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REGULAR ARTICLE

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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

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Abstract Growth differentiation factor-15/macrophage inhibitory cytokine-1 (GDF-15/MIC-1) is a new member of the transforming growth factor beta (TGF-β) superfamily, which has most recently been found in activated macrophages (MΦ). We have now investigated GDF-15/MIC-1 in human MΦ after exposure to oxidized low-density lipoproteins (oxLDL) related mediators in vitro and in arteriosclerotic carotid arteries. Using RT-PCR and Western blotting a pronounced induction of GDF-15/MIC-1 expression by oxLDL, C_6-ceramide, tumor necrosis factor (TNFα) and hydrogen peroxide (H_2O_2) was found in cultured human MΦ. In 11 human arteriosclerotic carotid arteries, immunohistochemical analyses supported by computer-assisted morphometry and regression analyses demonstrated a significant colocalization of GDF-15/MIC-1 immunoreactivity (IR) with oxLDL IR and manganese superoxide dismutase (MnSOD) IR in CD68 immunoreactive (ir) MΦ, which were also expressing AIF-IR (apoptosis-inducing factor), caspase-3-IR (CPP32), PARP-IR, c-Jun/AP-1-IR and *p53*-IR. Our data suggest that GDF-15/MIC-1 is inducible in human MΦ by oxLDL and its mediators in vitro and is supposed to contribute to oxidative stress dependent consequences in arteriosclerotic plaques, e.g. modulating apoptosis and inflammatory processes in activated MΦ.

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Introduction

Transforming growth factor betas (TGF-βs) have been shown to be strongly implicated in a number of pathophysiological processes including chronic vascular diseases (Topper 2000). Furthermore, TGF-βs constitute inflammatory markers of advanced arteriosclerosis (Erren et al. 1999; Czernichow and Hercberg 2001) and seem to be involved in the pathogenesis of ischemic heart disease in humans (Tashiro et al. 1997). High concentrations of TGF-βs have been described in early atherosclerotic lesions (fatty streaks), supporting the hypothesis that TGF-βs contribute to the pathogenesis of lipid-rich atherosclerotic lesions by activating proteolytic mechanisms in macrophages (MΦ) (Bobik et al. 1999).

MΦ-inhibitory cytokine-1 (MIC-1), which is identical to growth differentiation factor-15 (GDF-15) (Bootcov et al. 1997; Fairlie et al. 1999; Bauskin et al. 2000), a divergent member of the TGF-β superfamily (Strelau et al. 2000), is widely distributed in adult tissues, being most strongly expressed in epithelial cells and MΦ (Böttner et al. 1999b). GDF-15/MIC-1 expression is upregulated in mononuclear cells by a variety of stimuli including interleukin-1β, interleukin-2, tumor necrosis factor alpha (TNFα), phorbol myristate acetate or MΦ colony-stimulating factor and, thus, seems to be associated with MΦ activation (Bootcov et al. 1997; Bauskin et al. 2000; Fairlie et al. 2001; Schober et al. 2001). Moreover, GDF-15/MIC-1 has most recently been shown to be induced by *p53*, suggesting a role in apoptosis, too (Kannan et al. 2000). In the lesioned CNS, GDF-15/MIC-1 may have important anti-inflammatory functions, supplementing the roles of other members of the TGF-β superfamily. However, localization of GDF-15/MIC-1 in neurons within the lesion site has raised questions of putative

pro-apoptotic or anti-apoptotic functions of this protein (Schober et al. 2001; Subramaniam et al. 2003).

Activation of monocytes to MΦ is known to play a key role in normal and pathological processes of the arteriosclerotic vessel wall, including immune and inflammatory responses, largely by their capacity to phagocyte detrimental material and to secrete biologically active molecules. Apoptosis of these activated MΦ in arteriosclerotic lesions may considerably modulate their inflammatory response, affecting the plaque stability and growth, as has been suggested from human subjects and animal models (Libby et al. 1996; Kockx and Herman 2000; van Vlijmen et al. 2001). Most recently, MΦ have been shown to undergo apoptosis in conditions of lipid-rich plaque, pointing to a key role of lipid content and inflammatory cell viability in determining plaque thrombogenicity (Hutter et al. 2004). OxLDL and its mediators ceramide or TNFα are known to initiate apoptosis in human MΦ in vitro (Jovinge et al. 1996; Kinscherf et al. 1997a, b, 1998, 1999; Deigner et al. 2001). Signal transduction of oxLDL induced apoptosis includes activation of caspase-3 (CPP32) (Wintergerst et al. 2000), induction of manganese superoxide dismutase (MnSOD) as well as increase of *p53* (Kinscherf et al. 1997b, 1998) which all have also been shown in human arteriosclerotic plaques (Ihling et al. 1997; Kinscherf et al. 1997b; Mallat et al. 1997; Ihling et al. 1998; Kinscherf et al. 1998, 1999). In addition, poly (ADP-ribose) polymerase (PARP), c-Jun-AP-1 or apoptosis inducing factor (AIF) have also been detected in apoptotic cells (Grand et al. 1995; Martinet et al. 2002; Zhang et al. 2003).

