Biochimica et Biophysica Acta xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation

Shumei Liu [a,1], Pengyuan Yang [a,b,1], Hui Kang [a], Ling Lu [a], Yuefan Zhang [a], Jingwei Pan [c], Yao-Cheng Rui [a,*]

[a] Department of Pharmacology & School of Pharmacy, Second Military Medical University, Shanghai 200433, PR, China
[b] Institute of Immunology & National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai 200433, PR, China
[c] Department of Cardiology, Shanghai Jiaotong University Affiliated Sixth Peoples Hospital

ARTICLE INFO

Article history:
Received 6 July 2009
Received in revised form 25 September 2009
Accepted 28 September 2009
Available online xxxx

Keywords:
N-Myc downstream-regulated protein 2
Oxidized low density lipoprotein
Macrophage
Smooth muscle cell
Platelet-derived growth factor
Vascular endothelial growth factor
MAPK signal

ABSTRACT

During atherogenesis, macrophage foam cells produce prodigious growth factors, cytokines, and chemokines, which play the central roles in inflammatory process in atherosclerotic plaque formation. In the present study, we identified a new protein marker, N-Myc downstream-regulated protein 2 (NDRG2), which is significantly up-regulated in oxidized low density lipoprotein (oxLDL) treated macrophages and in human atherosclerotic plaques. Over-expression and siRNA knockdown studies showed that NDRG2 is a negative regulator of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) productions in macrophages. Furthermore, we investigated the effects of NDRG2 on MAPK signal activation. Our results showed ERK1/2 activation, but not P38 or JNK1/2 activation, is responsible for regulation of NDRG2 on VEGF and PDGF productions. Consistent with the PDGF levels, the vascular smooth muscle cell (VSMC) proliferation was also regulated by the conditional medium of the oxLDL treated macrophages with NDRG2 knockdown or over-expression. Neutralizing anti-PDGF antibody can significantly inhibit the enhanced VSMC proliferation by macrophage medium with NDRG2 knockdown. Our present results demonstrate that NDRG2 participates in oxLDL-induced macrophage activation and modulates ERK1/2-dependent PDGF and VEGF production, which has potential application in atherogenesis.

1. Introduction

Atherosclerosis, a narrowing of blood vessels caused by the deposition of inflammatory plaques in the vessel wall, is the leading cause of death in industrialized society. Oxidized low density lipoprotein (oxLDL) is thought to render the lipoprotein atherogenic. OxLDL is more readily taken up by macrophages via scavenger receptors. Lipid-laden macrophages (known as foam cells) are the major component of atherosclerotic lesions [1–3]. Macrophage foam cells also play important roles in regulating vascular smooth muscle cell (VSMC) migration and proliferation. During both early and late atherosclerotic lesions, macrophage-derived foam cells are prodigious secretary cells that can produce growth factors [2,4]. Among these growth factors, platelet-derived growth factor (PDGF) is a potent mitogen and chemo attractant for VSMCs, and vascular endothelial growth factor (vegf) is one of the most appreciated genes involved in angiogenesis [5–8]. Recent studies showed mitogen-activated protein kinase (MAPK) signaling plays central roles in growth factor productions in foam cells induced by oxLDL [9,10]. MAPK is serine-threonine kinase functioning as mediators of cellular responses to a variety of extracellular stimuli [11,12], which includes the extracellular signal-regulated kinases (ERK1/2, also termed p42/44 MAPK), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), BMK1 (also termed ERK5), and p38 MAPK (also termed CSBP). It is also reported that ERK1/2 and JNK/SAPK activation is responsible for PDGF expression [13].

Recently, our laboratory used several proteomic and gene expression array methods to explore new protein markers in oxLDL treated macrophages [14,15]. In this study, we found a new protein marker in foam cells—N-Myc downstream-regulated protein 2 (NDRG2), a member of NDRG1–4 family. NDRG2 has been reported the implication in stress responses and the regulation of cell proliferation and differentiation. Although Ndrg2 is in fact N-Myc-independent [16,17], it also appears to inhibit cell proliferation [18] and promote differentiation [19]. NDRG2 was reported to be up-regulated in the patients' brains with Alzheimer disease [20] and could induce the differentiation of dendritic cells [21]. Meanwhile, Takahashi and co-workers reported that NDRG2 might facilitate

Abbreviations: NDRG2, N-Myc downstream-regulated protein 2; VSMC, vascular smooth muscle cell; oxLDL, oxidized low density lipoprotein; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinases

* Corresponding author. No.325 GuoHe Road, Department of Pharmacology & School of Pharmacy, Second Military Medical University, Shanghai 200433, China. Tel./fax: +86 21 81871273.
E-mail addresses: ruiyaocheng@yahoo.com, ruiyc@smmu.edu.cn (Y.-C. Rui).
[1] Contributed equally to this study.

1388-1981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbalip.2009.09.022

neurite outgrowth of nerve growth factor-differentiated PC12 cells [22], suggesting the involvement of NDRG2 in differentiation. The expression of NDRG2 in human glioblastoma tissues is significantly lower than that in the normal brains [23], which showed NDRG2 as a candidate for tumor suppressor. These findings implicate NDRG2 as being associated with cell growth and differentiation. However, it remains unclear whether NDRG2 participates in macrophage activation and its functions in cardiovascular diseases.

In this study, we initially confirmed the enhanced expression of NDRG2 in oxLDL treated macrophages both in protein and mRNA levels. We constructed plasmid encoding NDRG2 sequence and synthesized siRNA to investigate the role of NDRG2 in oxLDL-induced macrophage activation. We found the significant inhibitory effect of NDRG2 on PDGF and VEGF productions in macrophages. The NDRG2 regulated PDGF production is contributed to the inhibition of VSMC proliferation in conditional medium of macrophages.

2. Materials and methods

2.1. Animals and reagents

Animals were maintained in accordance with guidelines of the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication No. [NIH] FS-23) on Animal Care. C57/6BL mice were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade II, Certificated SCXK Hu2002-0010). SB203580, a p38 inhibitor, PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, SP600125, a c-Jun NH2-terminal kinase (JNK) inhibitor. Neutralizing monoclonal anti-mouse PDGF antibody, mouse TNF-α, IL-6, IL-1β, IL-10, PDGF and VEGF ELISA kit were purchased from R&D Systems (Minneapolis, MN). NDRG2 antibody (E-20), Anti-phospho-ERK mAb, anti-ERK polyclonal antibody, anti-phospho-p38 MAP kinase mAb, anti-phospho-JNK mAb, and their respective horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA).

