GDF-15 protects from macrophage accumulation in a mouse model of advanced atherosclerosis

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Abstract

Background: The cytokine growth differentiation factor-15 (GDF-15), a member of the TGF beta superfamily, has recently been discovered to play an important role in cardiovascular diseases. It is mostly expressed in macrophages of atherosclerotic lesions, but its impact on advanced atherosclerosis is still unknown. This study was performed to evaluate the effects of GDF-15 in an established mouse model of advanced atherosclerosis.

Methods: Thirty-eight LDL receptor deficient mice received a lethal body radiation. Half of the group was transplanted with bone marrow of GDF-15 deficient mice. Nineteen mice were transplanted with bone marrow from wild-type controls. After 24 weeks on an atherogenic diet, animals were euthanized and sections of the aortic sinus were prepared. Lesion size and lesion composition, as well as macrophage content, were evaluated.

Results: While demonstrating no difference in lesion size, LDL-receptor knockout mice transplanted with bone marrow from GDF-15 deficient mice showed enhanced macrophage accumulation and features of atherosclerotic plaque destabilization, such as thinning of fibrous caps. Immunostaining against intercellular adhesion molecule-1 further revealed an increased expression in mice receiving GDF-15-deficient bone marrow.

Conclusions: This is the first study that demonstrates a protective role of GDF-15 in advanced atherosclerosis and macrophage accumulation, possibly due to the reduced expression of adhesion molecules.
Methods

Animals and bone marrow transplantation
Eight-week-old female LDL-receptor−/− mice (LDLr−/−, background C57/BL/6; Jackson Laboratory, Bar Harbor, USA; n=38) received lethal body irradiation at a dose of 9 Gy. Half of the group (n=19) was transplanted with bone marrow of mice (n=5) with a GDF-15 knock-out [18]. Nineteen LDLr−/− mice were used as controls, which were transplanted with bone marrow of wild-type mice (C57/BL/6CR). After transplantation, mice were fed a high fat western-type diet (Altromin, Lage/Germany; Nr. 11320010: 0.15% cholesterol) for 24 weeks. Animals were kept within the animal care facility of the University of Heidelberg. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The housing and care and procedures in the study were performed in accordance with the guidelines and regulations composed by the Animal Care Committee of the University of Heidelberg and approved by the Regierungspraesidium Karlsruhe.

Animal sacrifice and preparation of tissues
After 24 weeks on a high cholesterol Western-type diet, mice were heavily sedated (Avertin, Aldrich, Milwaukee, USA), blood was collected from the inferior vena cava, and the animals were sacrificed by exsanguination (fasted 3 h prior to sacrifice). The animals were perfused with 10 mL phosphate-buffered saline, followed by a perfusion with 4% buffered formalin via the left ventricle. The entire heart from each animal was dissected out, embedded in paraffin, and the aortic sinus was serially sectioned (5 μm). Every fifth section was stained with a modified Movat’s pentachrome stain [19].

Assessment of chimerism
The reconstitution of the transplanted bone marrow was determined by PCR on liver and spleen tissue.

Determination of plasma lipid concentration
Total serum cholesterol, high-density lipoprotein (HDL), LDL cholesterol, and triglycerides were determined enzymatically in heparinized plasma.

Evaluation of lesion size and lesion composition
Two investigators who were blinded to the study protocol determined the cross-sectional area of the lesion in each section by using computer-assisted morphometry (Image Pro, Media Cybernetics, Silver Spring, USA); this is reported as mean plaque area per animal (data expressed in μm²). We further evaluated each section for characteristic features of plaque morphology/composition: thickness of the fibrous cap (presented as μm), size of the necrotic core (a large necrotic core was defined as occupying more than 50% of the plaque’s volume and was measured by computer). Calcification was determined using von Kossa staining [20].

