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Angiopoeitin-2 modulates Survivin expression in OxLDL-induced endothelial cell apoptosis

Rongsong Li [a], David Mittelstein [a], Karen Fang [a], Tyler Beebe [a], Katherine Quigley [a], Judith Berliner [a,b], Tzung K. Hsiai [a,*]

[a] Biomedical Engineering and Cardiovascular Medicine, University of Southern California, Los Angeles, CA, United States
[b] Department of Pathology and Medicine, University of California, Los Angeles, CA, United States

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ABSTRACT

Angiopoeitin-2 (Ang-2) antagonizes Angiopeitin-1 (Ang-1)-mediated Tie-2 signaling. Ang-1 is reported to up-regulate anti-apoptotic Survivin expression. Here, we investigated the interplay between Ang-2 and Survivin in response to oxidized low density lipoprotein (OxLDL)-induced apoptosis. We demonstrate that treatment of human aortic endothelial cells (HAEC) with 100 μg/ml of OxLDL down-regulated Ang-2 expression as early as 4 h after treatment and persisted up to 24 h ($p < 0.05$, $n = 3$), but did not down-regulate Survivin until the 24 h point. Further, treatment of HAEC with recombinant Ang-2 up-regulated Survivin expression (at Ang-2 $\geqslant$200 ng/ml, $p < 0.05$, $n = 3$) and attenuated the OxLDL-mediated down-regulation of Survivin ($p < 0.05$, $n = 3$). Knockdown of Ang-2 further down-regulated Survivin expression, whereas over-expression of Survivin attenuated OxLDL-induced HAEC apoptosis ($p < 0.05$, $n = 3$). Hence, Ang-2 mediated Survivin expression in response to OxLDL-induced endothelial apoptosis.

1. Introduction

Apoptosis of vascular cells is intimately related to atherosclerosis. Survivin, a Wnt/β-catenin target protein, is a member of the inhibitors of apoptosis protein (IAP) family, and plays an important role in cytokinesis and tumorigenesis [1,2]. While IAP protein blocks apoptosis via inhibition of effector caspases, oxidized low density lipoprotein (OxLDL) differentially regulates the members of IAP family [3,4]. OxLDL has been reported to decrease the expression of cellular inhibitor of apoptosis protein 1 (cIAP-1) in endothelial cells [5], and prolonged OxLDL treatment down-regulated Survivin expression in macrophages [6]. In advanced atherosclerotic lesions, Survivin expression is absent despite an increase in both x-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein 2 (cIAP2) [6]. Whether OxLDL induces apoptosis via down-regulation of Survivin expression in vascular endothelial cells remains unknown.

Angiopoeitin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are antagonistic ligands that bind to Tie-2 receptors [7,8,9]. Ang-1 is constitutively released from pericytes and smooth muscle cells, promoting endothelial cell survival via Akt/Survivin signaling pathway [10], whereas Ang-2 stored in the endothelial Weibel-Palade bodies is rapidly released upon inflammatory responses, disrupting protective Tie-2 signaling [10,11]. However, the role of Ang-2 on Survivin expression has not been elucidated.

In this study, we examined the interplay between Ang-2 and Survivin in response to OxLDL in human aortic endothelial cells (HAECs). We demonstrate that OxLDL treatment downregulated the expression of Ang-2 expression in HAEC as early as 4 h post treatment, whereas Survivin was not down-regulated until 24 h. Ang-2 modulated Survivin expression and over-expression of survivin attenuated OxLDL induced apoptosis. Our findings indicate that Ang-2 mediates Survivin expression in OxLDL-induced apoptosis in HAEC.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) were cultured with endothelial cell growth media (Cell Application). The cells were utilized between passages 5 and 11. OxLDL was prepared as previously described [12]. HAEC were incubated with or without specific concentration of OxLDL in DMEM (Invitrogen Inc.)/1% FBS (Phenix Research) for specified intervals.

2.2. Apoptotic assay by FACS

The apoptosis of HAEC was examined by FACS analysis with PE-Annexin-V (BD Biosciences). HAEC were treated with or without

* Corresponding author. Address: Department of Biomedical Engineering and Division of Cardiovascular Medicine, University of Southern California, Los Angeles, CA 90089, United States. Fax: +1 213 740 0343.
E-mail address: hsiai@usc.edu (T.K. Hsiai).