Because the plasma concentration of GDF-15/MIC-1 has most recently been found to be a potential marker for cardiovascular events in women (Brown et al. 2002), we were interested about its presence in arteriosclerotic plaques and its induction and involvement in oxLDL-mediated apoptosis of human MΦ in vitro.

Materials and methods

In vitro experiments: isolation of peripheral blood mononuclear cells and cell culture of MΦ

Buffy coats of healthy human volunteers were obtained from the center of blood donation of the University of Heidelberg. PBMCs were isolated by Histopaque (Sigma, Taufkirchen, Germany) density gradient centrifugation and cultured in supplemented RPMI 1640 medium at 37°C in humified CO_2 (5%) atmosphere as described earlier (Kinscherf et al. 1997b, 1998; Schober et al. 2001). The differentiated MΦ were either kept in supplemented RPMI 1640 medium (control medium) or were incubated in lipoprotein-deficient serum for 24 h before exposition (4 h) to oxLDL (50 μg/ml), C_6-ceramide (30 μM), recombinant human TNFα (3 ng/ml) or H_2O_2 (100 μM) (Sigma, Taufkirchen, Germany) as described earlier (Kinscherf et al. 1997b, 1998; Deigner et al. 2001).

Measurement of apoptotic MΦ

Apoptotic cells were identified by YO-PRO-1 staining (Idziorek et al. 1995) in combination with the Hoechst 33342 dye (Mobitec Company, Goettingen) as previously published (Deigner et al. 2001). The percentage of apoptotic cells was counted using an inverse fluorescence microscope and a computer-assisted morphometry system developed by our group (VIBAM 0.0-VFG 1 frame grabber) (Kinscherf et al. 1997b, 1998; Deigner et al. 2001).

Reverse transcription-polymerase chain reaction

RNA extraction from 1×10^6 cells using Trizol LS reagent as well as denaturating agarose gel electrophoresis and RT-PCR were performed routinely as previously described (Kinscherf et al. 1997b, 1998; Deigner et al. 2001; Schober et al. 2001).

After reverse transcription, cDNA samples were subjected to PCR amplification using sequence-specific primers based on the human coding sequence of GDF-15/MIC-1: 5′-ACT GCT GGC AGA ATC TTC GT-3′ (forward) and 5′-AAT GAG CAC CAT GGG ATT GT-3′ (reverse), generating a 352 base pair (bp) fragment. Amplification of part of the β-microglobulin gene was used as a positive control using primers 5′-TGT CGG ATT GAT GAA ACC CAG-3′ (forward) and 5′-CTC GCG CTA CTC TCT CTT TCT-3′ (reverse). A PCR reaction profile with initial denaturation for 3 min at 94°C, followed by 30 s 94°C, 30 s 50°C, 45 s 72°C 32–36 cycles and a final extension step for 10 min at 72°C, was performed using a Genius thermal cycler (Techne Inc., Cambridge, UK). The O'Range Ruler 50 bp DNA Ladder (MBI Fermentas, St-Leon-Rot, Germany) was used. Amplification products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Western blotting

GDF-15/MIC-1 expression in cultured MΦ was measured by Western blotting as recently described (Schober et al. 2001) with minor modifications: MΦ were scraped off, boiled and the protein concentration was quantified. After SDS-PAGE gel electrophoresis, blotting was performed with a semi-dry system (Milliblot; Millipore, Eschborn, Germany) and PVDF membrane for ECL (Amersham Pharmacia, Freiburg, Germany). After protein transfer non-specific sites were blocked with TRIS-buffered saline, and rabbit anti-human GDF-15/MIC-1 antibodies were added (Schober et al. 2001). Protein loading was tested by the use of a monoclonal mouse anti-human α-tubulin antibody (1:200; Dianova, Hamburg, Germany). A polyclonal sheep anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate and the chemiluminescence ECL detection kit (Amersham Pharmacia) were used for visualization. A hyperfilm ECL (Amersham Pharmacia)

served for documentation. Washing steps after every incubation period were performed using TBS.

Human arteriosclerotic tissue

Specimen of human arteriosclerotic carotid arteries (n=11) obtained at surgery were shock-frozen in liquid nitrogen-cooled isopentane and kept in a −70°C freezer until used (Kinscherf et al. 1997b, 1998, 1999). The patients provided informed written consent before participation.

Immunohistochemistry and computer-assisted morphometry

Immunohistochemistry of human arteriosclerotic carotid arteries was routinely performed as described earlier (Kinscherf et al. 1997b, 1998, 1999). Cryostat sections (6 μm) were exposed to acetone (10 min, −20°C) and dried thereafter (30 min). Non-specific sites were blocked with 1% normal swine serum (Life Technologies) in PBS. The following primary monoclonal mouse (mab) and polyclonal antibodies were used: polyclonal rabbit anti-human GDF-15/MIC-1 (1:100), mab anti-human MnSOD (1:100; Alexis, Grünberg, Germany), mab anti-human *p53* (Ab5, 1:50; Calbiochem, Bad Soden/Schwalbach, Germany), polyclonal rabbit anti-human oxLDL (1:2,000; Immundiagnostik, Bensheim, Germany), polyclonal rabbit anti-human c-Jun/AP-1 (1:10; Calbiochem, Schwalbach, Germany), mab anti-human AIF (1:100; Chemicon, Hofheim, Germany), mab anti-human CPP32 (caspase-3) (1:100; Transduction Laboratories, Lexington, Ky., USA), mab anti-human PARP (1:50; BioMol, Hamburg, Germany), mab anti-CD68 (1:1,000; DakoCytomation, Glostrup, Denmark), mab anti-human α-actin (1:800; Roche Mannheim, Germany) and mab anti-human CD31 (1:1000; Immunotech, Marseille, France).