Immunohistochemistry
Detection of monocytes/macrophages was performed using monoclonal goat anti-mouse antibody (anti-Mac-2, Accurate, NY, USA) and detection of ICAM-1 by using a polyclonal antibody (Santa Cruz Biotech, CA, USA). Sections were incubated with the biotinylated secondary antibody, rinsed three times with PBS, and incubated for 10 minutes with streptavidin at room temperature. AEC chromogen substrate (Invitrogen, Karlsruhe, Germany) was used for visualization. The extent of positive staining within the lesions was determined using computer-assisted morphometry and is presented as ratio stained area/total lesion area (Image Pro, Media Cybernetics, Silver Spring, USA).

Statistical analysis
All data were expressed as mean ± SEM. Differences between means in plasma lipid profiles were determined with the two-tailed unpaired student’s t-test. For analysis of plaque morphology and areas of positive staining, groups were compared using the two-tailed Mann–Whitney U test. For evaluation of plaque morphology, groups were compared using the χ²-test. A p value <0.05 was considered statistically significant.

Results
Effect of bone marrow transplantation
Polymerase chain reaction analysis of the bone marrow demonstrated a complete conversion of the original LDLr−/− genotype to the donors’ type, indicating that the bone marrow population had been reconstituted (data

Table 1 Distribution of body weight, total serum cholesterol, LDL cholesterol, HDL cholesterol, and serum triglycerides of recipients of GDF-15−/− bone marrow (GDF-15−/−) and wild-type controls (GDF-15+/+) (p was non-significant)

|                       | Body weight (g) | Total cholesterol (mg/dL) | LDL cholesterol (mg/dL) | HDL cholesterol (mg/dL) | Triglycerides (mg/dL) | Lesion size (μm²) |
|-----------------------|-----------------|---------------------------|-------------------------|-------------------------|-----------------------|------------------|
| GDF-15−/− (WT) (n=15) | 28±1            | 351±28                    | 293±26                  | 25±2                    | 159±20                | 246,566±14,788²  |
| GDF-15−/− (KO) (n=17) | 29±1            | 364±15                    | 309±14                  | 28±3                    | 134±5                 | 203,079±17,898¹  |

Quantitative evaluation of lesion size within the aortic root also showed no significant difference between the groups (p=0.08); Means±SEM; ²p=0.08.

WT: wild-type (recipients of bone marrow from C57/Black 6 control mice); KO: knock-out (recipients of bone marrow from GDF-15 negative mice).
There were no differences in body weight and mortality between the groups.

**Effect on plasma lipid level and body weight**

There were no significant differences in total cholesterol, LDL, HDL, and triglycerides between mice that received GDF-15\(^{-/-}\) bone marrow and controls. Furthermore, there was no difference in body weight (Table 1).

**Mean lesion area**

After 24 weeks on the western type diet, the extent of atherosclerotic lesion development in the aortic sinus was evaluated. We could not detect any significant difference in lesion size (GDF-15\(^{+/+}\)2246.566±14.788 \(\mu\)m\(^2\) versus GDF-15\(^{-/-}\)203.079±17.898 \(\mu\)m\(^2\), \(p=0.08\); Table 1).

**Enhanced macrophage content in GDF-15 deficient mice**

After 24 weeks on the western-type diet, we were able to demonstrate macrophage rich lesions and enhanced foam cell formation evaluated by macrophage staining in both groups. Mice transplanted with bone marrow of GDF-15\(^{-/-}\) donors showed enhanced macrophage accumulation within atherosclerotic lesions (\(0.51\) versus \(0.31\); \(p<0.01\); Figures 1A and 2A).

**Mice transplanted with GDF-15 deficient bone marrow showed enhanced expression of intercellular adhesion molecule-1 (ICAM-1)**

ICAM-1 staining was enhanced in atherosclerotic lesions of chimeric mice (\(0.41\) versus \(0.25\) in wild-type controls, \(p<0.01\); Figures 1B and 2B).

**Features of lesion composition**

Video-microscopic evaluation of features of lesion-destabilization showed significantly more thinning of the fibrous cap in LDLr\(^{-/-}\) mice transplanted with bone marrow from GDF-15\(^{-/-}\) mice than in controls (48.5 \(\mu\)m
versus 30.5 μm, p<0.01; Figure 1C). We could not detect any difference in size of the necrotic core or calcification within the lesions (data not shown).