2.2. OxLDL preparation

LDL ($d = 1.019$ to 1.063 kg/L) was purchased from Sigma Co (ST. Louis). OxLDL was prepared as described previously [4,14,15]. LDL was sterilized by filtration through 0.45-μm Millipore membranes, and stored at 4 °C as described previously. After ehylenediamine tetraacetic acid disodium salt was removed by dialysis, LDL was oxidized by incubating in $CuSO_4$ 10 μmol/L for 16 h at 37 °C, and then dialyzed in phosphate buffered saline (PBS) containing ethylenediamine tetraacetic acid disodium salt 0. 1 mmol/L for 24 h at 4 °C. TBARS is determined colorimetrically by using Malondialdehyde (MDA) as a standard, which showed 0.13 nmol of MDA/mg Protein in starting LDL and 38. 3 nmol of MDA/mg Protein in oxLDL. Endotoxin (LPS) levels in these ODNs were <0.015 endotoxin units/mg ODN, measured by Limulus Amoebocyte Lysate assay.

2.3. Clinical tissues

Segments of atherosclerotic plaque specimens and adjacent aortic wall tissue samples of control normal thoracic aortic tissues without atherosclerotic changes within 5 cm were collected from 11 patients undergoing graft surgery described previously [24]. For humans, the study conforms with the Declaration of Helsinki.

2.4. Plasmids and small interfering RNA fragment

NDRG2 construct [25], tagged with flag and encoded in pcDNA3.1 plasmid, was kindly provided by Prof Yao in XiAn, China. The siRNA specific for murine NDRG2 (siR1: 5′-CCAAACGCCCGGCAATATTtt-3′, siR2: 5′-GCCCGGCAATATTCACCTAtt-3′, and siR3: 5′-CTACATTCTGTCGCGATATTtt-3′) were designed using siRNA fragments template design tool (Ambion). The silence efficiency was determined by real-time PCR. Control nonsense RNA (5′-TTC TCC GAA CGT GTC ACG T-3′), which has no specific target sequence to any genes, was determined to have no effect on silencing of Ndrg2 gene expression. All the siRNA fragments were synthesized in GeneChem Co. in Shanghai, China.

2.5. Cell culture and transiently transfection

The murine macrophage cell line RAW264.7 macrophages were commercially obtained from American Type Culture Collection (Manassas, VA). Female C57BL/6J mice (5–6 weeks old) were used for the preparation of primary mouse macrophages, and thioglycolate-elicited mouse peritoneal macrophages were prepared as described [26]. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. The mouse aortic VSMCs were cultured in Dulbecco's Modified Eagle Medium (PAA) as described previously [27]. Six to eight passage cells were used. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, OR).

2.6. Western blot analysis

Cells were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail and protein concentrations of the extracts were measured by bicinchoninic acid (BCA) assay (Pierce). Forty micrograms of the protein was used either for immunoprecipitation or loaded per lane, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes. Activated p-ERK1/2, p-p38, p-JNK1/2 and p-P65 were detected as described previously. Unactivated total ERK1/2, p38, JNK1/2 and P65 in each sample were also detected to show equal protein loading. Western blot data is quantified by Quantity One software from Bio-Rad Co (Version 4.5.2 Windows and Macintosh).

2.7. Real-time PCR

RNA was separated by TriReagent (Invitrogen, Burlington, ON, USA). The quality of samples was determined by analysis on 1.2% agarose electrophoresis gels. Total RNA was reverse-transcribed to single-strand cDNA using the SYBR ExScript RT-PCR kit (Takara).

The primers used for SYBR Green PCR are listed as follows:

NDRG2: Forward: 5′-CAGGACAAACACCCGAGACT-3′
Reverse: 5′-AGCCATAAGGTGTCTCCACAG-3′
PDGF-A chain: Forward: 5′-CACCAGCAGCGTCAAGTG-3′
Reverse: 5′-TTCCTGACATACTCCACTTTGG-3′

SYBR Green PCR amplifications were performed in a DNA Engine Peltier Thermal cycler coupled with a Chromo 4 Real-Time PCR Detector (Bio Rad, U.S.A.). Each sample was replicated two to three times. All reactions were repeated at least three times independently to ensure the reproducibility of the results.

2.8. ELISA analysis

RAW264.7 macrophages and primary peritoneal macrophages were stimulated with oxLDL for indicated time periods. The concentrations of PDGF in culture supernatants were measured using mouse TNF-α, IL-6, IL-1β, IL-10, PDGF and VEGF ELISA Kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

2.9. Proliferation analysis

VSMC proliferation was detected by a commercial WST-1 Kit (Beyotime Institute of Biotechnology) [28]. Conditional medium was

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022

derived from supernatant of RAW264.7 macrophages. The macrophages were firstly transfected with NDRG2 knockdown siRNA or overexpression plasmid for 24 h, and treated with oxLDL for 12 h, then, cultured with fresh medium. The supernatant was harvested as the conditional medium. To determine the VSMC proliferation, the VSMCs were separated from the upper chamber lates, and then mixed with WST-1 reagent. After standard culture conditions, the absorbance was determined as the proliferation rate using a 96-well plate reader at 450 nm. The NDRG2 overexpressed RAW264.7 cell viability was also monitored by measurement with WST-1 Kit.

2.10. Statistical analysis

Data is shown as means ± S.E. of the mean for separate experiments. Statistical significance was determined by Student's *t* test with a value of $P<0.05$ considered as statistically significant. The numbers of repeated experiments in each group were shown as n in figure legends.

3. Results

3.1. NDRG2 is up-regulated in oxLDL-treated macrophages and atherosclerotic plaques

We firstly confirmed the NDRG2 expression in macrophages. With incubation with oxLDL 10, 20, 40, 80 mg /L separately, we measured the NDRG2 expression levels by Western blotting and real-time PCR. The GAPDH and β-actin levels were detected to show the total amount of protein and mRNA. As shown in Fig. 1A and B, both protein and mRNA levels of NDRG2 were significantly enhanced by oxLDL in a dose-dependent manner. The results confirmed that NDRG2 were up-regulated in macrophages induced by oxLDL.

After oxLDL stimulation, macrophages enter into the intima and form into foam cells, which are the major component of atherosclerotic lesions. Therefore, we determined the NDRG2 levels in atherosclerotic plaque from human aortas. All eleven clinical AS plaque tissues from 11 patients undergoing graft surgery express higher levels of NDRG2 than control normal aorta tissues. This indicates that NDRG2 participates in atherosclerotic lesions (Fig. 1C). Therefore, we concluded that NDRG2 expression was up-regulated in macrophages and atherosclerotic plaques, and the up-regulation of NDRG2 may play roles during atherogenesis.

