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Estradiol blocks the induction of CD40 and CD40L expression on endothelial cells and prevents neutrophil adhesion: An ERα-mediated pathway

Pedro Geraldes [a,b], Stéphanie Gagnon [a,b], Sofiane Hadjadj [a], Yahye Merhi [a,b], Martin G. Sirois [a,c], Isabelle Cloutier [a], Jean-François Tanguay [a,b,*]

[a] *Research Center of Montreal Heart Institute, Université de Montréal, Montréal, Québec, Canada*
[b] *Department of Medicine of Montreal Heart Institute, Université de Montréal, Montréal, Québec, Canada*
[c] *Department of Pharmacology, Université de Montréal, Montréal, Québec, Canada*

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Abstract

Objective: Interferon gamma (IFN-γ) was shown to induce CD40 and CD40L expression on endothelial cells (ECs) and consequently to promote neutrophil adhesion. The pro- and anti-inflammatory effects of estrogens are well recognized but their role on the regulation of CD40 and CD40L expression on ECs remains undefined.

Methods and results: Treatment of porcine aortic endothelial cells (PAEC) with IFN-γ for 24 h enhanced CD40 and CD40L expression by 97% and 78%, respectively. Pretreatment of PAEC with 17-beta-estradiol (17βE) for 24 h prevented the latter expression of CD40/CD40L. Treatment of PAEC with antisense oligomers targeting ERα mRNA attenuated the ability of 17βE to inhibit the IFN-γ-induced CD40 and CD40L protein expression. The IFN-γ activation pathway of CD40 is known to involve the phosphorylation of the Janus activated kinase (JAK) and the signal transducer and activator of transcription 1 (Stat1). 17βE, acting via the estrogen receptor α (ERα), abrogated IFN-γ-mediated effects on Stat1 but failed to inhibit Jak1 and Jak2 phosphorylation. Furthermore, 17βE prevented neutrophil adhesion induced by IFN-γ.

Conlusion: In summary, 17βE binding to ERα blocked IFN-γ-induced Stat1 phosphorylation, CD40 and CD40L protein expression, and neutrophil adhesion onto ECs.

Keywords: Restenosis; Estrogens; Cytokines; Receptors; Endothelial function

1. Introduction

The pathophysiology of restenosis involves early elements of direct injury to ECs, smooth muscle cells (SMCs) and thrombus deposition. The widespread use of coronary stents has fundamentally altered the vascular response to injury by causing a more intense and prolonged inflammatory state [1]. Elevated levels of inflammatory markers such as CD40–CD40L were shown to predict future cardiovascular diseases [2]. CD40 is a cell surface receptor belonging to the tumour necrosis factor (TNF) receptor superfamily. It is expressed primarily on antigen presenting cells, but also by a variety of nonimmune cells such as SMCs, fibroblasts, platelets and ECs [3]. The corresponding ligand, CD40 ligand (CD40L, also known as CD154), a transmembrane protein originally identified on CD4+ T cells, is likewise expressed on SMCs, platelets and ECs [3,4]. Under pro-inflammatory conditions, cytokines such as IFN-γ induce CD40 and CD40L expression on ECs [3]. The binding of

* Corresponding author. Research Center, Montreal Heart Institute, 5000 Belanger Street, Montréal, Québec, Canada H1T 1C8. Tel.: +1 514 376 3330x3250; fax: +1 514 593 2596.
E-mail address: pascale.geoffroy@icm-mhi.org (J.-F. Tanguay).

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IFN-γ to its receptors (IFNGR1 and IFNGR2) activates the receptor-associated Janus tyrosine kinases (JAKs), Jak1 and Jak2. JAKs tyrosines phosphorylate the latent cytoplasmic protein Stat1 which translocates to the nucleus and induces CD40 expression [5]. A previous study demonstrated that the administration of a neutralizing anti-CD40L monoclonal antibody reduced the initiation and progression of atherosclerotic lesions in hypercholesterolemic mice and further, modulated plaque architecture or plaque stability in such a manner suggesting a lower risk of thrombosis [6]. Collectively, these findings suggest an important role of CD40/CD40L interaction in the physiopathology of restenosis formation and related vascular diseases. Furthermore, this association may represent a key event to increase the expression of cellular adhesion molecules (CAMs) such as E-selectin, intercellular adhesion molecules (ICAM-1) and vascular cellular adhesion molecules (VCAM-1), and/or secretion of cytokines which contribute to leukocyte adhesion to the endothelium [7].