Incubation times at room temperature and secondary antibody dilutions were as follows: (1) single staining was performed by incubation of primary antibody with biotinylated anti-rabbit IgG (1:100; 2 h; Vector Lab, USA); endogenous peroxidase activity was suppressed with 3% H_2O_2 in PBS (5 min); afterwards the sections were incubated with peroxidase-conjugated streptavidin (1:100, 2 h) and staining reaction was achieved with diaminobenzidine (DAB) solution (Pierce, Rockford, Ill., USA). Nuclei were counterstained with hematoxylin. (2) Double-stainings were performed by adding two primary antibodies sequentially to the same section and using Cy2-conjugated or Cy3-conjugated streptavidin (1:1000, 2 h; Jackson ImmunoRes. Lab, USA) for visualization. Anti-GDF-15/MIC-1 antibodies were applied in a three-step procedure using Cy3 (or Cy2) fluorochrome followed by a two step procedure for CD68, MnSOD, oxLDL, CPP32, AIF, PARP, c-Jun/AP-1, α-actin or CD31 visualized by Cy2 (or Cy3)-conjugated anti-rabbit or anti-mouse IgG (1:500, Jackson ImmunoRes.). Between first and second staining, sections were rinsed in PBS. Nuclei were counterstained with DAPI (Sigma, Munich, Germany). Finally, all sections were rinsed 3 times in PBS and embedded in fluorescent mounting medium. Negative controls for the GDF-15/MIC-1 polyclonal antibody were performed as described most recently (Schober et al. 2001), using routine methods, i.e. omission of the first antibody, which abolished the immunoreactivity completely (e.g. Kinscherf et al. 1997b). Furthermore irrelevant, isotype-matched monoclonal antibodies as well as mouse/rabbit serum (of non-immunized animals) were used to exclude a cross-reaction of the antibodies due to a Fc-receptor-mediated reaction. The use of this number of antibodies did not reveal any labeling of plaque cells.

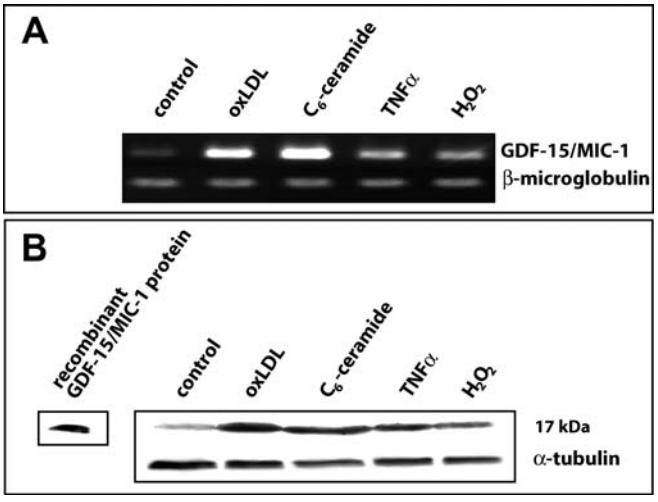

Fig. 1 GDF-15/MIC-1 expression in human MΦ. RT-PCR **A** and Western blot **B** analyses showing pronounced GDF-15/MIC-1 induction in cultured human MΦ after exposure to oxLDL (50 μg/ml), C_6-ceramide (30 μM), TNFα (3 ng/ml) or H_2O_2 (100 μM) for 4 h in comparison to medium (control). For Western blot analyses, recombinant GDF-15/MIC-1 protein (band refers to 200 ng), produced in our laboratory, was used as positive control. α-Tubulin or β-microglobulin was used as an internal control

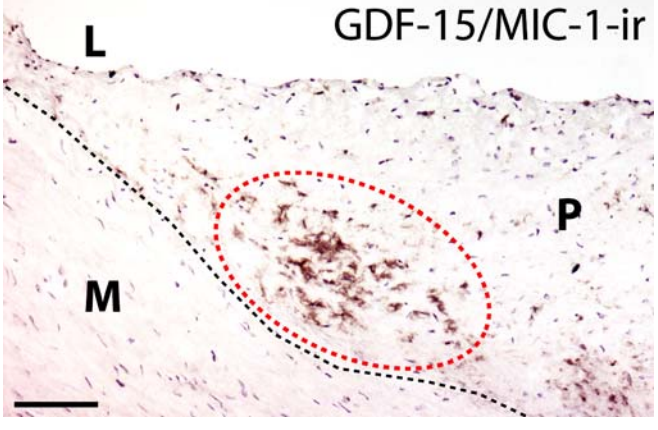

Fig. 2 Immunohistochemistry of an atherosclerotic human carotid artery. GDF-15/MIC-1 immunoreactivity (*ir*) is seen in several plaque cells (*red circle*). Nuclei were counterstained with hematoxylin. *Dotted line* marks the border between media (*M*) and plaque (*P*). *L* lumen; *Bar* 100 μm