3.2. NDRG2 antagonizes PDGF and VEGF productions in oxLDL-induced macrophages

It is reported that NDRG2 can inhibit glioblastoma cell proliferation [18] and can promote apoptosis in A549 lung cancer cells [23]. Therefore, we determined the effect of NDRG2 on cell viability on macrophages. As shown in Suppl Fig. 1., with NDRG2 overexpression, macrophage RAW264.7 cells cannot be determined any changes in number of cell viability. To clarify the functions of NDRG2 in

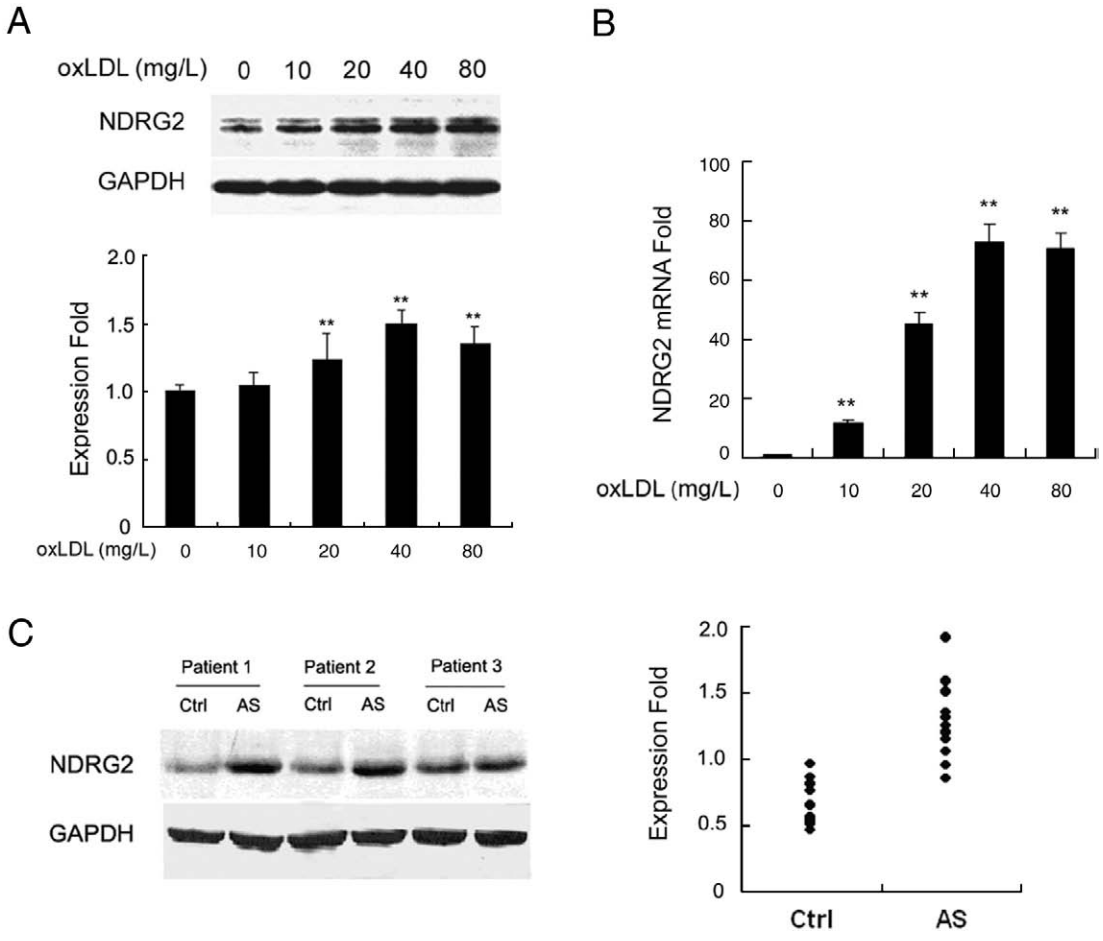

Fig. 1. Expression of NDRG2 in oxLDL-induced macrophages and human atherosclerotic plaques. (A) NDRG2 expression levels were enhanced in macrophage-derived foam cells. RAW264.7 cells were treated with oxLDL (0–80 mg /L) in 24 h, and then NDRG2 expression levels were determined by Western Blotting. Relative expression fold was calculated by the ratio of levels NDRG2 to GAPDH. Data is shown as mean ± SD ($n=4$) of one representative experiment (**$P<0.01$). (B) NDRG2 expression levels were determined by real-time PCR. Relative expression fold at below was normalized to β-actin. Data are shown as mean ± SD (n = 4) of one representative experiment (**$P<0.01$). (C) Expressions of NDRG2 protein levels in human atherosclerotic plaque tissues and adjacent control tissue samples determined by Western blot. Relative expression fold at below was calculated by the ratio of NDRG2 to GAPDH ($n=11$).

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022

macrophage activation, we determined its effects on cytokines TNF-α, IL-6, IL-1β, IL-10, and growth factors PDGF and VEGF productions. Although it seems NDRG2 cannot affect cytokine expressions (Fig. 2C–F), we found the negatively regulation of NDRG2 on PDGF and VEGF productions. The oxLDL-induced PDGF and VEGF productions were significantly inhibited in macrophage cells after transfected transiently with 400 ng of NDRG2 (Fig. 2A–B).

To further confirm the modulation role of NDRG2 on PDGF and VEGF production, we silenced NDRG2 expression using RNA-mediated interference. We confirmed the knockdown of endogenous NDRG2 in RAW264.7 cells using Western blot (Fig. 3A) after transfected with NDRG2 siRNA. The endogenous NDRG2 expression was decreased by siRNA in a dose dependent manner. Then, the PDGF and VEGF productions were measured by ELISA. NDRG2 siRNA transfection resulted in 3-fold increase in PDGF production in macrophages (Fig. 3B). As shown in Fig. 3C, NDRG2 knockdown also significantly increased oxLDL-induced VEGF production in macrophages. We also determined the effect of NDRG2 knockdown on oxLDL-induced growth factor productions in mouse primary macrophages. As shown in Fig. 3D and E, the similar up-regulation of oxLDL-induced PDGF-BB and VEGF productions were detected with NDRG2 knockdown in mouse primary macrophages. These results provided further evidence that NDRG2 negatively regulates PDGF and VEGF productions in oxLDL-induced macrophages.