Healthy women with increased plasma levels of soluble CD40L were shown to be at risk for cardiovascular events [8]. Estrogens regulate a variety of pathways such as nitric oxide (NO) production which can directly influence the inflammatory process. The effects of estrogens are primarily mediated by estrogen receptors (ER) alpha and beta (ERα and ERβ). Clinical trials suggested that enhance estrogen levels correlated with increased C-reactive protein (CRP), a marker of inflammation associated with elevated risk of future cardiovascular events [9]. By contrast, estrogens induce anti-inflammatory effects by blocking monocyte/macrophage production of cytokines such as TNF-α and monocyte chemoattractant protein-1 and inhibit interleukin-1-induced expression of CAMs on ECs [10–12]. The effects of estrogens on CD40 and CD40L are not fully understood. A recent study demonstrated that CD40 and CD40L were upregulated in platelets of ovariectomyzed pigs and the response was abrogated with estrogen treatment [13]. Previous studies also reported that 17βE has dual effects on the expression of CD40 and CD40L in inflammatory cells [14,15]. However, the role of estrogens on CD40 and CD40L protein expression on ECs is unknown. Thus, to better understand the mode of action of 17βE, we characterized the specific role of ERα and ERβ on CD40 and CD40L expression and neutrophil adhesion on ECs under IFN-γ stimulation. Moreover, Stat1, Jak1 and Jak2 phosphorylation state was examined as those molecules are possible intracellular targets of 17βE in the activation cascade of IFN-γ.

2. Materials and methods

2.1. Cell culture

All experimental procedures were done following NIH guidelines for animal care. Porcine aortic endothelial cells (PAEC) were isolated from freshly harvested aortas, cultured and characterized as described previously [16]. PAEC were used between passages 1 and 3.

2.2. Antisense oligonucleotide gene therapy

The specific role of either ERα and ERβ in the 17βE-mediated effects on CD40L and CD40 expression on PAEC was assessed by using antisense oligonucleotides complementary to porcine ERα and ERβ mRNA (GeneBank accession numbers Z37167 and AF164957, respectively) as previously described [17].

2.3. Analyses of CD40 and CD40L expression

PAEC were plated at 1×10^5 cells/well in 6-well tissue culture plates (Becton-Dickinson, Rutherford, NJ) in Dulbecco's modified eagle medium (DMEM; Life Technologies Inc. Carlsbad, CA) containing 5% of fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and antibiotics (penicillin and streptomycin, Sigma, St-Louis, MO) for 24 h at 37 °C. G_0 synchronization was achieved by starving the cells for 48 h in DMEM containing 0.1% FBS. Cells were then grown for 16 h in DMEM with 1% FBS before the administration of 17βE-water soluble (10^{-10} to 10^{-6} mol/L, Sigma) and cells were incubated for an additional 24 h. Cells were rinsed and incubated in DMEM with recombinant porcine IFN-γ (1000 U/mL, R&D systems, Minneapolis, MN) at 37 °C for 24 h. The efficacy and specificity of our antisense oligomers to prevent the expression of targeted proteins were previously demonstrated [17]. For antisense treatment, oligomers (10^{-6} mol/L) were added daily as previously described [17]. For Western blot analysis, equal amount of total protein (100 μg) was loaded and migrated on 10% SDS-PAGE (Protean II kit; Bio-Rad, Hercules, CA) under reducing conditions and transblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were incubated overnight with one of the following antibodies; rabbit polyclonal anti-human-CD40, anti-human-CD40L (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-phospho-Stat1, anti-phospho-Jak1 or anti-phospho-Jak2 (1:1000 dilution, Cell Signaling Technology, Beverly, MA). Visualization of protein bands was achieved with an anti-rabbit IgG conjugated to horseradish peroxidase (1:20,000 dilution, Santa Cruz Biotechnology) and a chemiluminescence reagent (NEN Life Science Products, Boston, MA). Membranes were stripped with Re-Blot Plus (Chemicon International Inc., Temecula, CA) and total protein expression was determined with a rabbit polyclonal anti-β-actin (1:2000 dilution, Santa Cruz Biotechnology), rabbit polyclonal anti-Stat1, anti-Jak1 or anti-Jak2 (1:1000 dilution, Cell Signaling Technology). Results are presented as the relative expression of CD40, CD40L, phospho-Stat1, phospho-Jak1 and phospho-Jak2 proteins normalized with the expression of β-actin, Stat1, Jak1 or

Jak2 using Digital image densitometry (PDI Bioscience, Aurora, ON). Western blot analyses were performed in triplicate and results of image densitometry are representative of these experiments.

