**Preclinical research**

**Elevated expression of PDGF-C in coxsackievirus B3-induced chronic myocarditis**

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Received 5 February 2004; revised 14 December 2004; accepted 13 January 2005; online publish-ahead-of-print 9 March 2005

See page 642 for the editorial comment on this article (doi:10.1093/eurheartj/ehi201)

**Aims**

Coxsackievirus B3 (CVB3) is a frequent cause of human chronic myocarditis and subsequent fibrosis, leading to dilated cardiomyopathy. The molecular processes underlying the development of fibrosis are poorly understood. Enhanced levels of platelet-derived growth factors (PDGFs), especially PDGF-C, have recently been linked with the development of different forms of fibrosis. Therefore, the expression of PDGF was analysed in hearts of CVB3-infected major histocompatibility complex class II knockout mice. The latter were recently established as mouse model mimicking the chronic inflammation and fibrosis characteristic for this disease.

**Methods and results**

Expression of PDGF was analysed by reverse transcription–polymerase chain reaction, *in situ* hybridization, and immunohistochemistry. Hearts of C57BL/6 mice served as controls because infection of these animals leads to acute cardiac inflammation, but the hearts heal without signs of chronic inflammation. In uninfected hearts, basal expression of PDGF, notably PDGF-C, was detectable throughout the heart. The chronic inflammatory process was associated with elevated and sustained expression of all tested PDGF isoforms. Immunostaining and *in situ* hybridization analysis localized enhanced PDGF levels to areas with highest virus load and inflammatory infiltrations, adjacent to fibrotic areas.

**Conclusion**

PDGF may participate in fibrosis development in CVB3-induced myocarditis. Therefore, PDGF signalling may be considered a target for therapeutic interference.

**KEYWORDS**

Chronic myocarditis; Coxsackievirus B3 (CVB3); Platelet derived growth factor (PDGF) isoforms; PDGF-C; Major histocompatibility complex (MHC) class II knockout mouse

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**Introduction**

Human pathogenic coxsackievirus B3 (CVB3) is considered as the most frequent viral cause of myocarditis.¹ Acute myocarditis as a consequence of CVB3 infection is followed by either complete healing or transition to a progressive disease. This chronic stage is, according to the Dallas criteria,² characterized by chronic inflammation and sometimes by accumulation of connective tissue in heart muscle, i.e. fibrosis. In these cases, fibrosis may contribute to a loss of cardiac function, which is also characteristic for dilated cardiomyopathy (DCM), a frequent consequence of chronic myocarditis in men.

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The molecular mechanism of fibrosis development in chronic myocarditis and DCM is currently not well understood.

In general, fibrosis is characterized by substantial proliferation of resident fibroblasts with subsequent deposition of extracellular matrix components like collagen, fibronectin,\textsuperscript{3,4} or tenascin.\textsuperscript{5} Both proliferation as well as matrix protein synthesis are stimulated by a set of cytokines and growth factors, including transforming growth factor-\(\beta\), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1 (IL-1), and platelet-derived growth factors (PDGF).\textsuperscript{3,6} Immune cells invading tissue upon inflammation are a substantial source of these growth factors.

Isoforms of the PDGF family are potent mitogens for fibroblasts and other mesenchymal cells. The PDGF family comprises several disulfide-linked dimeric growth factors including PDGF-AA, -BB, -AB, and the recently discovered molecules PDGF-C (also designated Fallotein) and PDGF-D.\textsuperscript{7–10} Their activity is exerted by activation of PDGF receptor (R)-\(\alpha\) or PDGFR-\(\beta\), two structurally related receptor tyrosine kinases. Investigations in different tissues have suggested an important role for the PDGF system in fibrotic processes, including fibrosis of lung and kidney.\textsuperscript{6,11–15} So far, little is known about the role of PDGF in cardiac fibrosis. PDGF-C overexpression in the myocardium of transgenic mice leads to substantial fibrosis\textsuperscript{7,16} suggesting that PDGF-C may be an important player in this disease. Similar studies with PDGF-A and -B have not yet been reported.