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OxLDL for 24 h. The cells were then trypsinized and collected. After incubation with PE-Annexin-V, cells were re-suspended in PBS/2% paraformaldehyde, and analyzed with LSR II flow cytometer (BD Biosciences). The percentage of PE-positive cells was quantified as the degree of apoptosis. In the apoptotic assay with Survivin over-expression, percentages of PE-positive cells in GFP positive cells were quantified.

2.3. Caspase-3 activity assay

HAEC were treated with or without OxLDL for 24 h or with 10 μM of Camptothecin (CPT, positive control) for 4 h. The cells were then lysed with passive lysis buffer (PLB, Promega). After centrifugation, the clear lysate was used for Caspase-3 activity assay (Caspase-3 colorimetric assay kit: Genscript) performed according to the manufacturer instructions. The protein concentration of the lysate was measured and the relative Caspase-3 activities were normalized to protein concentration.

2.4. Western blot

HAEC were grown to confluence and treated with or without OxLDL for 6 h for Ang-2 expression (Anti-Angiopoietin-2 and Anti-β-Tubulin: Millipore) or for 24 h for Survivin expression (Ant-Survivin: Cell Signaling) in DMEM/1% FBS. Cell lysate preparation and Western blot were performed as previously described [12].

2.5. Quantitative RT-PCR

The expression of Survivin and Ang-2 mRNA were quantified (qRT-PCR: Applied Biological Materials Inc.). Total RNA was isolated (Bio-Rad kit), and potential genomic DNA contamination was removed with on-column DNase I digestion (R&D System). Total RNA (0.5–1 μg) was reverse transcribed (Bio-Rad's iScript cDNA synthesis kit), and qRT-PCR was performed as previously described [13]. The following primers were used for qRT-PCR: for Survivin: forward: 5′-CCTGGCAGCCCTTTCTCAAGGACCA-3′, reverse: 5′-CCAG CCTTCCAGCTCCTTGAAGCAG-3′; for Ang-2: forward: 5′-GACCACG AGACTTGAACTTCAG-3′, reverse: 5′-GGATGATGTGCTTGTCTTCCATA G-3′; for GAPDH: forward 5'-CCTCAAGACATCAGCAATG CCTC CT-3', reverse 5'-GGTCATGAGTCCTTCCACGATACCAA-3'. The differences in C_T values versus control were used to determine the relative expression of genes of interest normalized to GAPDH.

2.6. siRNA Transfection

siRNA transfection was performed with Lipofectamine RNAiMax (Invitrogen). HAEC were plated in six well plates without antibiotics on the day prior to transfection. The cells were transfected with 50 nM Ang-2 siRNA (Qiagen). Transfection media were changed to normal growth media after 5 h of transfection. Cells were used for confirmation of gene knockdown or function assay at 48 h after transfection.

2.7. Survivin over-expression

Survivin over-expression was established with transduction of Adenovirus-GFP-Survivin (Adv-Survivin). Adenovirus-GFP (Adv-GFP) was used as control. HAEC were infected with Adv-Survivin or Adv-GFP at MOI (Multiple of Infection) 100 for 24 h. The cells were then used for expression measurement or treatment with or without OxLDL for apoptosis assay. Survivin recombinant adenovirus was kindly provided by Dr. Altieri of University of Massachusetts Medical School.

2.8. Statistical analysis

Experiments were performed in three or more trials. Data were expressed as mean ± standard deviation (SD). For comparison between two groups, student *t*-test was used. For comparison among multiple values, one-way analysis of variance (ANOVA) was performed. A p value <0.05 was considered statistically significant.

3. Results

3.1. OxLDL induced HAEC apoptosis

To induce HAEC apoptosis, we treated primary HAEC with high-dose OxLDL (50 and 100 μg/ml) for 24 h. Flow cytometry analysis revealed that OxLDL at 50 and 100 μg/ml increased apoptotic (PE-Annexin V positive) cells from 7.8% (control) to 18% and 23%, respectively ($n = 3$, $p < 0.01$) (Fig. 1A). OxLDL also significantly induced Caspase-3 activities (Control = 0.097 ± 0.006; at 50 μg/ml: 0.144 ± 0.025; at 100 μg/ml: 0.175 ± 0.008; $p < 0.05$, $n = 3$) (Fig. 1B).