2.4. Analyses of CD40 mRNA levels

To study the effect of 17βE on the expression of CD40 and CD40L at mRNA levels, PAEC were stimulated as described above. Total RNA was isolated using TRIZOL reagent according to the manufacturer's instructions (Life Technologies Inc.). For each sample, 1 μg of total RNA was reverse transcribed into cDNA using p(N6) primer random hexamer (Amersham Biosciences, Baie d'Urfé, QC, Canada). CD40 forward 5-TGTACCAACAGCGCCT-GTGA-3′ (nt 331–350) and reverse primers 5′-CTCTG-TCGCCATCTGCTTGA-3′(nt 411–392), and CD40L forward 5-ATTCACTTGGGCGGAGTCTTC-3′ (nt 667–687) and reverse primers 5-GTGGCTCACTTGGCTTG-GAT-3′ (nt 747–728) complementary to the pig CD40 and CD40L genes were synthesized (Life Technologies Inc.). The ABI 5700 Sequence Detection System PE of Applied Biosystems (Foster City, CA) was used to amplify cDNA and detect PCR products. Final reaction volume of 25 μL for each PCR reaction was set up with 15 μL of Master SYBER Green I Kit (PE of Applied Biosystems), 9×10^{-7} mol/L of each of the forward and reverse primers, and 5 μL of cDNA. Standard curves were generated from serial dilutions (0 to 1000 ng) of total RNA obtained from IFN-γ treated cells. The values for CD40 mRNA were normalized to the level of 18S rRNA expressed in each sample. Each experiment was performed in triplicate. At the end of the amplification, 10 μL of PCR products were analyzed by electrophoresis on a 4% agarose gel to confirm products size (80 bp).

2.5. Neutrophil isolation and purification

As previously described [18], neutrophils were isolated from swine blood by red blood cell sedimentation with dextran 4% (ICN Biomedicals Inc., Aurora, OH), followed by Ficoll-Paque Plus (Amersham Biosciences) density gradient centrifugation. Using Coulter counter and by Wright–Giemsa staining, the purity of neutrophils was evaluated to be up to 97%. Viability was found to be greater than 99% as assessed by trypan blue dye exclusion assay.

2.6. Neutrophil adhesion assay

PAEC were seeded in 96-well tissue culture plates coated with 0.25% of gelatin and treated with or without antisense oligomers and/or 17βE before stimulation with IFN-γ as described above. Relative importance of CD40 and CD40L was evaluated by treatment of PAEC for one hour either with neutralizing anti-CD40 (5 μg/mL, clone 82102), anti-CD40L (1 μg/mL, clone 40804) or both and anti-ICAM-1 (10 μg/mL, clone BBIG-I1) (R&D systems) after IFN-γ stimulation. Normal rabbit IgG (Santa Cruz Biotechnology) was used as negative control. Neutrophils (1×10^5 cells) were then added to PAEC. After one hour incubation, wells were flicked dry, washed 3 times with Hank's Balanced Salt Solution, and adherent cells were estimated using the myeloperoxydase (MPO) assay of Grisham, Benoit, and Granger with a minor modification [19]. Each experiment is means obtained from 6 wells for each treatment and performed in triplicate. The absorbance of PAEC without neutrophils was subtracted from all the data as background value to exclude any potential effect of nNOS produce by PAEC. Cell counts were extrapolated from the absorbance of experimental samples using a standard curve which was established over a range of 1.0×10^4 to 1.4×10^5 neutrophils with an R-value of 0.988. Data are reported as relative mean of neutrophil adhesion compared to baseline.