Immunoreactive cells in arteriosclerotic carotid arteries were recorded by a video camera (Olympus HCC-3600 P high gain) and quantified using a computer-assisted image analysis system developed by our group (VIBAM 0.0-VFG 1 frame grabber) (e.g. Kinscherf et al. 1997b, 1999). Finally, sections were photographed using an Axioplan2 imaging microscope (Carl Zeiss GmbH, Jena, Germany) and the digital high resolution imaging system AxioCam/AxioVision (Carl Zeiss). Digitalized images were processed, arranged and lettered by standard imaging software (Adobe Photoshop 6.0/Illustrator 10.0).

Statistical analyses

The results were calculated as mean±SEM. Statistical procedures were performed by the Mann–Whitney *U*–Wilcoxon Rank Sum *W*-test or by the Student's *t*-test for unpaired data using the SPSS Base 11.5 for windows. Correlation between different parameters was graphically described by scatterplots and linear regression lines. The dependency was quantitatively assessed by Pearson's product correlation coefficient *r* and by a statistical test for the existence of a positive or negative slope. A *P* value of 0.05 or less was chosen for statistical significance.

Results

Induction of apoptosis and GDF-15/MIC-1 expression in human MΦ

After exposure (4 h) to oxLDL (50 μg/ml), C_6-ceramide (30 μM), TNFα (3 ng/ml) or H_2O_2 (100 μM), the number of apoptotic MΦ significantly increased about 3.41±0.013-fold, 2.91±0.032-fold, 2.34±0.015 or 3.20±0.034-fold in