3.2. OxLDL down-regulated Survivin and Ang-2 expression

OxLDL decreased Survivin mRNA expression in a dose-dependent manner with significant effects at OxLDL ⩾ 50 μg/ml (Data

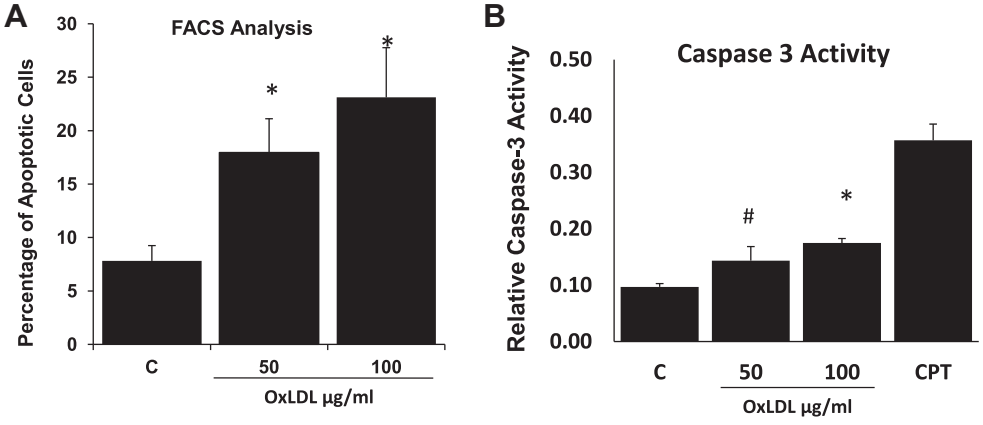

Fig. 1. OxLDL induced apoptosis of human aortic endothelial cells (HAEC). HAEC were treated with OxLDL at the indicated concentration for 24 h. (A) FACS analysis with PE-Annexin V staining revealed that OxLDL induced apoptosis. (B) Caspase-3 activities were measured as described in Section 2. Camptothecin (CPT) at 10 μM for 4 h treatment was used as positive control for apoptosis (C = control. * vs. C, $p < 0.01$, $n = 3$; # vs. C, $p < 0.05$, $n = 3$).

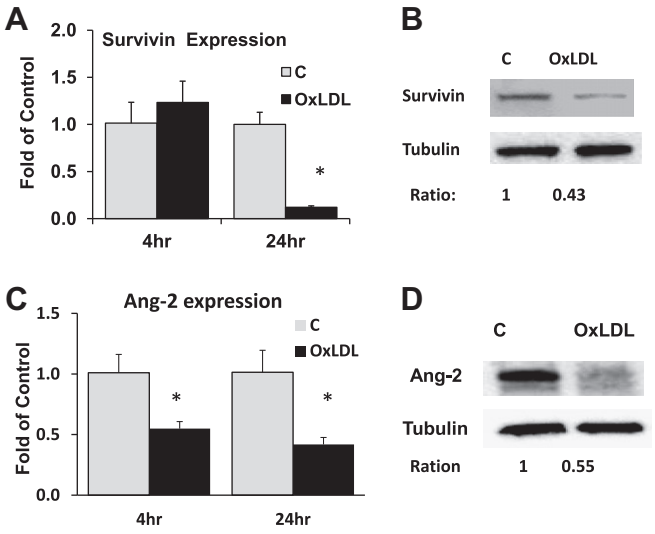

Fig. 2. OxLDL down-regulated Survivin and Angiopoietin-2 expression. HAEC were treated with OxLDL at 100 μg/ml for 4 h or 24 h. (A) Survivin and (C) Ang-2 mRNA expression was measured by quantitative RT-PCR. (B) Survivin protein expression in HAEC was measured by Western blot after 24 h treatment. (D) Ang-2 protein expression was down-regulated 6 h after OxLDL treatment at 100 μg/ml (C = control. * vs C, $p < 0.05$, $n = 3$).