3. Statistical analysis

Data are mean±SEM. Statistical comparisons were determined by ANOVA followed by a Bonferroni's test correction for multiple comparisons. A P value <0.05 was considered as significant. *, P<0.05 as compared PBS-

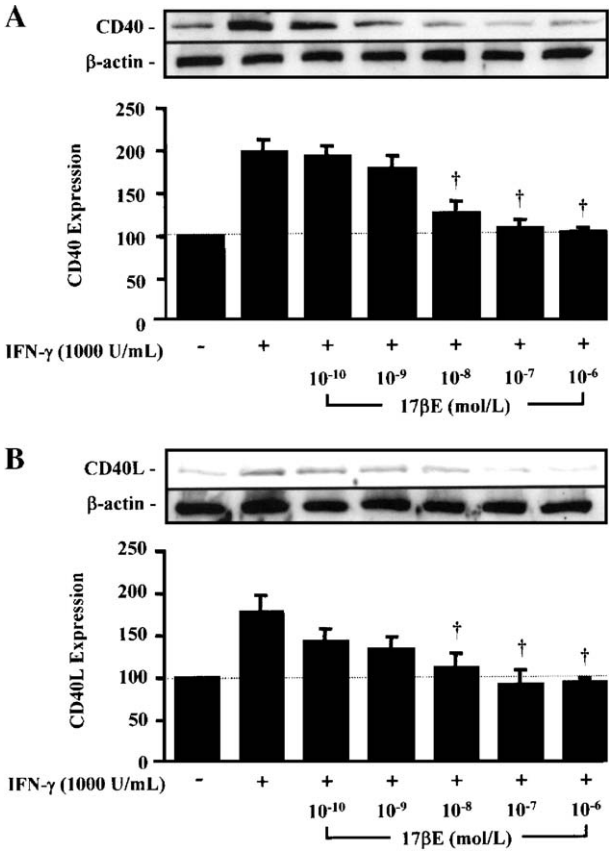

Fig. 1. Effect of 17βE on CD40 and CD40L expression induced by IFN-γ. Cells were pretreated ±17βE for 24 h and then stimulated with IFN-γ for 24 h as described in Methods. Protein expression of CD40 (A) and CD40L (B) was detected by Western blot analyses. Values are mean±SEM of at least 3 experiments.

treated cells; †, $P<0.05$ as compared to cells treated with IFN-γ (1000 U/mL); ‡, $P<0.05$ as compared to cells treated with 17βE (10^{-7} mol/L) and IFN-γ.

4. Results

4.1. Effect of 17βE on CD40 and CD40L mRNA and protein expression

IFN-γ-induced CD40 and CD40L expression were reported for human ECs [3]. We first assessed if treatment with IFN-γ could influence CD40 and CD40L mRNA and protein expression on PAEC. In unstimulated cells, we observed a minimal basal expression level of both proteins and mRNA. We performed a concentration-dependent stimulation of PAEC with IFN-γ and found that maximal effect on CD40 and CD40L protein expression was achieved after 24 h of treatment with a concentration of 1000 U/mL (data not shown). The 24 h treatment with IFN-γ (1000 U/mL) increased the protein expression of CD40 and CD40L by 97% and 78%, respectively (Fig. 1). Pretreatment with 17βE (10^{-10} to 10^{-6} mol/L) for 24 h provided a concentration-dependent and complete inhibition of CD40 and CD40L expression mediated by IFN-γ (Fig. 1). CD40 and CD40L mRNA levels were also increase by 200% and 87% with IFN-γ stimulation (Fig. 2) while, pretreatment with 17βE (10^{-7} mol/L) for 24 h prevented this latter induction. Protein expression of CD40 and CD40L were equivalent between cells from passages 1 to 3. In PAEC treated with 17βE alone (10^{-7} mol/L), CD40 and CD40L mRNA and protein levels were comparable to PBS-treated cells (data not shown).

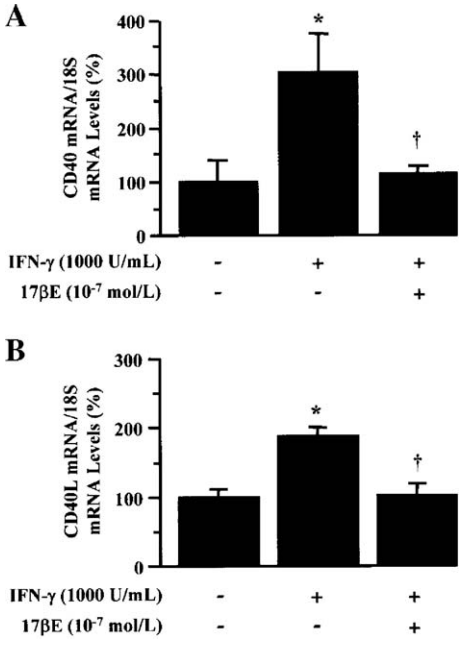

Fig. 2. Effect of 17βE on CD40 and CD40L mRNA expression induced by IFN-γ in PAEC. Cells were pre-treated ±17βE for 24 h and then stimulated with IFN-γ for 24 h as described in Methods. Quantitative analysis of CD40 (A) and CD40L (B) expression was performed by real-time RT-PCR. Values are mean±SEM of at least 3 experiments.