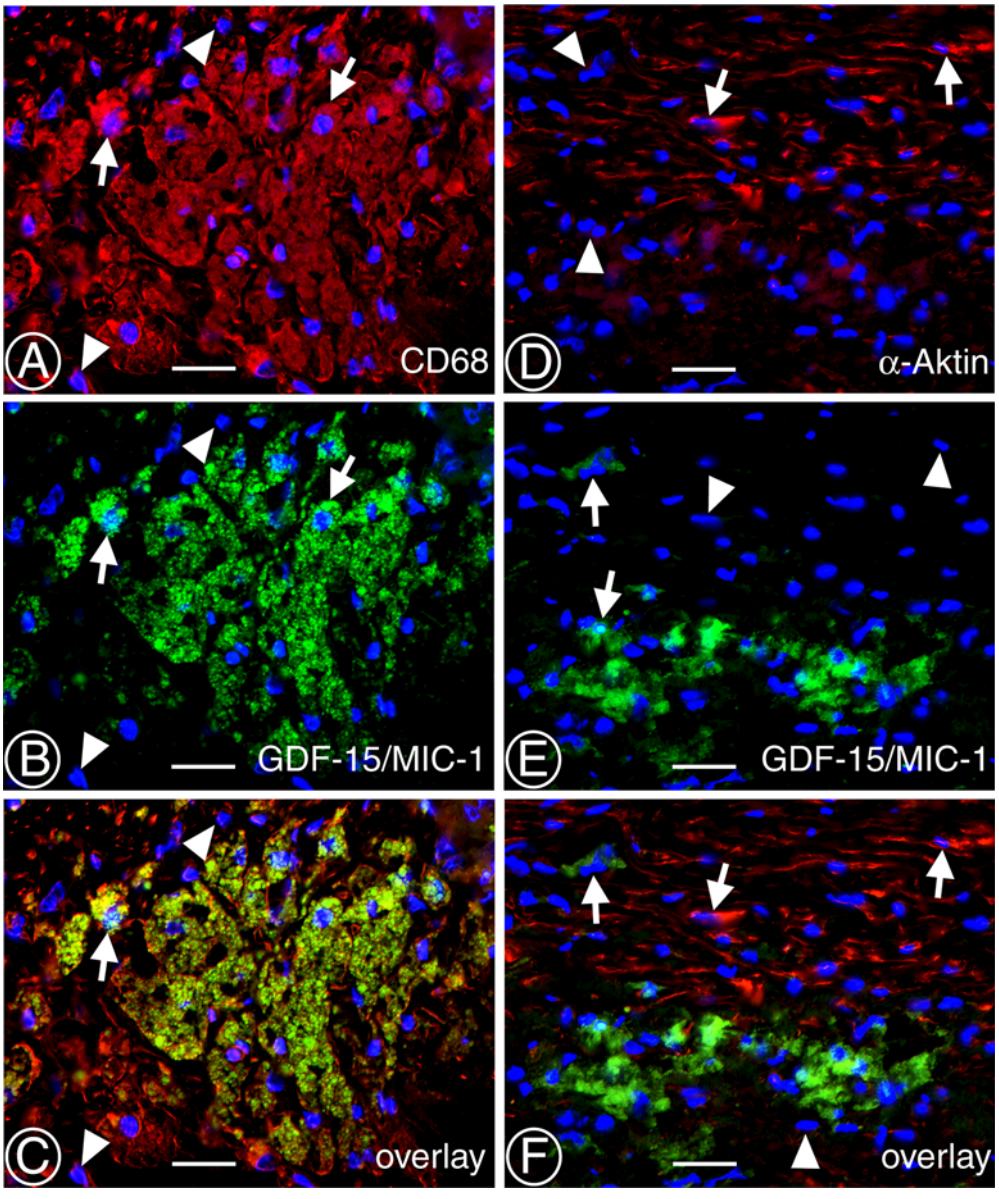

Fig. 3 Double staining of MΦ in an arteriosclerotic plaque of a human carotid artery. CD68 ir (**A**; *arrows*) is colocalized with GDF-15/MIC-1 ir (**B**; *arrows*) in most MΦ as seen in the overlay **C** (*yellow color; arrows*). α-Actin ir (**D**; *arrows*; =smooth muscle cells) is not colocalized with GDF-15/MIC-1 ir (**E**; *arrows*) as also demonstrated in the overlay **F**. Nuclei are counterstained with DAPI. *Arrowheads* show cells, which are neither CD68-actin nor α-actin or GDF-15/MIC-1 ir. *Bar* 25 μm

comparison to the control, which revealed an absolute apoptosis rate of 9.2%±1.3% (five independent experiments). As shown by RT-PCR (Fig. 1A) and Western blotting (Fig. 1B), a pronounced GDF-15/MIC-1 expression in human MΦ was simultaneously induced by oxLDL, C_6-ceramide, TNFα or H_2O_2.

Localization of GDF-15/MIC-1 IR in apoptotic MΦ in arteriosclerotic lesions of human carotid arteries

Immunohistology shows that GDF-15/MIC-1 ir cells are located in arteriosclerotic lesions of human carotid arteries (Fig. 2). GDF-15/MIC-1 ir cells were found to be localized in superficial and deeper regions of the plaques. Double immunostainings revealed a colocalization of GDF-15/MIC-1 IR in CD68-ir MΦ (Fig. 3A–C), but neither in α-actin ir smooth muscle cells (Fig. 3D–F) nor in CD31 ir endothelial cells (not shown). GDF-15/MIC-1 IR was also colocalized in oxLDL ir MΦ (Fig. 4A–C) and MnSOD ir MΦ (Fig. 5A–C). Moreover, GDF-15/MIC-1 ir was also found in apoptotic, CPP32 (caspase-3)-ir (Fig. 5D–F) AIF-ir, c-Jun/AP-1-ir, PARP-ir or *p53*-ir MΦ (not shown).

Morphometric analyses of immunoreactivities in arteriosclerotic human carotid arteries demonstrated a significant positive correlation between the percentage of GDF-15/MIC-1-ir and CD68-ir, oxLDL-ir, MnSOD-ir, CPP32-ir, AIF-ir, c-Jun/AP-1-ir, PARP-ir, or *p53*-ir MΦ (Fig. 6).

Discussion

GDF-15/MIC-1 expression and oxidative stress in human MΦ in vitro

GDF-15/MIC-1, a member of the TGF-β superfamily, has recently been induced in phorbolester-stimulated, activated MΦ (Bootcov et al. 1997; Schober et al. 2001). The present study, which focused on cell associated GDF-15/MIC-1, demonstrates for the first time that GDF-15/MIC-1 expression in human MΦ is upregulated by oxLDL and its mediators C_6-ceramide, TNFα or H_2O_2. Simultaneously, the expression of several other substances (cytokines) like TNFα, macrophage migration inhibitory factor or transcription factors, e.g. nuclear factor kappa B, as well as processes such as apoptosis and inflammation, have been found to be enhanced (Jovinge et al. 1996; Kinscherf et al. 1997b, 1998; Mikita et al. 2001; Burger-Kentischer et al. 2002). OxLDL and its mediators are known to increase oxidative stress in MΦ, as indicated, e.g. by an induction of the MnSOD and *p53* expression (Kinscherf et al. 1997b, 1998). We therefore assume that oxidative stress might also be involved in the increased GDF-15/MIC-1 expression. This hypothesis is supported by the finding that phorbol ester, which stimulates superoxide anion production in microglia cells or MΦ (Colton et al. 1998; Wagner et al. 2000), or H_2O_2 alone (this study), induce GDF-15/MIC-1 expression. Recent findings showing the regulation of GDF-15/MIC-1 gene induction by, e.g.