Discussion

Recent studies have hypothesized a crucial role of the cytokine GDF-15 in cardiovascular diseases. While clinical investigations demonstrate GDF-15 as a parameter for risk stratification in myocardial infarction and heart failure, experimental studies show a cardio-protective effect in ischemia and reperfusion [7,10-13]. Furthermore, GDF-15 is correlated with systemic inflammation [21]. These data suggest an involvement of GDF-15 in the initiation and progression of atherosclerosis. Recently, de Jager et al. demonstrated an anti-atherosclerotic effect of GDF-15 deficiency in a mouse model of atherosclerosis [17]. The authors used LDLr−/− mice transplanted with GDF-15-deficient bone marrow. In this study, GDF-15 deficiency resulted in a reduction of early atherosclerotic lesion size after 4 weeks on a high cholesterol western-type diet. After 12 weeks, no differences in lesion size could be detected. Using mice following 24 weeks on a western-type diet, we focused on more advanced and complex lesions to model late-stage disease. It is known that lesions in mice become quite complex with increased duration of feeding [22]. We could not detect any differences in lesion size, but in contrast to the findings of de Jager et al., our data demonstrated a pro-inflammatory plaque phenotype in mice transplanted with bone marrow from GDF-15−/− donors with enhanced macrophage accumulation [17]. In the present study macrophages were identified by using a Mac-2 antibody, which is an appropriate staining used in many LDLr−/− mouse studies. We cannot exclude that staining for other macrophage markers will identify different subpopulations of macrophages with different results. The increase seen in our study was accompanied by enhanced expression of ICAM-1 within lesions.

Monocyte/macrophage recruitment is dependent on adhesion molecules [23]. ICAM-1 is mostly expressed by endothelial cells but also in macrophages within atherosclerotic lesions and it is supposed to be involved in foam cell transformation of monocytes and therefore contributes to changes in lesion vulnerability [24,25].

Our data also confirm a correlation between enhanced macrophage content and signs of the vulnerable plaque determined by the thickness of fibrous caps. This is in line with autopsy findings of ruptured plaques in human [26]. Macrophages excrete an excess of matrix-degrading enzymes and macrophage-rich lesions, and therefore most likely undergo thinning of the fibrous caps and subsequent enhanced vulnerability followed by plaque rupture [27,28]. However, our findings of an association between GDF-15 deficiency and reduced plaque stability
are in contrast to the findings of de Jager et al., where a decreased necrotic core formation in GDF-15−/− chimera is reported [17]. It is known that at one point in atherosclerotic lesion development, changes in plaque composition but not progression of size are dominating. The increase in macrophage and the subsequent increase in inner-plaque inflammation finally results in a reduction of plaque stability. Other than the effect on fibrous caps, we could not detect any differences in other features of lesion destabilization, which might also be due to the duration of the study and the animal model since it is known that differences in necrotic core and hemorrhage are more common in brachiocephalic arteries in apoE−/− mice [29].

There are several limitations to our study. Investigating atherosclerotic lesions in LDLr−/− mice is mostly done in the aortic root, which is not a typical lesion location. It is known as a model of early stages in atherosclerosis and does not show much progress in late-stage disease [30,31]. We did not focus on the onset of atherosclerotic changes within the vascular wall such as lipid accumulation in younger mice. Evaluation of fibrous caps was performed morphometrically as in many LDLr−/− mouse studies. Given the amount of tissue obtained, we were not able to stain for other parameters such as the differences in collagen content. Further, we do not know if bone-marrow transplantation has an effect on other cytokines, the immunosystem, or metabolism, which is an important factor in atherosclerosis. Recently, it has been shown that GDF-15 is a key regulator in anoxemia, and weight and fat loss [32]. However, lipid levels and body weight in our study were equally distributed. We could not detect any further change in lethality after transplantation.

Conclusions
In conclusion, this is the first study evaluating the effects of GDF-15 in advanced stages of atherosclerosis. We were able to demonstrate a GDF-15-dependent inhibition of macrophage adhesion and accumulation in an atherosclerotic LDLr−/− mouse model. This effect may contribute to changes in lesion vulnerability such as thinning of fibrous caps and potential plaque rupture.