4.2. Role of ERα and ERβ on CD40 and CD40L expression

To evaluate the specific contribution of ER subtypes in the regulation of CD40 and CD40L protein expression, we used an antisense oligomer gene therapy approach. As previously demonstrated [17], the antisense oligomers AS1-ERα and AS2-ERα (10^{-6} mol/L) blocked PAEC ERα protein expression by 94% and 95%, while AS1-ERβ and AS2-ERβ (10^{-6} mol/L) suppressed ERβ protein expression by 90% and 97%, respectively. Treatment of PAEC with AS1-ERα and AS2-ERα reduced the inhibitory effect of 17βE on IFN-γ-induced CD40 protein expression by 53% and 61%, and CD40L protein expression by 74%, 71%, respectively (Fig. 3). In contrast, treatment with antisense oligomers targeting ERβ mRNA or scrambled oligomers had no detectable effect (Fig. 3). In the same series of experiments, we also evaluated the expression of ERs after IFN-γ treatment alone to ensure that ERα and ERβ expression levels were not affected. Western blot analyses confirmed that IFN-γ did not modulate the expression of ERα and ERβ (Fig. 3).

4.3. Effect of ERα on IFN-γ signalling pathway

The most well defined signalling cascade for type II IFN/IFN receptor involves JAK–STAT pathway. The following experiments examined whether the inhibitory effect of 17βE on IFN-γ-mediated induction of CD40 and CD40L was associated with concomitant inactivation of JAK/STAT pathway. As expected, stimulation with IFN-γ for 24 h induced an intense phosphorylation of Jak1, Jak2 and Stat1 in PAEC (Fig. 4A and 4B). Treatment of ECs with 17βE (10^{-7} mol/L) did not interfere with Jak1 and Jak2 activation induced by IFN-γ (Fig. 4A). However, pretreatment with 17βE (10^{-7} mol/L) completely blocked the phosphorylation of Stat1 mediated by IFN-γ (Fig. 4B). Treatment with antisense oligomers demonstrated that the down regulation of ERα protein expression abrogated the inhibitory effects of 17βE on Stat1 phosphorylation, whereas the inhibition of ERβ protein expression did not influence the 17βE activity on Stat1 phosphorylation (Fig. 4B).

4.4. 17βE inhibits neutrophil adhesion

Only one group has previously reported that human neutrophils expressed CD40L [20]. We confirmed by Western blot analyses and confocal microscopy that both CD40 and CD40L are present on porcine neutrophils (P.G. and J-F.T, unpublished data, November 2004). Upregulation of CD40 and CD40L expression on ECs increases neutrophil adhesion to the endothelium [3]. In this regard, we evaluated the relative role of CD40 and CD40L on neutrophil–EC adhesion by using antibodies able to block either CD40 or