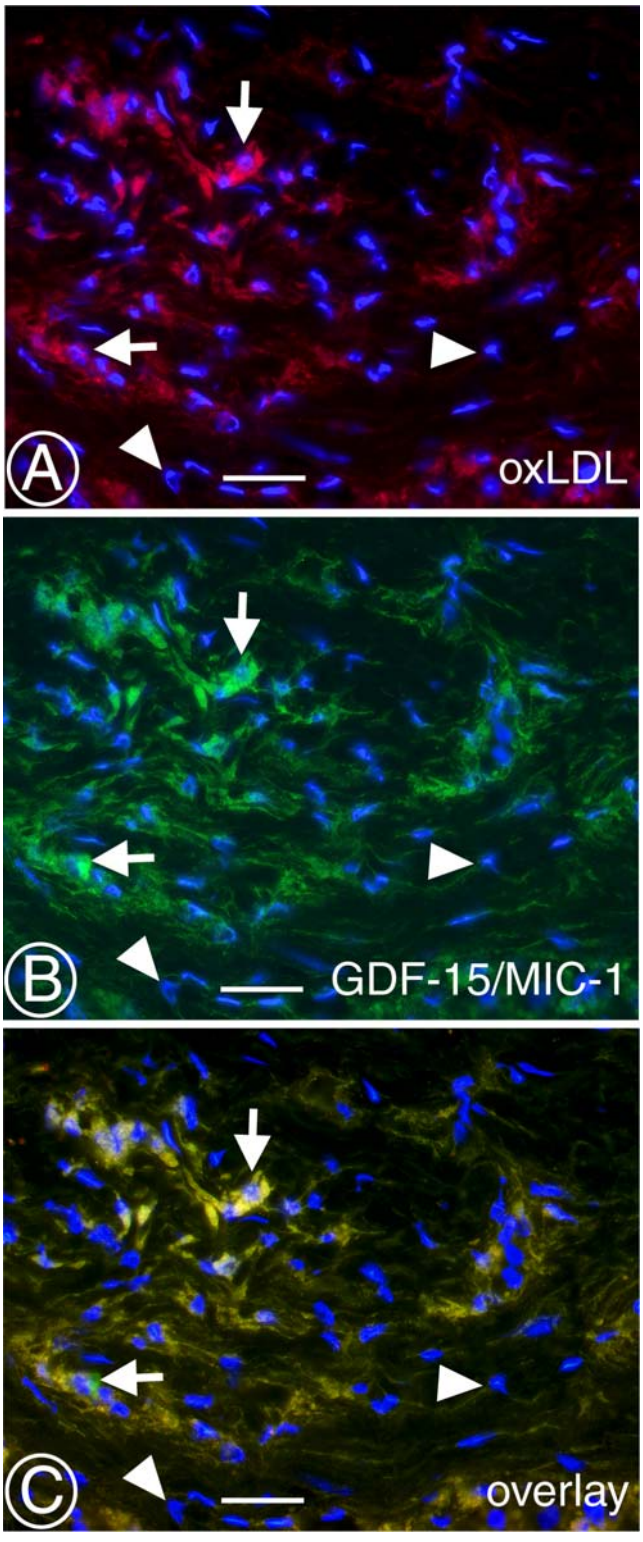

Fig. 4 Double staining of MΦ in an arteriosclerotic plaque of a human carotid artery. OxLDL ir (**A**; *arrows*) is colocalized with GDF-15/MIC-1 ir (**B**; *arrows*) in most MΦ, as seen in the overlay (**C**; *yellow color; arrows*). Nuclei are counterstained with DAPI. *Arrowheads* show cells, which are neither oxLDL ir nor GDF-15/MIC-1 ir. *Bar* 25 μm