Abbreviations
GDF-15: Growth differentiation factor-15; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TGF-β: Transforming growth factor-β.

Competing interest
All authors declare that they have no competing interests.

Authors’ contribution
MB and CA carried out the immunostaining, PCR and lesion analyses. MB performed the body irradiation. MRP, MB, EB, and FB participated in the design of the study and performed the statistical analysis. MP, HAK, and FB conceived the study and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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References
1. Grim HR, Orsi NM, Homr-Vennasinkam S: An overview of cytokine interactions in atherosclerosis and implications for peripheral arterial disease. Vasc Med 2007, 12:299–307.
2. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK: Disruption of TGF-beta signalling in T cells accelerates atherosclerosis. J Clin Invest 2003, 112:1342–1350.
3. Topper JN: TGF-beta in the cardiovascular system: molecular mechanisms of a context-specific growth factor. Trends Cardiovasc Med 2000, 10(1):132–137.
4. Bootcov MR, Bauskin AR, Valenzuela SM, Bansal M, He XY, Zhang HP, Donnellan M, Maher S, Pryor K, Walsh BJ, Nicholson RC, Fairlie WD, Por SB, Robbins JM, Brett SN: MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proc Natl Acad Sci U S A 1997, 94:11154–11159.
5. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, Ward RL, Hawkins NJ, Quinn DJ, Russell PJ, Sutherland RL, Brett SN, Moskaluk CA, Friesen HF, Jr, Hampton GM: Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. Proc Natl Acad Sci U S A 2003, 100:3410–3415.
6. Schittenhardt D, Schober A, Streulau J, Bonartera GA, Schmidtw M, Unsicker K, Metz J, Kirschner R: Involvement of growth differentiation factor-15 /macrophage inhibitory cytokine-1 (GDF-15/MIC-1) in oxLDL-induced apoptosis of human macrophages in vitro and in arteriosclerotic lesions. Cell Tissue Res 2004, 318:325–333.
7. Kempf T, Eden M, Streulau J, Nagub M, Willenbockel C, Tonniger J, Heineke J, Kotlarz D, Xu J, Molkentin JD, Niessen HW, Drexler H, Wollert KC: The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. Circ Res 2006, 98:351–360.
8. Xu J, Kimball TR, Lorenz JN, Brown DA, Bauskin AR, Kleivstoy R, Hewett TE, Brett SN, Molkentin JD: GDF15/MIC-1 functions as a protective and anti hypertrophic factor released from the myocardium in association with SMAD protein activation. Circ Res 2006, 98:342–350.
9. Frank D, Kuhn C, Brors B, Hanselmann C, Ludde M, Katus HA, Frey N: Gene expression pattern in biomechanically stretched cardiomyocytes: evidence for a stretch-specific gene program. Hypertension 2008, 51:309–318.
10. Wollert KC, Kempf T, Peter T, Ollofsson S, James S, Johnston N, Lindahl B, Hom-Wichmann R, Brabant G, Simon M, Armstrong PW, Califf RM, Drexler H, Wallentin L: Prognostic value of growth-differentiation factor-15 in patients with non-ST-elevation acute coronary syndrome. Circulation 2007, 115:962–971.
11. Wallentin L, Wallentin L: Growth differentiation factor-15 improves risk stratification and selection of an invasive treatment strategy in non ST-elevation acute coronary syndrome. Circulation 2007, 116:1540–1548.
12. Kempf T, Björklund E, Ollofsson S, Lindahl B, Alhoff T, Peter T, Tongers J, Wollert KC, Wallentin L: Growth-differentiation factor-15 improves risk stratification in ST-segment elevation myocardial infarction. Eur Heart J 2007, 28:2858–2865.
13. Kempf T, von Haehling S, Peter T, Alhoff T, Cicinora M, Doehner W, Ponikowski P, Filippatos GS, Rzzenytny P, Drexler H, Anker SD, Wollert KC: Prognostic utility of growth differentiation factor-15 in patients with chronic heart failure. J Am Coll Cardiol 2007, 50:1054–1060.
14. Mallat Z, Gojova A, Marchiol-Fourniquat C, Esposito B, Kamaté C, Merval R, Fradelizi D, Tedgui A: Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. Circ Res 2001, 89:930–934.