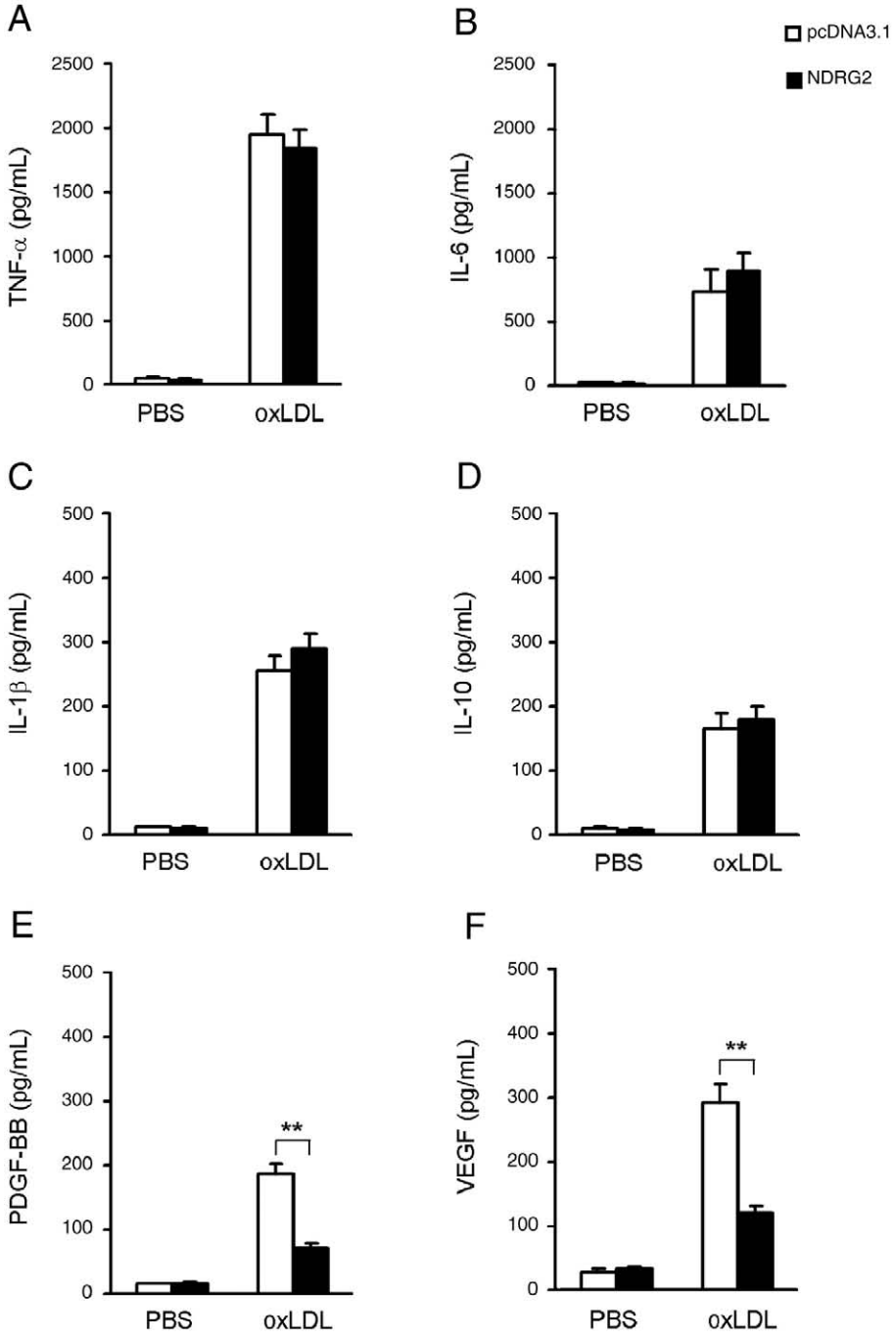

Fig. 2. Effect of NDRG2 on cytokine TNF-α (A), IL-6 (B), IL-1β (C), IL-10 (D), and growth factor PDGF (E) and VEGF (F) productions induced by oxLDL in macrophages. 2×10^5 RAW264.7 cells were transfected transiently with 400 ng of NDRG2 or pcDNA3.1 by LipofectAMINE 2000 followed by 24 h incubation. Then the cells were stimulated by oxLDL 80 mg /L. Cell culture supernatant was collected and was detected by ELISA kits. All data represent means ± SD ($n=3$) $^{**}P<0.01$.