In the present study, the possible involvement of PDGF in the development of cardiac fibrosis was investigated in a previously established mouse model of CVB3-induced chronic myocarditis.\textsuperscript{17} Immunodeficient C57BL/6 major histocompatibility complex (MHC) class II knockout mice (genetic background H-2\(^b\)) were kindly provided by Professor Blüthmann (Roche Center for Medical Genomics, Basel, Switzerland). C57BL/6 mice were purchased from the Laboratory Animal Centre, Friedrich Schiller University Jena, Jena, Germany. Experiments were performed in accordance with the animal protection law (registration no. 02-30/99).

Five experiments were performed using identical protocols: In each experiment, we started with groups of five 8–13-week-old male C57BL/6 and B6-Aa\(^2\)/Aa\(^2\) MHC class II knockout mice.\textsuperscript{18} The mice were inoculated intraperitoneally with 10\(^4\) pfu of CVB3-Mu\(^/J\) and sacrificed under ether narcosis at days 7, 21, and 56 post-infectionem (p.i.), respectively. A clearly visible necrosis of the exocrine pancreas was taken as indication of successful virus infection because this organ is the first target of CVB3 infection.\textsuperscript{19} Those animals (‘responders’) (usually two to five out of five) were included into the further studies. Non-responders were excluded.

Table 1 describes the number of totally analysed animals per group, 9–12 mice were used as non-infected controls.

<table>
<thead>
<tr>
<th>Table 1 Number of animals per experimental group</th>
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<tr>
<td>C57BL/6 mice</td>
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<td>Naive control mice</td>
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<tr>
<td>12</td>
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<td>7d p.i.</td>
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<td>16</td>
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<td>21d p.i.</td>
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<tr>
<td>16</td>
</tr>
<tr>
<td>56d p.i.</td>
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<tr>
<td>14</td>
</tr>
<tr>
<td>MHC class II knockout mice</td>
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<tr>
<td>Naive control mice</td>
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<tr>
<td>9</td>
</tr>
<tr>
<td>7d p.i.</td>
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<tr>
<td>17</td>
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<tr>
<td>21d p.i.</td>
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<tr>
<td>18</td>
</tr>
<tr>
<td>56d p.i.</td>
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<tr>
<td>24</td>
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It has to be mentioned that not all four analyses from one heart could be performed because the fixation of tissue is different for different analyses (see Methods). The choice which animal was used for a certain analysis was done at random at the time of sacrificing.

\(a\)Sum of all animals studied in five experiments.

\(b\)Number of animal samples analysed with the given method.
GmbH, München-Halbergmoos, Germany) was used for image capturing and pictures were processed with the Axiosview image processing system (Carl Zeiss, München-Halbergmoos, Germany). For each myocardial sample, histological indicators of myocarditis were graded semi-quantitatively according to the extent of cellular infiltration/fibrosis on a four-point scale ranging from 0 (no infiltration, no fibrosis) to 3 (strong infiltration, strong fibrosis).17

Virus titre

The CVB3 titre of the heart tissue was determined on HeLa cells17 by the tissue culture infectious dose 50 (TCID50) assay method according to Reed and Muench20 (see Supplementary material online).