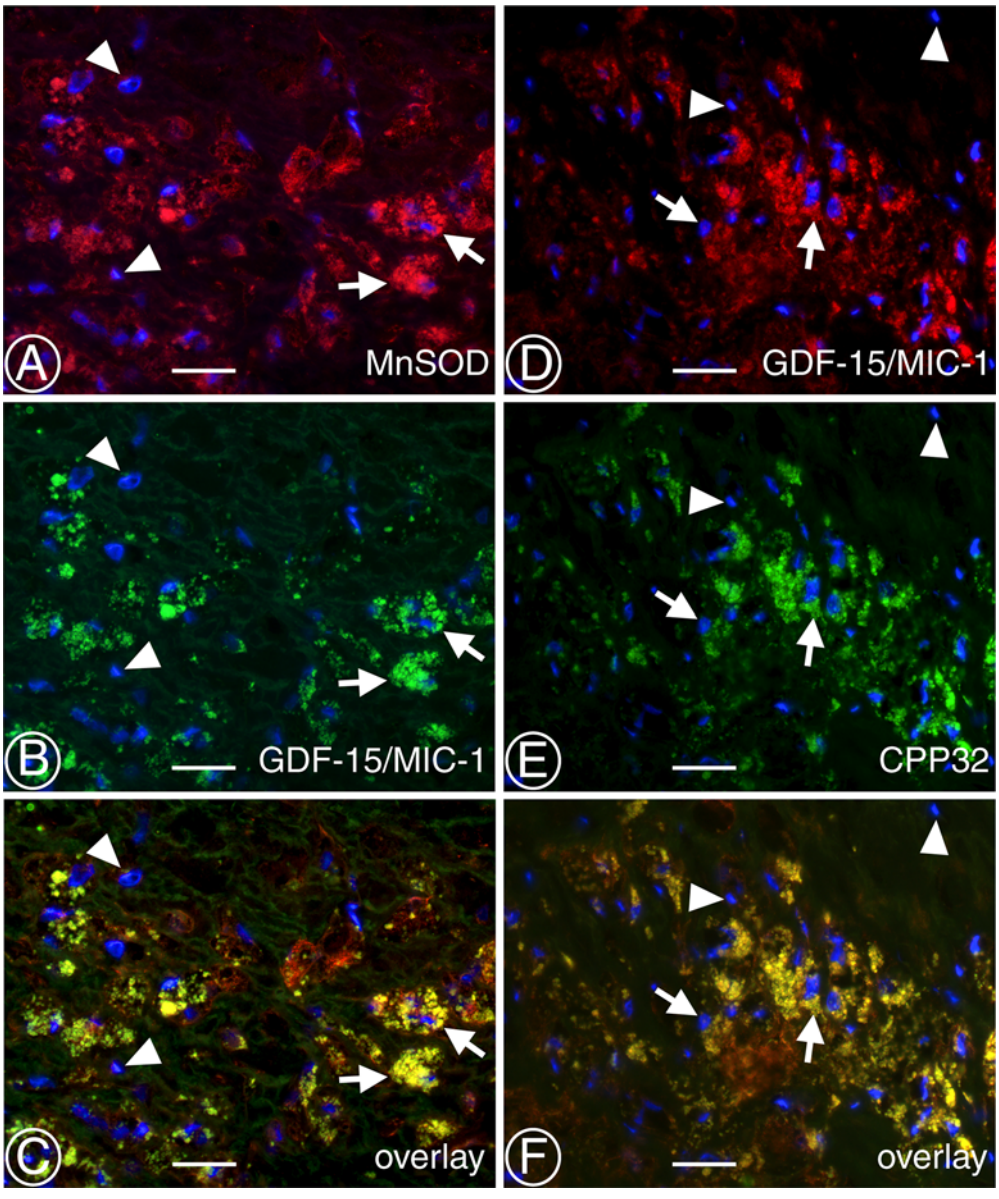

Fig. 5 Double staining of MΦ in an arteriosclerotic plaque of a human carotid artery. MnSOD ir (**A**; *arrows*) and GDF-15/MIC-1 ir (**B**; *arrows*) are colocalized in most MΦ as seen in the overlay **C** (*yellow color; arrows*). GDF-15/MIC-1 (**D**; *arrows*) ir is colocalized with CPP32 (caspase-3) ir (**E**; *arrows*) in most MΦ as seen in the overlay (**C**; *yellow color; arrows*). Nuclei are counterstained with DAPI. Cells that are neither MnSOD nor GDF-15/MIC-1 ir or CPP32 ir are also seen (*arrowheads*). *Bar* 25 μm

TNFα, LPS and *p53* (Bootcov et al. 1997; Kannan et al. 2000), which all are known to induce or to be associated with oxidative stress, lend further support (De la Fuente and Victor 2001). Based on these findings, GDF-15/MIC-1 has been supposed to limit the later phases of MΦ activation (Bootcov et al. 1997). A recent analysis of the rat promoter of the GDF-15/MIC-1 gene has indicated the presence of multiple regulatory elements, including a TATA-like sequence as well as several SP1, AP-1 and AP-2 sites (Böttner et al. 1999a), which are all well-known to be redox-regulated (Das et al. 1995; Deigner et al. 2001; Zhao et al. 2001).

GDF-15/MIC-1 ir apoptotic MΦ in human arteriosclerotic lesions

TGF-βs have already been shown to be multifunctional cytokines playing key roles in cell cycle control, development, repair processes, and apoptosis (e.g. Böttner et al. 2000; Unsicker and Strelau 2000; Schuster and Krieglstein 2002). Several earlier studies report that many members of the TGF-β superfamily are involved in inflammatory processes, such as those in arteriosclerotic lesions (Corradin et al. 1993; Letterio and Roberts 1998; Bobik et al. 1999; Reckless et al. 2001). We here show for the first time GDF-15/MIC-1-ir cells in human arteriosclerotic carotid arteries, which have been identified as MΦ. Double staining procedures excluded smooth muscle or endothelial cells to contain GDF-15/MIC-1. Most of the GDF-15/MIC-1-ir MΦ revealed a colocalization with MnSOD IR, which has been used as a marker for increased oxidative