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022

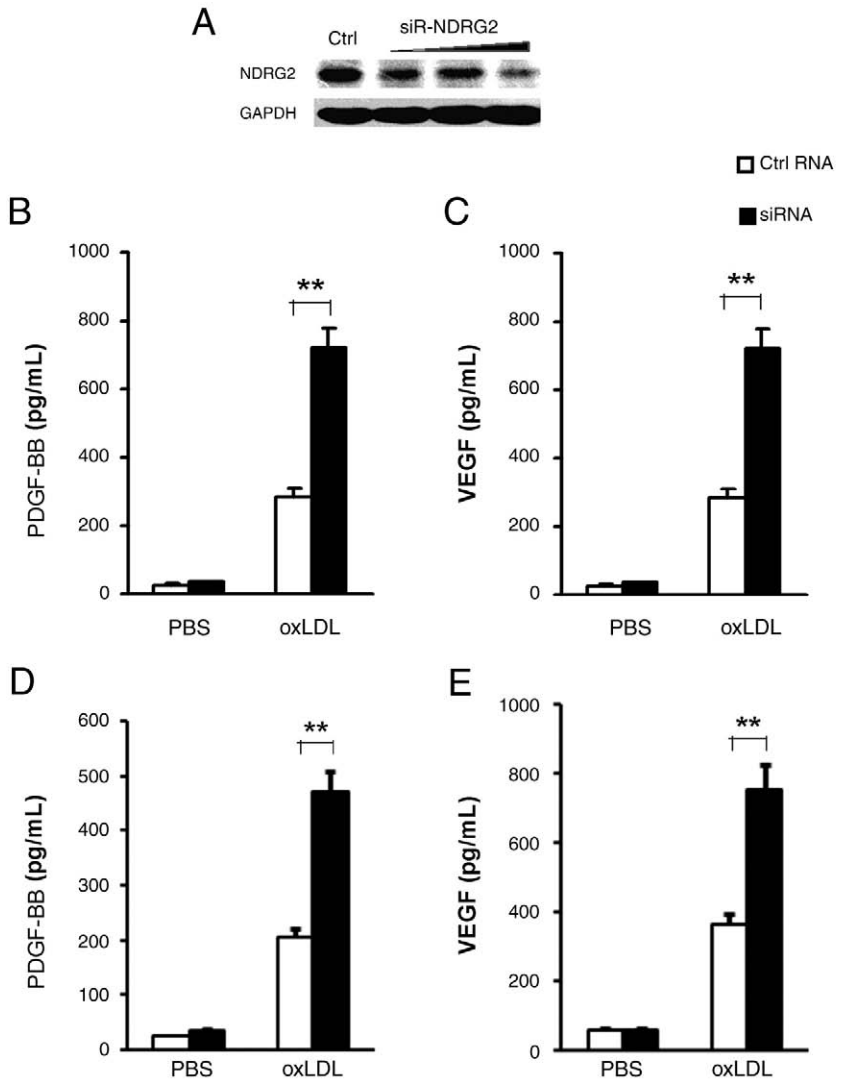

Fig. 3. Knocking down of NDRG2 enhances PDGF and VEGF productions in macrophages. 2×10^5 RAW264.7 cells were transfected with 100 nmol, 200 nmol, 400 nmol per ml of NDRG2 siRNA (5′-CTACATTC TGTCGCGATAT) or control siRNA by LipofectAMINE 2000 followed by 36 h incubation. (A) Cell lysates were analyzed by Western blotting with anti-NDRG2 and anti-GAPDH antibodies. Relative expression fold was calculated by the ratio of NDRG2 to GAPDH. After transfection of NDRG2 siRNA (400 nmol) followed by 36 h incubation, RAW264.7 cells were treated with oxLDL 80 mg/L or control DMEM. Then, PDGF-BB (B) and VEGF (C) productions were determined by ELISA. In cultured mouse primary macrophages, effects of NDRG2 on oxLDL-induced PDGF-BB (D) and VEGF (E) productions were also determined. All data represent means ± SD ($n=3$) $^{**}P<0.01$.

3.3. NDRG2 inhibits p42/44 ERK pathway activation in macrophages

It is well known that activation of MAPK is necessary for oxLDL-induced growth factors production [9]. We investigated the effects of NDRG2 overexpression on oxLDL-induced MAPK phosphorylation (Fig. 4A–D). Stimulation with oxLDL resulted in phosphorylation of ERK1/2, JNK1/2, and P38 kinases in RAW264.7 cells. Compared with mock transfection, NDRG2 transfection significantly inhibited oxLDL-induced ERK1/2 phosphorylation. However, neither oxLDL-induced JNK1/2 phosphorylation nor P38 kinas phosphorylation was remarkably affected by overexpression of NDRG2. These results demonstrated that NDRG2 selectively controls oxLDL-induced ERK1/2 activation in macrophages.

3.4. Increased ERK1/2 activation is responsible for the regulatory effect of NDRG2 on PDGF and VEGF productions

To confirm the effect of NDRG2 on MAPK/ERK activation, we used NDRG2 siRNA to silence endogenous NDRG2 expression. Then, RAW264.7 cells were treated with PD98059, an ERK1/2 pathway inhibitor after transfected with NDRG2 siRNA. As shown in Fig 5, PD98059 treatment could inhibit PDGF/VEGF productions in NDRG2 knockdown macrophages, indicating ERK1/2 activation is necessary for NDRG2 to regulate PDGF and VEGF productions. In contrast, neither SB203580, a P38 inhibitor, nor SP600125, a JNK1/2 inhibitor, can affect the NDRG2 knockdown macrophages-derived high PDGF and VEGF productions. Therefore, our results suggest that ERK1/2 activation, but not P38 or JNK1/2 activation, is responsible for high PDGF and VEGF productions in NDRG2 knockdown macrophages.

3.5. Regulation of macrophage-derived PDGF contributes to the effects on VSMC proliferation

PDGF was originally identified in platelets and in serum as a mitogen for SMC in culture. Therefore, the regulation of PDGF production by NDRG2 led us the intention to testify whether NDRG2 overexpression or knockdown in macrophage foam cells can modulate VSMC proliferation. We cultured VSMCs by using the conditional medium of oxLDL treated macrophages with NDRG2 plasmid or siRNA transfection. As shown in Fig 6A, the enhanced VSMC proliferation

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022

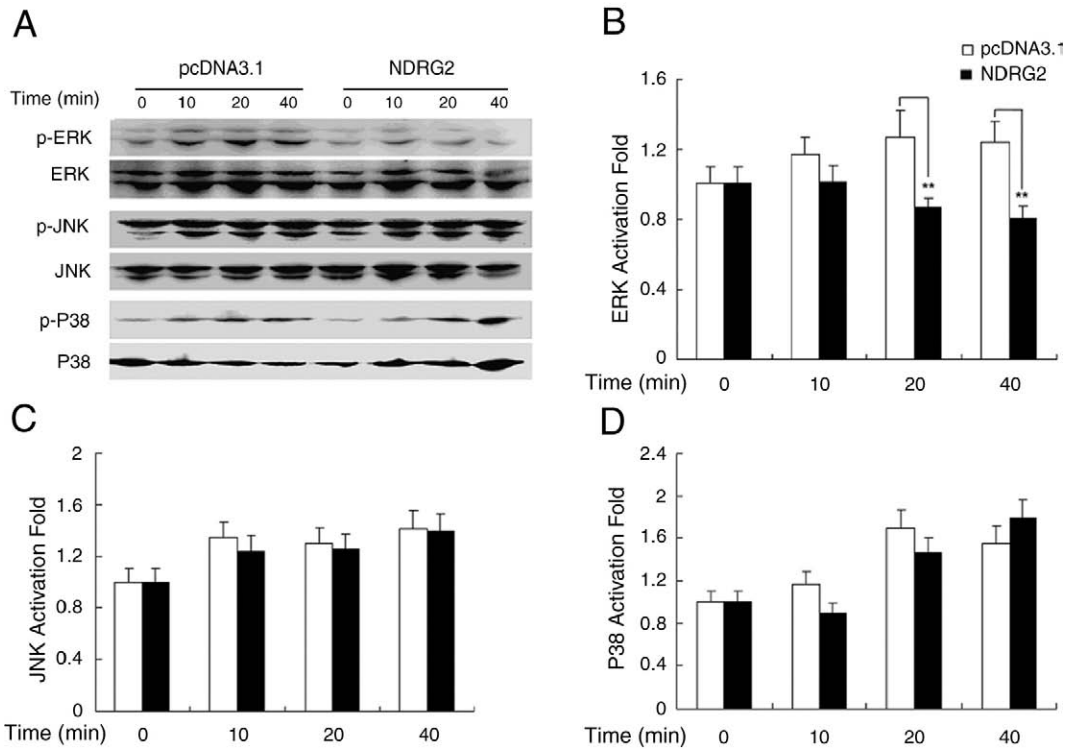

Fig. 4. NDRG2 inhibits oxLDL-induced p42/44 ERK activation in macrophages. 1×10^6 RAW264.7 cells were plated in 6-well plates and transfected with 1 μg of pCDNA3.1 or NDRG2. After stimulation with 80 mg oxLDL for 10, 20, or 40 min, the cells were lysed and levels of ERK, JNK, and p38 were detected by Western blotting (A). Levels of the phosphorylated proteins at the time points after oxLDL stimulation were detected by Western blot and quantitated by measuring band intensities. The values of the activation fold were normalized to the total ERK (B), JNK (C), or p38 values (D) respectively. Relative phosphorylation levels of the proteins in the control cells were set as 1 $^{**}P<0.01$.

induced by oxLDL treated macrophage-derived conditional medium was significantly decreased in NDRG2 overexpression macrophage medium treatment groups. Consistently, the medium from NDRG2 knockdown macrophages increased the VSMC proliferation significantly. (Fig 6B)

To confirm the PDGF contribution. we used the mouse PDGF antibody to neutralize PDGF bioactivity. As shown in Fig 6B, after PDGF bioactivity was neutralized, the enhanced VSMC proliferation, induced by NDRG2 knockdown macrophage medium, was greatly decreased, while the control isotype antibody has no effect. Our results demonstrated that the regulation of macrophage-derived PDGF by NDRG2 contributes to the effects on VSMC proliferation rate.