Semi-quantitative PCR

Total RNA was isolated according to Chomczynski and Sacchi.21 Reverse transcription was performed with 10 μg total RNA with oligo (dT) and 500 U MML V reverse transcriptase (Superscript II, Invitrogen) corresponding to the manufacturers protocol. Heart cDNA was adjusted to equal concentrations by competitive polymerase chain reaction (PCR) between β-actin cDNA and a corresponding control fragment.22 A constant amount of experimental cDNA was used as template for any amplification. The 50 μL PCR reactions contained 0.2 mM of each dNTP (Promega), 20 nM of each primer, 1.25 U HotStar Taq polymerase, 5 μL 10X concentrated PCR buffer, and 10 μL solution Q and MgCl2 (all reagents from Qiagen). The final MgCl2 concentration was 2.5 mM for amplification of β-actin, VP1, TNF-α, PDGF-A, -B, and -C cDNAs, and 1.5 mM for all other cDNAs. Amplification was performed for 30 cycles according to the manufacturer’s protocol. Primer sequences are mentioned in the Supplementary material online. The primers always correspond to different exons. Therefore, amplification of genomic DNA is either precluded or would lead to formation of clearly larger products. Fifteen microlitres of the PCR products were analysed on 1.5% agarose gels.

In situ hybridization

Detection of viral RNA in the myocardium by in situ hybridization using a digoxigenin (DIG)-labelled DNA probe detecting the CVB3 capsid protein gene VP1 was performed according to Fleming et al.23 (see Supplementary material online).

A probe for the detection of PDGF-C mRNA was generated by PCR amplification with Pfu polymerase (Stratagene) using PDGF-C cDNA (‘Fallotein’ cDNA, kindly provided by Dr Yuan-Jang Tsai, Division of Reproduction and Endocrinology, Department of Medical Research, Mackay Memorial Hospital, Tamshui, Taiwan) as template and the primers 5’-TCC AGC GAC AAG GAA CAG AAC G-3’ (forward) and 5’-GGA GAT TCA GAT TCA CCA CTG TGC-3’ (reverse). The resulting 625 bp blunt-end product was cloned into EcoRV-digested pBluescript vector II KS (+). For synthesis of antisense and sense RNA probes, the plasmid was linearized and in vitro transcription was performed with the DIG-RNA Labelling Kit (Roche) according to the instructions of the manufacturer using T3 and T7 polymerase, respectively. The linearized probes were precipitated with 2.5 mM LiCl–ethanol, aliquoted, and stored at −80°C until use in in situ hybridization according to Dijkman et al.24 For further details see Supplementary material online. Immunological detection of the bound probes was performed as described for VP1 (see Supplementary material online), except that the anti-DIG-antibody was used in a dilution of 1:200.

Immunohistochemistry

Heart tissue was mounted in Cryomatrix (Thermo Shandon, Pittsburgh, PA, USA), snap-frozen, and stored at −80°C until use. Immunohistochemistry was performed as described earlier.17 Incubation with primary antibodies was performed at 4°C overnight using goat anti-mPDGF-A (I-20; Santa Cruz Biotecology), 1:100; rabbit anti-mPDGF-B (H-55; Santa Cruz Biotecology), 1:100; rabbit anti-mPDGF-C (kindly provided by Dr Y.J. Tsai; 1:300 in the absence or presence of blocking peptide CDCVCRGNAAG-amide), rabbit anti-PDGFR-α (951; Santa Cruz Biotecology) 1:200; rabbit anti-phospho Akt (Ser473) (Cell Signaling Technology, Inc., Cummings Centre Beverly, USA), 1:100. Rabbit IgG (4 μg/mL) (SC-2027; Santa Cruz Biotecology) was used as negative control. Sections were counter-stained with Hemalaun.

Results

CVB3 infection leads to chronic inflammation and progressive cardiac fibrosis in MHC class II knockout mice

Immunocompetent C57BL/6 mice or immunodeficient MHC class II knockout mice were infected with CVB3-Mü/J (Nancy strain) and hearts were analysed until day 56 p.i. As described earlier,17 CVB3 induced a severe acute myocarditis characterized by marked cell infiltration in the myocardium of both mouse strains.
The inflammatory infiltrates in hearts of acutely CVB3-infected C57BL/6 mice contain CD8⁺, CD4⁺, and CD11b⁺ cells. In the MHC class II knockout mice, CD8⁺ and CD11b⁺ cells, but no CD4⁺ cells, can be detected. Here, we show that in MHC class II knockout mice, the myocarditis was accompanied by a necrosis of cardiomyocytes indicated by a loss of nuclear staining, a focal increased eosinophilia of cardiomyocyte cytoplasm, and cardiomyocyte fragmentation resulting in a reduction of functional heart tissue (Figure 1A). The necrosis of grouped cardiomyocytes and the presence of infiltrating cells persisting until day 56 was accompanied by severe fibrosis as shown by staining with Sirius Red (Figure 1B). In contrast, the CVB3-induced myocarditis healed completely in immunocompetent C57BL/6 mice without any further interstitial inflammatory cells or signs of fibrosis (Figure 1C and D).