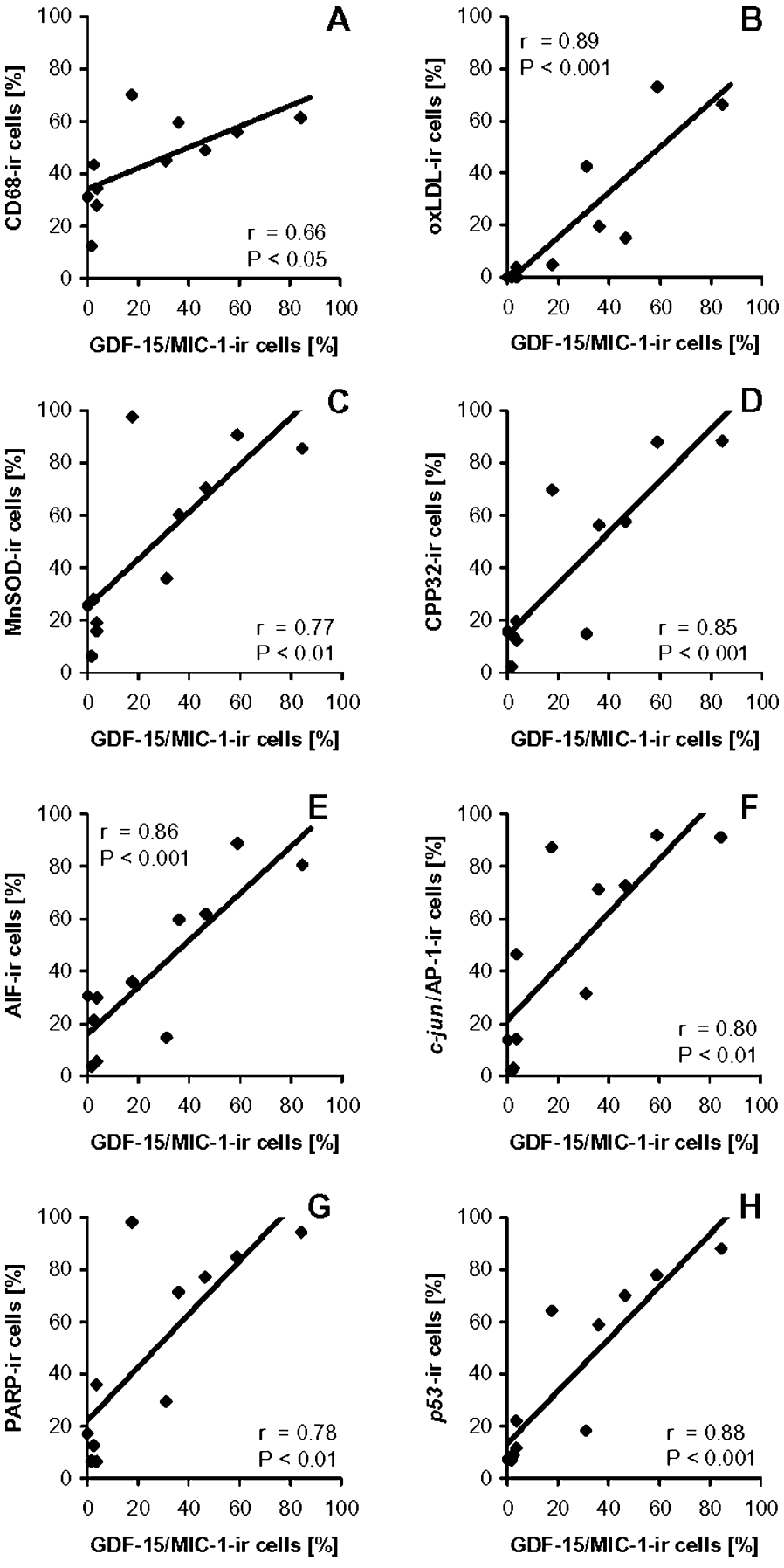

Fig. 6 Significant positive correlation between the percentage of GDF-15/MIC-1 ir MΦ and CD68 **A**, oxLDL **B**, MnSOD **C**, CPP32 (caspase-3; **D**), AIF **E**, c-Jun/AP-1 ir **F**, PARP **G**, *p53***H** ir MΦ in arteriosclerotic human carotid arteries. *P* statistical significance; *r* correlation coefficient

stress (Kinscherf et al. 1997b, 1998, 1999; Martinet et al. 2002; Zhang et al. 2003). We further found a significant colocalization with oxLDL IR, which was supported by a strong positive statistical correlation between the percentage of GDF-15/MIC-1-IR and CD68-IR or oxLDL IR in MΦ of the arteriosclerotic plaques. In the context with our in vitro experiments, we assume an induction of GDF-15/MIC-1 (as well as of MnSOD) by oxLDL (or its mediators) in MΦ of human arteriosclerotic lesions (Kinscherf et al. 1997b, 1998). GDF-15/MIC-1 IR is also colocalized with apoptosis markers like PARP, c-Jun/AP-1, CPP32 (caspase-3) or AIF IR. Thus, it might be involved in apoptosis signaling of plaque MΦ, as has already been suggested according to results in several cell lines (Baek et al. 2001; Subramaniam et al. 2003). The colocalization of GDF-15/MIC-1 IR with *p53* IR supports further evidence for a direct functional link, as has been supposed for the TGF-β superfamily (Li et al. 2000). Moreover, GDF-15/MIC-1 is suggested as an important downstream mediator of *p53* function, while acting as a target of cellular stress signaling (Albertoni et al. 2002). The death program might modulate inflammatory processes within MΦ of the atherosclerotic vessel wall (Schlittenhardt, Kinscherf unpublished results), however, putative cross talks between these pathways need to be addressed in more detail by future experiments. Potential autocrine effects of this protein on MΦ remain as long speculative as GDF-15/MIC-1 receptors have not been identified.

In summary, we found a pronounced induction of GDF-15/MIC-1 expression by oxLDL, C_6-ceramide, TNFα and H_2O_2 in cultured human MΦ. In human arteriosclerotic carotid arteries, GDF-15/MIC-1 IR was exclusively localized in MΦ and colocalized with oxLDL-IR, MnSOD-IR, AIF-IR, caspase-3-IR (CPP32), PARP-IR, c-Jun/AP-1-IR, and *p53*-IR. GDF-15/MIC-1 is supposed to contribute to modulation of apoptosis and inflammatory processes of activated MΦ.

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