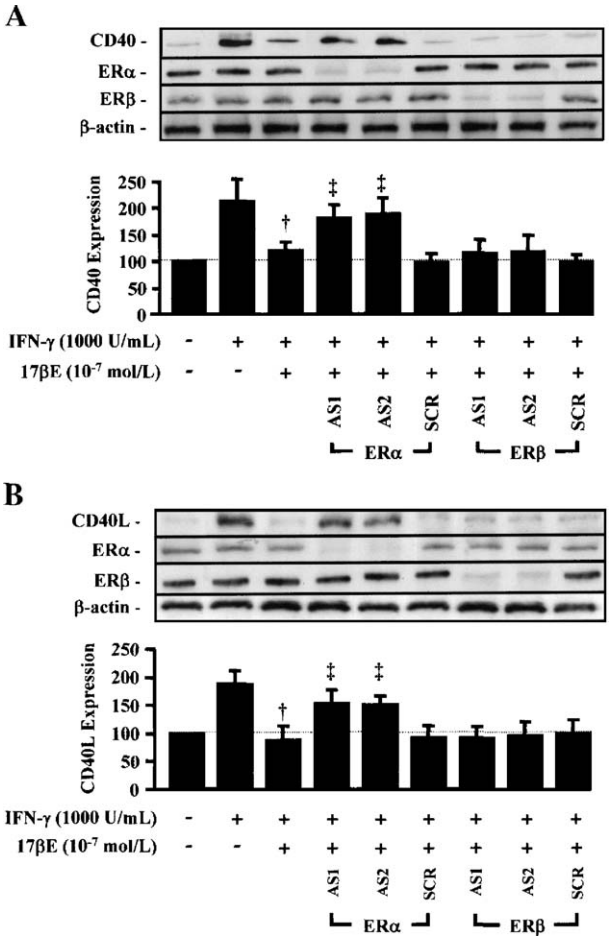

Fig. 3. Contribution of ERα and ERβ on the regulation of CD40 and CD40L expression in PAEC. Cells were treated either with antisense or scrambled oligomers (10^{-6} mol/L) as described in Methods. Cells were then treated ±17βE for 24 h and stimulated with IFN-γ for 24 h. Proteins expression of CD40 (A) and CD40L (B) was detected by Western blot analyses. Values are mean±SEM of at least 3 experiments.

CD40L. Treatment with IFN-γ (1000 U/mL) for 24 h increased by 2-fold the adhesion of neutrophils to activated ECs (Fig. 5). As shown by Lindmark et al. [21] in platelets and lymphocytes, the current study observed as well that maximal inhibition of neutrophil adhesion to EC was achieved using 5 μg/mL of anti-CD40 and 1 μg/mL of anti-CD40L antibodies prior to IFN-γ stimulation. In the present study, the use of an anti-CD40 antibody on ECs to prevent its binding onto CD40L expressed on neutrophils, reduced by 62% the adhesion of neutrophils onto IFN-γ-treated ECs. By the same way, the blocking anti-CD40L on ECs prevents its ligation to CD40 expressed on neutrophils, attenuated by 56% the adhesion of neutrophils to ECs following IFN-γ stimulation. Moreover, the combination of both blocking antibodies provided a greater inhibitory effect of IFN-γ-mediated adhesion of neutrophils to ECs (Fig. 5). Treatment with either anti-ICAM-1 or control IgG had no significant effect on the adhesion of neutrophils onto PAEC. In this assay, the background was obtained by measuring MPO activity in PAEC alone to exclude any potential nNOS

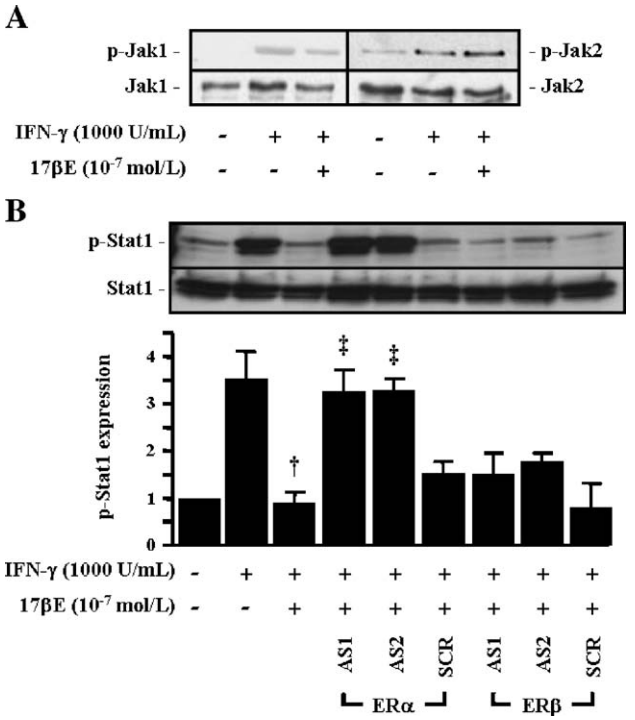

Fig. 4. Role of 17βE and ER on Jak1, Jak2 and Stat1 phosphorylation induced by IFN-γ. Cells were treated ±antisense or scrambled oligomers (10^{-6} mol/L) as described in Methods. Cells were then incubated ±17βE for 24 h and stimulated with IFN-γ (1000 U/mL) for another 24 h. Protein expression of (A) phospho-Jak1, Jak1, phospho-Jak2, Jak2, (B) phospho-Stat1 and Stat1 was detected by Western blot analyses. Values are mean± SEM of at least 3 experiments.