**Elevated expression of PDGF isoforms correlates with CVB3-induced myocarditis**

To evaluate the possible participation of PDGF in the fibrotic process, mRNA expression of different PDGF isoforms was monitored by semi-quantitative PCR. For comparison, mRNA expression of TNF-α, IL-1α, and IL-1β was also detected. Representative experiments are shown in Figure 2. The histological score for
myocarditis and fibrosis, the virus titre, as well as the presence of VP1 RNA are shown to characterize the disease state of the analysed animals.

High virus titres were detectable on day 7 after infection. Acute myocarditis in both mouse strains was characterized by strong infiltration of the heart muscle with inflammatory cells. In correlation with inflammation, a moderate to strong expression of the inflammatory cytokines TNF-α, IL-1α, and IL-1β could be detected in the myocardium. Notably, also the mRNAs of all three PDGF isoforms were clearly up-regulated. At days 21 and 56, the differences in the disease course between both mouse strains became apparent: the cytokines TNF-α, IL-1α, and IL-1β, as well as the virus titre, were already below the detection limit in C57BL/6 mice. In addition, the expression of the PDGF mRNAs was

Figure 3  In situ hybridization of PDGF-C mRNA and viral RNA in CVB3-infected hearts. (A) In situ hybridization of PDGF-C mRNA in paraffin-embedded heart tissue sections of MHC class II knockout mice (left column) and immunocompetent C57BL/6 mice (right column) from days 7, 21, and 56 after infection with CVB3. Insets show the controls with the sense probe. The sections are not counter stained. Dark blue to brown stained areas are positive for PDGF-C mRNA. It is possible to distinguish strongly positively reactive inflammatory cells (black arrows) and weakly reactive cardiomyocytes (white arrows). Original magnification ×200; co: non-infected controls. (B) In situ hybridization for detection of viral RNA in paraffin-embedded heart tissue sections of MHC class II knockout mice (left column) and immunocompetent C57BL/6 mice (right column) from days 7, 21, and 56 after infection with CVB3. Insets show negative controls without probe. Positive regions are coloured in dark blue to brown. Sections are counterstained with Haematoxylin. VP1 RNA is strongly detected in cardiomyocytes (white arrows). Original magnification ×400; co: non-infected controls.
down-regulated to levels of non-infected controls at day 56 p.i., only PDGF-A still seems to be slightly enhanced compared with the controls (Figure 2). In contrast to immunocompetent mice, virus infection and inflammation persisted in MHC class II knockout mice. The persisting inflammation was associated with a sustained expression of PDGF-A, -B, and -C mRNAs.

Elevated PDGF-C production occurs in the infiltrated heart tissue

To obtain information about the cellular origin of PDGF-C, we localized PDGF-C mRNA and protein by in situ hybridization and immunohistochemistry, respectively. For comparison, and to characterize the inflammatory process, virus RNA (VP1 gene) was also analysed by in situ hybridization. PDGF-C mRNA reactivity was detected in heart sections of MHC class II knockout mice using the RNA antisense probe (Figure 3A, left). The specificity of detection was verified by negative staining with the sense probe (Figure 3A, see insets). The main localization of this mRNA was found in areas of infiltrating inflammatory cells. However, basal expression of PDGF-C mRNA was also detectable in cardiomyocytes. A diffuse PDGF-C mRNA expression could also be shown in cardiomyocytes of cured C57BL/6 mice at 21 and 56 days p.i.
(Figure 3A, right). The inflamed areas are surrounded by RNA of the VP1 gene (Figure 3B). This distribution pattern of PDGF-C and VP1 was seen in hearts from MHC class II knockout mice at all time points of infection, as well as in hearts of C57BL/6 mice at day 7 p.i.

