaired (Limbourg *et al*., 2007). *In vitro* studies showed that VEGF and FGF synergistically induced Dll1 with corresponding Notch activation. This activation was necessary and sufficient to regulate ephrin-B2 expression and induced branching and vascular network formation (Limbourg *et al*., 2007). The group proposes a model of postnatal arteriogenesis in which the relationship of Dll1 induction by angiogenic growth factors activates Notch signalling and ephrin-B2 expression; this mediates remodelling and outward growth of the collateral artery towards an EphB4 enriched environment (Limbourg *et al*., 2007). The observed transient induction of Dll1 in connection with the previous finding of reciprocal Dll1 inhibition by activated Notch receptors might possibly explain the termination of arteriogenesis through Dll1 downregulation after persistent Notch activation (Artavanis-Tsakonas *et al*., 1999; Limbourg *et al*., 2007).