15. Lutgen E, Gibels M, Smook M, Heeringa P, Gotwals P, Koteliantsky VE, Daenen M: Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. Arterioscler Thromb Vasc Biol 2002, 22:975–982.

16. Ago T, Sadoshima J: GDF15, a cardioprotective TGF-beta superfamily protein. Circ Res 2006, 98:294–297.

17. de Jager SC, Bermúdez B, Bot I, Koenen RR, Bot M, Kavelaars A, de Waard V, Strelau J, Strzelczyk A, Rusu P, Bendner G, Wiese S, Diella F, Altick AL, von Bartheld CS, Klein R, Sendtner M, Unsicker K: Demonstration of all connective tissue elements in a single section; pentachrome stains. AMA Arch Pathol 1955, 60:289–295.

18. Yang PY, Rui YC: Intercellular adhesion molecule-1 and vascular inflammation and fibrosis during plaque progression. J Exp Med 2011, 208:217–225.

19. Strelau J, Strzelczyk A, Rusu P, Bendner G, Wiese S, Altick AL, von Bartheld CS, Klein R, Sendtner M, Unsicker K: Progressive postnatal motoneuron loss in mice lacking GDF-15. J Neurosci 2009, 29:13640–13648.

20. Movat HZ: Demonstration of all connective tissue elements in a single section; pentachrome stains. AMA Arch Pathol 1955, 60:289–295.

21. Puchtler H, Meloan SN: Demonstration of phosphates in calcium deposits: a modification of von Kossa’s reaction. Histochemistry 1978, 56:177–185.

22. Skipworth RJ, Dears DA, Tan BH, Sangster K, Paterson-Brown DA, Hunter M, Bret SN, Ross JA, Fearon KC: Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophageo-gastric cancer. Br J Cancer 2010, 102:665–672.

23. Duplaia C, Couffinhal T, Labat L, Moreau C, Petit-Jean ME, Doutre MS, Loree HM, Tobias BJ, Gibson LJ, Kamm RD, Small DM, Lee RT: Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. Circulation 2009, 119:208–217.

24. Yang PY, Rui YC: Intercellular adhesion molecule-1 and vascular endothelial growth factor expression kinetics in macrophage-derived foam cells. Life Sci 2003, 74:471–480.

25. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R: Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997, 336:1276–1282.

26. Davis MJ, Gordon JL, Gearing AJ, Pigott R, Woolf N, Katz D, Kyriakopoulos A: The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. J Pathol 1993, 171:223–229.

27. Sukhova GK, Schönbeck U, Rabkin E, Schoen FJ, Poole AR, Billinghurst RC, Yang PY: Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. Circulation 1999, 99:2003.

28. Jackson CL, Bennett MR, Biessen EA, Johnson JL, Krams R: Assessment of unstable atherosclerosis in mice. Arterioscler Thromb Vasc Biol 2007, 27:714–720.

29. Johnen H, Eitzman DT, Gordon D, Westrick R, Nabel EG, Ginsburg D: Atherosclerosis progression in LDL receptor-deficient and apolipoprotein E-deficient mice is independent of genetic alterations in plasminogen activator inhibitor-1. Arterioscler Thromb Vasc Biol 2000, 20:846–852.

30. Shiodo J, Itozuka T, Kato M, Nakai T, Tanaka T, Iwasaki H, Tominaga S, Yajima H, Nishimura M, Fujita T, Ohtsuki M, Nakamura Y, Ishida K: Apolipoprotein E deficiency protects against atherosclerosis by attenuating CCR2-mediated macrophage chemotaxis. J Exp Med 2011, 208:217–225.

31. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R: Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997, 336:1276–1282.

32. Breslow JL: Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. Nat Med 2007, 13:1333–1340.

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