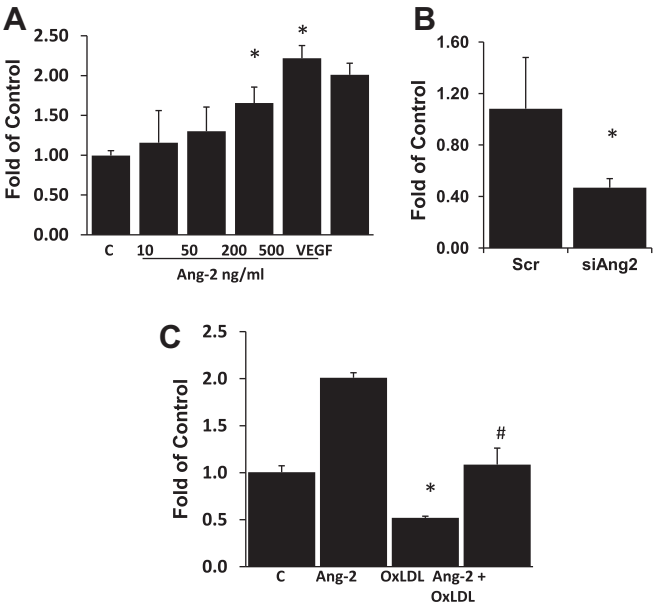

Fig. 3. Survivin as a target gene of Ang-2. (A) Ang-2 up-regulated Survivin expression. HAEC were treated with different concentrations of Ang-2 for 6 h and VEGF was used as a positive control. Survivin mRNA expression was measured by qRT-PCR. Ang-2 dose-dependently up-regulated Survivin expression. (B) Down-regulation of Ang-2 influenced Survivin expression. HAEC were transfected with 50 nM of control (Scr) or Ang-2 siRNA (siAng2) for 48 h. Survivin expression was measured. (C) OxLDL down-regulated Survivin expression via Ang-2. HAEC were treated with 100 μg/ml of OxLDL for 24 h in the presence or absence of 1 μg/ml of Ang-2. Ang-2 significantly attenuated OxLDL-induced down-regulation in Survivin mRNA expression (C = control. * vs. C, $p < 0.05$, $n = 3$; # OxLDL vs. Ang-2 + OxLDL: $p < 0.05$, $n = 3$).

not shown). OxLDL at 100 μg/ml down-regulated Survivin mRNA by 85% ($p < 0.05$, $n = 3$) and protein by 57%, respectively (Figs. 2A and B). These trends were consistent with those of OxLDL-induced apoptosis in Fig. 1.

OxLDL also decreased Ang-2 expression. While Survivin expression was significantly down-regulated at 24 h (Fig. 2A), Ang-2 mRNA expression was down-regulated by 46% as early as 4 h and 59% at 24 h ($p < 0.05$, $n = 3$) (Fig. 2C). OxLDL also down-regulated Ang-2 protein expression by 45% at 6 h (Fig. 2D).

3.3. Survivin as a target gene of Ang-2

We investigated whether Ang-2 was an upstream cytokine of Survivin expression. Treatment of HAEC with recombinant Ang-2 dose-dependently increased Survivin mRNA expression (significant at Ang-2 $\geqslant$200 ng/ml, $p < 0.05$, $n = 3$, Fig. 3A). Knockdown of Ang-2 expression by siRNA (siAng2) inhibited Ang-2 expression (data not shown) and Survivin mRNA by 59% and 53%, respectively ($p < 0.05$, $n = 3$, Fig. 3B). These findings support the notion that Survivin is a target gene of Ang-2.

3.4. Ang-2 mediated Survivin expression in response to OxLDL

To further examine the interplay between Ang-2 and Survivin, we assessed the effect of recombinant Ang-2 in OxLDL-treated HAEC. Recombinant Ang-2 significantly attenuated OxLDL-mediated down-regulation in Survivin expression (control = 1.0 ± 0.07, Ang-2 = 2.01 ± 0.52, OxLDL = 0.52 ± 0.02, Ang-2 + OxLDL = 1.09 ± 0.18; $p < 0.05$ for Ang-2 + OxLDL vs. OxLDL, $n = 3$) (Fig. 3C). This finding suggests that Ang-2 is implicated in down-regulation in Survivin expression in response to OxLDL.

3.5. Survivin over-expression attenuated OxLDL-induced apoptosis

Using recombinant Survivin adenovirus (Adv-Survivin) that also expresses green fluorescent protein (GFP), we over-expressed Survivin in HAEC (Fig. 4A), and used GFP-adenovirus (Adv-GFP) as a control. Over-expression of Survivin significantly attenuated OxLDL-induced HAEC apoptosis (PE-Annexin V positive) from