To detect PDGF-C protein, we used an antibody recognizing an epitope of the biologically active domain of PDGF-C. This antibody stained the regions of massive immune cell infiltration likewise (Figure 4A), confirming the expression of PDGF-C in these areas. No signals were obtained in controls either omitting the primary antibody or upon blocking of anti-PDGF-C antibody by the corresponding peptide antigen (data not shown). PDGF-B and -A were also expressed in areas of infiltration as revealed by immunohistochemistry (Figure 4B and C).

In summary, these data suggest that the inflammatory cells are the main source of elevated PDGF levels in the CVB3-infected hearts. The cognate receptor for PDGF-C, PDGF-Rα, is also detectable by immunohistochemistry in the regions of inflammation and fibrosis (Figure 4D). The expression qualitatively parallels the expression of PDGF-C. The receptor expression is prominent not in the cardiomyocytes, but in small cells within and also outside the inflammatory lesions, which represent invaded immune cells and probably also resident fibroblasts. Visualization of activated receptors in situ is not possible until today. In order to obtain an indication for the activation status of PDGF-R, we, therefore, used an important down-stream signalling mediator of PDGF receptors, Akt/PKB kinase. Antibodies recognizing only

![Figure 4](https://education.oup.com/eurheartj/article-abstract/26/7/728/455982) Immunohistochemical detection of PDGF-C, -B, and -A, PDGF-Rα, and activated Akt/PKB. Frozen myocardial sections of CVB3-infected MHC class II knockout mice (left column) or immunocompetent C57BL/6 mice (right column), respectively, from days 7, 21, or 56 after infection were reacted with antibodies to PDGF-C (A), -B (B), or -A (C), PDGF-Rα (D), and phospho-Akt (Akt/PKB) (E). The respective PDGF immunoreactive cells in inflammatory regions and outside are indicated by black arrows. Insets show isotype controls (non-immune IgG as primary antibody). Original magnification ×200.
the activated form of Akt/PKB again revealed signals in the hearts of infected MHC class II knockout animals with a similar distribution as in case of PDGF-Rα staining (Figure 4E). This finding is consistent with a possible PDGF-R activation in resident cardiac cells.

Discussion

The molecular mechanisms involved in the transition of an acute myocarditis into an ongoing chronic disease are still not clear. Human chronic myocarditis is sometimes characterized by the development of progressive fibrosis,2 whose molecular mechanisms are not well understood. To analyse the underlying mechanisms, we employed a mouse model of chronic myocarditis, which is accompanied by fibrosis. The fibrotic process comprises the replacement of normal tissue by mesenchymal cells and the extracellular matrix produced by these cells, and thereby leads to distortion of normal tissue function.3 Different cytokines and growth factors can stimulate mesenchymal cell proliferation and matrix synthesis and may be involved in the pathological fibrosis, which accompanies chronic myocarditis. For example, transgenic expression of TNF-α in mouse hearts leads to severe myocarditis, fibrosis, and cardiomegaly.25,26 Persistent expression of cytokines in the chronic stage of CVB3-induced myocarditis has been described for various mouse models,27,28 as well as for human DCM.29 Although chronic inflammation is characteristic for the human disease as well as for our mouse model, fibrosis is more prevalent in the latter.

Here, we explored the possibility that factors of the PDGF family are involved in the pathology of chronic
myocarditis. A model of CVB3-induced myocarditis in mice was used for this study. This mouse model comprises several hallmarks of the human disease. For example, the course of the disease depends strongly on the immune status of the recipient mice. We took advantage of this fact by comparing two mice strains in our expression analysis: immunodeficient MHC class II knockout mice develop a chronic myocarditis upon CVB3 infection, whereas immunocompetent C57BL/6 wild-type mice exhibit only an acute myocarditis, which is completely reversible.