4. Discussion

Recent studies showed that the lipid-laden foam cell was considered to be “an elusive target for therapeutic intervention” [29]. Several findings since then have indicated new approaches for altering the inflammatory responses during lipid accumulation in macrophages [1,30]. In the present study, we found a new up-regulated gene, NDRG2, in macrophages induced by oxLDL. The function study showed that NDRG2 plays important roles in regulating growth factor PDGF and VEGF productions in macrophages.

Although NDRG2 has also been reported to play roles in other functions, such as insulin action [17], aldosterone-mediated epithelial sodium channel (ENaC) function [19], dendritic cell (DC) differentiation [20,21] and oncogenesis [23,31], there is no report about the expression or function in macrophages. To confirm the expression of NDRG2, we investigate its expression both in protein and mRNA levels. The NDRG2 level was up-regulated in macrophages by oxLDL in a dose dependent manner. Since macrophage foam cell has been considered to be the main source of the cytokines and growth factors in atherogenesis, we determined the role of NDRG2 on the cytokines TNF-α, IL-6, IL-1β, IL-10, and growth factors PDGF and VEGF productions in macrophages. Although, we cannot find the effect on several cytokines, we found that NDRG2 can antagonize PDGF and VEGF productions. The up-regulation of NDRG2 in oxLDL treated macrophages may play the roles in negative feedback mechanism in plaque proliferation during atherogenesis.

The PDGF family consists of disulfide-bonded dimeric isoforms [6,32,33]: PDGF-AA, -AB, -BB, -CC and -DD. The currently known PDGF genes and polypeptides belong to a family of structurally and functionally related growth factors including the vascular endothelial growth factors (VEGFs). PDGF/VEGF growth factors are conserved throughout the animal kingdom and form part of a large superfamily of proteins containing cystine knots. In the present study, we determined the PDGF-BB subtype isoforms in oxLDL-induced macrophages and the effects of NDRG2.

Interestingly, the antagonistic action of NDRG2 on PDGF and VEGF productions is dependent on the inhibitory effect on ERK1/2 activation, but not P38 or JNK1/2 activation. ERK activation is selective inhibited in NDRG2 over-expressed macrophages. Our results provided the evidence that NDRG2 selectively controls oxLDL-induced ERK1/2 activation in macrophages. ERK1/2 activation, but not P38 or JNK1/2 activation, is responsible for NDRG2 siRNA-induced PDGF/VEGF production.

The mechanism of the selective effects of NDRG2 on ERK1/2 activation remains unclear. NDRG2 inactivation has recently been found to have an important role in some tumorigenesis. Bioinformatics analysis of NDRG2 revealed several binding sequences for different transcription factors [16], which are mostly involved in growth regulation and early differentiation of cells. Some of those factors, such as Wilms tumor gene 1 (WT1) protein, hypoxia-induce factor-1a (HIF-1a), and glucocorticoids, up-regulate NDRG2 expression. However, the function of NDRG2 in intracellular signal transduction pathways remains poorly defined. The potential roles

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022

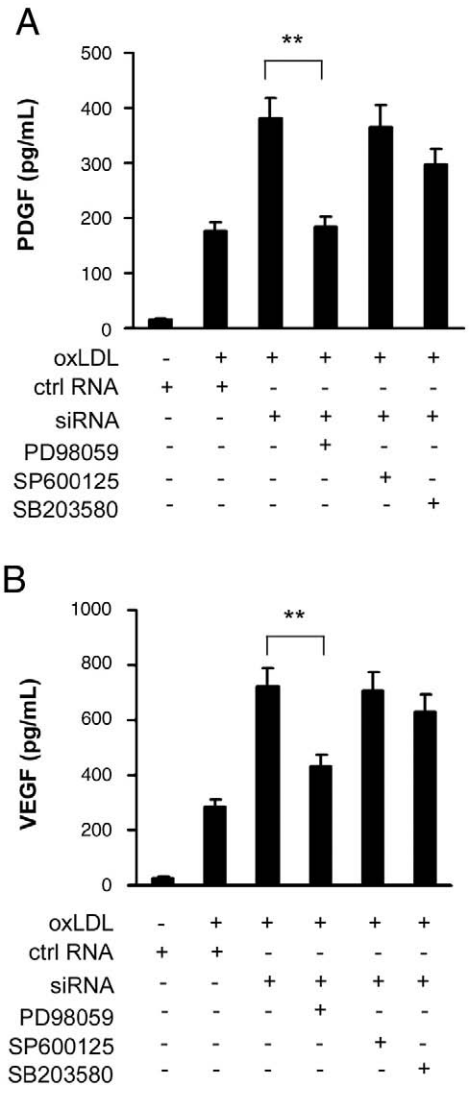

Fig. 5. P42/44 ERK activation is responsible for antagonism of NDRG2 on growth factor productions. After transfection of NDRG2 siRNA or control RNA followed by 24 h incubation, cells were treated with 10 μmol/ml PD98059, 10 μmol/ml SB203580 or 50 μmol /ml SP600125 for 30 min, then treated with 80 mg /L oxLDL for 24 h. PDGF (A) and VEGF (B) productions were determined by ELISA. All data represent means ± SD ($n = 3$) $^{**}P<0.01$.