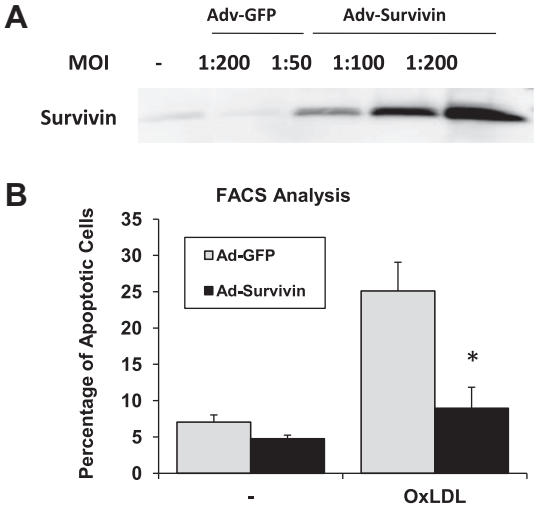

Fig. 4. Over-expression of Survivin significantly attenuated OxLDL-induced endothelial apoptosis. (A) HAEC were infected with recombinant Survivin adenoviruses (Adv-Survivin) or GFP adenovirus (Adv-GFP, control) for 24 h at different MOI. Survivin expression was measured by Western blot. (B) HAEC were infected with Adv-Survivin or Adv-GFP at MOI 100 for 24 h and the cells were treated with or without 100 μg/ml of OxLDL. Over-expression of Survivin significantly inhibited OxLDL-induced apoptosis (* OxLDL + Survivin vs. OxLDL + GFP: $p < 0.05$, $n = 3$).

25.1 ± 3.96% to 8.97 ± 2.88% ($p < 0.05$, $n = 3$) (Fig. 4B). Survivin further reduced background level of apoptosis (Fig. 4B). These findings corroborate that OxLDL induces apoptosis via down-regulation of Survivin expression in vascular endothelial cells.

4. Discussion

This study presents the novel finding that Ang-2 expression is intimately concordant with Survivin expression. We demonstrate that OxLDL down-regulated Ang-2 as early as 4 h post-treatment, followed by Survivin at 24 h. Recombinant Ang-2 significantly attenuated OxLDL-mediated down-regulation in Survivin expression. Furthermore, Survivin over-expression attenuated OxLDL-induced apoptosis. In this context, our findings support the notion that Survivin is a target gene of Ang-2.

Survivin is highly expressed in breast, lung, colorectal, and prostate cancer [14]. However, Survivin expression is developmentally regulated in healthy tissues, and its expression is relatively low in the vast majority of terminally differentiated tissues [1,14,15]. Increasing evidence reveals that Survivin is implicated in both regulatory [14] and survival mechanisms [16,17]. In atherosclerosis-prone areas, the rate of endothelial cell turnover and apoptosis is significantly elevated [18]. Moran et al. reported an elevated Survivin, cIAP2, and XIAP expression in smooth muscle cells from patients with carotid stenosis [19]. The immuno-activity of Survivin expression was present in CD68 positive monocyte/macrophages in the endoluminal fatty streaks, and was also elevated in the fibrous cap of atherosclerotic lesions [19]. However, Survivin expression was down-regulated in the advanced lesions [6]. Our finding of OxLDL-mediated down-regulation of Survivin may support the absence of Survivin in the advanced lesions.

Ang-1 and Ang-2 are well-characterized ligands binding to Tie-2 receptor [20,21]. Both in vitro and animal models establish Ang-1 as the natural activator of Tie-2, and Ang-2 as the antagonist to Ang-1[9,22]. Ang-1 was reported to up-regulated Survivin expression via Akt activation. Here we report Ang-2 also stimulated Survivin expression. While Ang-2 is an antagonist to Tie-2 in the presence of Ang-1, several studies support the notion that Ang-2 may also be an agonist to Tie-2. For instance, Ang-2 was implicated as a Tie-2 activator for post-natal retinal vascular remodeling in the Ang-2 knockout mouse model [23]. Furthermore, both Ang-1 and Ang-2 were reported to induce HUVEC tube formation via Tie-2 receptor, suggesting that both Ang-1 and Ang-2 activate Tie-2 signaling in the absence of pericytes or smooth muscle cells [24].

Both Ang-1 and Ang-2 promote endothelial cell survival [11,25–27]. Ang-2 has protective effects in stressed endothelial cells, whereas down-regulation of Ang-2 in endothelial cells promotes apoptosis [28]. While Ang-1 is primarily expressed in pericytes and smooth muscle cells, Ang-2 is selectively expressed in endothelial cells [9,22]. Our findings suggest that Ang-2 may play an autocrine role in mediating Survivin expression. In sum, we provide a new molecular insight into the interplay between Ang-2 and Survivin in response to OxLDL-induced apoptosis.

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