Interestingly, all analysed isoforms of PDGF-A, -B, and -C were up-regulated in close correlation with the inflammatory process. High levels of the growth factors persisted only in CVB3-infected MHC class II knockout mice.

Localization of elevated PDGF expression in the CVB3-infected hearts showed that PDGF expression occurs in the areas of inflammatory cell invasion. Thus, infiltrating immune cells are likely to be the source of PDGF in this setting. In this context, PDGF-C is of particular interest. Earlier, it has been found that PDGF-AA is a very potent mitogen for cardiac fibroblasts, suggesting that its cognate receptor, the PDGF<sub>A</sub>-receptor, drives their proliferation. PDGF-C is another recently discovered ligand for the PDGF<sub>A</sub>-receptor and therefore potentially important for proliferation of cardiac fibroblasts. In line with such a presumed role, selective transgenic expression of PDGF-C in the mouse heart induces strong proliferation of interstitial cells as cardiac fibroblasts. These data convincingly support the concept that PDGF-C can play a causative role for myocardial fibrosis development. Evidence for elevated PDGF receptor signalling in resident cardiac cells as consequence of PDGF overexpression is still elusive. PDGF-R<sub>A</sub> is expressed in the
inflamed hearts, suggesting that up-regulation of PDGF-C may be functional. Further, we were able to detect activation of Akt/PKB, a major known signalling mediator of PDGF receptors, in the regions of inflammatory cell invasion. Although this finding is consistent with possible resident cell activation by PDGF, further work is required to better characterize the cell types involved in PDGF expression and perception of PDGF signals.

A role for members of the PDGF family in development of fibrosis has previously been proposed also for lung and kidney. For example, AG1295, a selective inhibitor of the PDGF receptors attenuated fibrosis in rat kidney after unilateral obstruction suggesting a causal role of this signalling system. Very recently, Eitner et al. demonstrated up-regulation of PDGF-C in fibrotic processes associated with tubulo-interstitial kidney injuries, further supporting an important role of this factor for fibrosis. That activation of resident PDGF receptors may critically contribute to fibrosis development has also been concluded from recent experiments with the PDGF receptor blocker STI571 (Glivec). In chronic myeloid leukemia (CML) patients, treatment with this compound reduced fibrosis in bone marrow. In a rat model of liver fibrosis, STI571-treatment attenuated the disease, concomitant with an inhibition of myofibroblast conversion. Our observation that enhanced mRNA and protein levels of PDGF-C are associated with CVB3-induced myocarditis and fibrosis is consistent with the possible participation of PDGF-C in initiation or maintenance of the cardiac fibrotic process. Notwithstanding that other growth factors and cytokines may also contribute to the disease, it seems tempting to speculate in the light of existing literature data that PDGF-C up-regulation plays a role for disease development rather than being merely a secondary phenomenon.
As an obvious continuation of these experiments, it will be necessary to directly test this hypothesis. Inhibiting PDGF receptor signal transduction in the available mouse model of CVB3-induced chronic myocarditis with selective kinase inhibitors should be one possible approach. This should help to better understand the disease and may potentially reveal a novel therapeutic strategy for the prevention of fibrosis in chronic myocarditis.

Supplementary material
Supplementary material is available at European Heart Journal online.

Acknowledgements
The authors would like to thank Birgit Meißner, Birgit Schikowski, and Heike Urban for excellent technical assistance and Carl Zeiss, Jena GmbH, for technical support. The study was supported by a grant from Deutsche Forschungsgemeinschaft (DFG: BO 1043/5-1) and partially by the Thuringian Ministry of Science, Research, and Art (TMWFK) and the IZKF of the University Jena (FKZ: B307-04004).

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