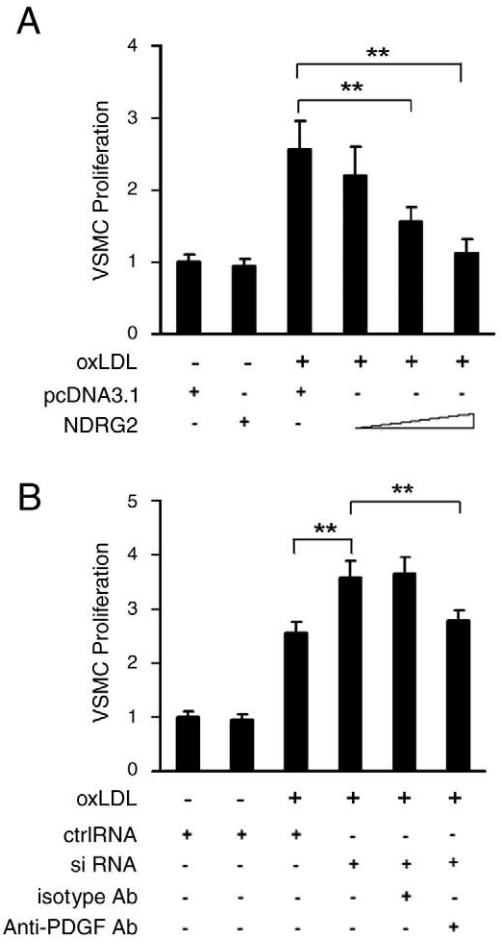

Fig. 6. VSMC proliferation rate was regulated by conditional medium of macrophages with NDRG2 over-expression or knockdown through modulating PDGF production. The RAW264.7 macrophages were transfected with NDRG2 plasmid (A) or siRNA (B) followed by 24 h incubation, treated with oxLDL for 12 h, and then cultured with fresh medium. The supernatant was harvested as the conditional medium. To determine the VSMC proliferation, the VSMCs were separated from the upper chamber layers and mixed with WST-1 reagent. After standard culture conditions, the absorbance was determined as the proliferation rate using a 96-well plate reader at 450 nm. In some experiments, before incubation with VSMC, RAW264.7 cells were pretreated with neutralizing monoclonal anti-mouse PDGF antibody (5 μg/ml) for 1 h, then the conditional medium were added to VSMC (B).The isotype antibody (5 μg/ml) was used as the control. All data represent means ± SD ($n = 3$). $^{*}P<0.05$, $^{**}P<0.01$.

of NDRG2 may associate with c-raf or ras, which is critical in ERK activation. However, we have pulled down the NDRG2 interacting proteins, and the Western Blot results did not reveal c-raf or ras.

The PDGF [6,7,34,35] initiates signal transduction pathways associated closely with atherosclerosis. Rabbits immunized against PDGF demonstrate significantly smaller regions of cholesterol-induced atherosclerotic lesions [36]. In the pathogenesis of atherosclerosis, PDGF is thought to mediate the proliferation of vascular smooth muscle cells in injured arteries. Since NDRG2 can regulate PDGF production, we also determined the VSMC proliferation rate induced by NDRG2 overexpression or silence macrophages. Consistent with the PDGF levels, the vascular smooth muscle cell (VSMC) proliferation was also regulated by the conditional medium of the oxLDL treated macrophages with NDRG2 knockdown or overexpression. PDGF neutralizing antibody can significantly inhibit the enhanced VSMC proliferation by macrophage medium with NDRG2 knockdown. Given these results, we propose that regulation of macrophage-derived PDGF by NDRG2 contributes to the effects on VSMC proliferation rate. Although it is reported that NDRG2 can regulate several cancer cell proliferation, our results suggest a growth factor regulation mechanism of NDRG2 in macrophage activation, which may give new insight in the NDRG2 function.

In conclusion, we demonstrated that NDRG2 participates in oxLDL-induced macrophage activation and atherosclerotic lesion. NDRG2 also plays important roles in modulating PDGF and VEGF productions in macrophages via inhibition of P42/44 ERK1/2 activation. The PDGF regulated by NDRG2 contributes to the effects of macrophage on VSMC proliferation.

Conflict of interest statement
None declared.

Acknowledgments

We would like to thank Dr. Jianxin Sun and Dr. Xinsheng Chen for critical reading of the manuscript, and Dr. Li-Bo Yao for kindly providing plasmids for reconstruction of NDRG2 expression plasmid.

This study is supported by National Basic Research Program of China (973 Program, No. 2009CB521900), Shanghai Key Natural Science Fund (08431900300), and National Natural Science Funds of China (No. 30572189, No. 30672455).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbalip.2009.09.022.

References

[1] M.A. Cascieri, The potential for novel anti-inflammatory therapies for coronary artery disease, Nat. Rev. Drug Discov. 1 (2002) 122.
[2] P. Shashkin, B. Dragulev, K. Ley, Macrophage differentiation to foam cells, Curr. Pharm. Des. 11 (2005) 3061.
[3] C.K. Glass, J.L. Witztum, Atherosclerosis. the road ahead, Cell 104 (2001) 503.
[4] P.Y. Yang, Y.C. Rui, Intercellular adhesion molecule-1 and vascular endothelial growth factor expression kinetics in macrophage-derived foam cells, Life Sci. 74 (2003) 471.
[5] H. Bujo, Y. Saito, Modulation of smooth muscle cell migration by members of the low-density lipoprotein receptor family, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1246.
[6] A.D. Hughes, G.F. Clunn, J. Refson, C. moliou-Mason, Platelet-derived growth factor (PDGF): actions and mechanisms in vascular smooth muscle, Gen. Pharmacol. 27 (1996) 1079.
[7] A. Simm, M. Nestler, V. Hoppe, Mitogenic effect of PDGF-AA on cardiac fibroblasts, Basic Res.Cardiol. 93 (Suppl 3) (1998) 40.
[8] E. Song, N. Ouyang, M. Horbelt, B. Antus, M. Wang, M.S. Exton, Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts, Cell Immunol. 204 (2000) 19.
[9] Q. Jing, S.M. Xin, W.B. Zhang, P. Wang, Y.W. Qin, G. Pei, Lysophosphatidylcholine activates p38 and p42/44 mitogen-activated protein kinases in monocytic THP-1 cells, but only p38 activation is involved in its stimulated chemotaxis, Circ. Res. 87 (2000) 52.
[10] J.W. Zmijewski, A. Landar, N. Watanabe, D.A. Dickinson, N. Noguchi, V.M. rley-Usmar, Cell signalling by oxidized lipids and the role of reactive oxygen species in the endothelium, Biochem. Soc. Trans. 33 (2005) 1385.
[11] A.G. Turjanski, J.P. Vaque, J.S. Gutkind, MAP kinases and the control of nuclear events, Oncogene 26 (2007) 3240.
[12] B.D. Cuevas, A.N. Abell, G.L. Johnson, Role of mitogen-activated protein kinase kinase kinases in signal integration, Oncogene 26 (2007) 3159.
[13] P.K. Mehta, K.K. Griendling, Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system, Am. J. Physiol. Cell Physiol. 292 (2007) C82.
[14] P.Y. Yang, Y.C. Rui, P.Y. Yang, Y.L. Yu, Proteomic analysis of foam cells, Methods Mol. Biol. 357 (2007) 297.
[15] Y.L. Yu, Z.Y. Huang, P.Y. Yang, Y.C. Rui, P.Y. Yang, Proteomic studies of macrophage-derived foam cell from human U937 cell line using two-dimensional gel electrophoresis and tandem mass spectrometry, J. Cardiovasc. Pharmacol. 42 (2003) 782.
[16] J. Zhang, F. Li, X. Liu, L. Shen, J. Liu, J. Su, W. Zhang, Y. Deng, L. Wang, N. Liu, W. Han, J. Zhang, S. Ji, A. Yang, H. Han, L. Yao, The repression of human differentiation-related gene NDRG2 expression by Myc via Miz-1-dependent interaction with the NDRG2 core promoter, J. Biol. Chem. 281 (2006) 39159.
[17] X. Qu, Y. Zhai, H. Wei, C. Zhang, G. Xing, Y. Yu, F. He, Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family, Mol. Cell. Biochem. 229 (2002) 35.
[18] Y. Deng, L. Yao, L. Chau, S.S. Ng, Y. Peng, X. Liu, W.S. Au, J. Wang, F. Li, S. Ji, H. Han, X. Nie, Q. Li, H.F. Kung, S.Y. Leung, M.C. Lin, N-Myc downstream-regulated gene 2 (NDRG2) inhibits glioblastoma cell proliferation, Int. J. Cancer 106 (2003) 342.
[19] M.O. Wielputz, I.H. Lee, A. Dinudom, S. Boulkroun, N. Farman, D.I. Cook, C. Korbmacher, R. Rauh, NDRG2 stimulates ammiloride-sensitive Na+ currents in *Xenopus laevis* oocytes and Fisher rat thyroid cells, J. Biol. Chem. 282 (38) (2007) 28264.
[20] C. Mitchelmore, S. Buchmann-Moller, L. Rask, M.J. West, J.C. Troncoso, N.A. Jensen, NDRG2: a novel Alzheimer's disease associated protein, Neurobiol. Dis. 16 (2004) 48.
[21] S.C. Choi, K.D. Kim, J.T. Kim, J.W. Kim, D.Y. Yoon, Y.K. Choe, Y.S. Chang, S.G. Paik, J.S. Lim, Expression and regulation of NDRG2 (N-myc downstream regulated gene 2) during the differentiation of dendritic cells, FEBS Lett. 553 (2003) 413.
[22] K. Takahashi, M. Yamada, H. Ohata, K. Honda, M. Yamada, Ndrg2 promotes neurite outgrowth of NGF-differentiated PC12 cells, Neurosci. Lett. 388 (2005) 157.
[23] L. Wang, N. Liu, L. Yao, F. Li, J. Zhang, Y. Deng, J. Liu, S. Ji, A. Yang, H. Han, Y. Zhang, J. Zhang, W. Han, X. Liu, NDRG2 is a new HIF-1 target gene necessary for hypoxia-induced apoptosis in A549 cells, Cell. Physiol. Biochem. 21 (2008) 239–250.
[24] J.W. Pan, M. Wei, P.Y. Yang, X. Zheng, J.B. Li, Z.G. Lu, X.X. Zhao, H. Wu, H. Kang, Y.C. Rui, Regulation of Nogo-B expression in the lesion of aortic aneurysms, Clin. Exp. Pharmacol. Physiol. 34 (2007) 856–860.
[25] J. Zhang, J. Liu, X. Li, F. Li, L. Wang, J. Zhang, X. Liu, L. Shen, N. Liu, Y. Deng, A. Yang, H. Han, M. Zhao, L. Yao, The physical and functional interaction of NDRG2 with MSP58 in cells, Biochem. Biophys. Res. Commun. 352 (2007) 6.
[26] M. Karaghiosoff, R. Steinborn, P. Kovarik, G. Kriegshauser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, et al., Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock, Nat. Immunol. 4 (2003) 471–477.
[27] T. Fujino, K. Yuhki, T. Yamada, A. Hara, O. Takahata, Y. Okada, C.Y. Xiao, H. Ma, H. Karibe, Y. Iwashima, J. Fukuzawa, N. Hasebe, K. Kikuchi, S. Narumiya, F. Ushikubi, Effects of the prostanoids on the proliferation or hypertrophy of cultured murine aortic smooth muscle cells, Br. J. Pharmacol. 136 (2002) 530.
[28] K.I. Kim, H.J. Cho, J.Y. Hahn, T.Y. Kim, K.W. Park, B.K. Koo, C.S. Shin, C.H. Kim, B.H. Oh, M.M. Lee, Y.B. Park, H.S. Kim, Beta-catenin overexpression augments angiogenesis and skeletal muscle regeneration through dual mechanism of vascular endothelial growth factor-mediated endothelial cell proliferation and progenitor cell mobilization, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 91.
[29] A.C. Li, C.K. Glass, The macrophage foam cell as a target for therapeutic intervention, Nat. Med. 8 (2002) 1235.
[30] J.L. Ma, P.Y. Yang, Y.C. Rui, L. Lu, H. Kang, J. Zhang, Hemin modulates cytokine expressions in macrophage-derived foam cells via heme oxygenase-1 induction, J. Pharmacol. Sci. 103 (2007) 261.
[31] Y. Qiu, Y.C. Rui, T.J. Li, L. Zhang, P.Y. Yao, Inhibitory effect of extracts of *Ginkgo biloba* leaves on VEGF-induced hyperpermeability of bovine coronary endothelial cells in vitro, Acta Pharmacol. Sin. 25 (2004) 1306.
[32] C.H. Heldin, B. Westermark, Mechanism of action and in vivo role of platelet-derived growth factor, Physiol. Rev. 79 (1999) 1283.
[33] L. Fredriksson, H. Li, U. Eriksson, The PDGF family: four gene products form five dimeric isoforms, Cytokine Growth Factor Rev. 15 (2004) 197.
[34] H. Bostrom, A. Gritli-Linde, C. Betsholtz, PDGF-A/PDGF alpha-receptor signaling is required for lung growth and the formation of alveoli but not for early lung branching morphogenesis, Dev. Dyn. 223 (2002) 155.
[35] F.S. Santiago, L.M. Khachigian, Ets-1 stimulates platelet-derived growth factor A-chain gene transcription and vascular smooth muscle cell growth via cooperative interactions with Sp1, Circ. Res. 95 (2004) 479.
[36] D.J. Lamb, T.Y. Avades, G.A. Ferns, Endogenous neutralizing antibodies against platelet-derived growth factor-aa inhibit atherogenesis in the cholesterol-fed rabbit, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 997.

Please cite this article as: S. Liu, et al., NDRG2 induced by oxidized LDL in macrophages antagonizes growth factor productions via selectively inhibiting ERK activation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbalip.2009.09.022