impact. We then investigated the effects of 17βE on neutrophil adhesion to ECs. Pretreatment of ECs with 17βE (10^{-7} mol/L) reduced by 79% neutrophil adhesion to

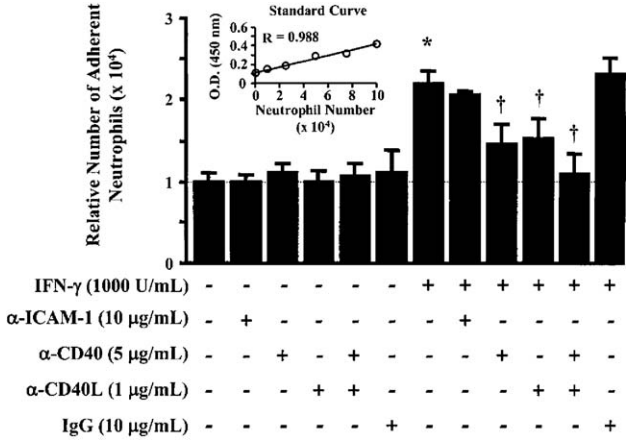

Fig. 5. Contribution of ICAM, CD40 and CD40L on neutrophil adhesion on PAEC. Cells were stimulated with INF-γ before the addition of either blocking antibodies against ICAM-1 (10 μg/mL), CD40 (5 μg/mL), CD40L (1 μg/mL) or a control IgG for 1 h and neutrophils (1×10^5 cells) were added for another 1 h. Neutrophil adhesion was evaluated by MPO assay as described in Methods. Values are mean±SEM of at least 7 experiments. *, $P<0.05$ as compared IgG-treated cells; †, $P<0.05$ as compared to cells treated with IFN-γ (1000 U/mL).

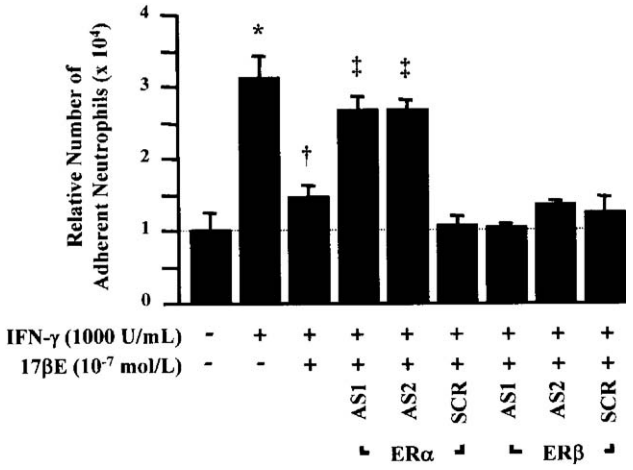

Fig. 6. Effects of 17βE on neutrophil adhesion on PAEC. Cells were treated either with antisense or scrambled oligomers as described in Methods. Cells were then treated ±17βE for 24 h and stimulated with IFN-γ for 24 h. Neutrophil adhesion was evaluated by MPO assay as described in Methods. Values are mean±SEM of at least 7 experiments.

PAEC mediated by IFN-γ (Fig. 6). 17βE alone does not affect the cell surface IFN-γ receptor expression level (data not shown). In order to characterize the implication of ERs in neutrophil–ECs interaction, we treated ECs with antisense oligomers before the addition of 17βE. We demonstrated that ERα protein expression but not ERβ protein expression is essential for the 17βE-mediated control of neutrophil adhesion (Fig. 6).

5. Discussion

Vascular inflammatory response plays an important role in the pathogenesis of restenosis and may contribute to the increased risk of unstable angina. It is well known that estrogens have both anti-inflammatory and pro-inflammatory effects. Estrogens were shown to modulate the expression of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 in response to cytokine stimulation in cultured human ECs [22]. Moreover, in human umbilical vein endothelial cells (HUVEC), estrogens reduced the level of MCP-1 and IL-8 expression mediated by TNF-α [12]. By contrast, estrogens increased CRP expression suggesting a potential role in the proinflammatory response. In recent findings, CD40 and CD40L emerge as crucial mediators not only in the initial events of restenosis but also predict late restenosis following coronary stenting [23]. Therefore, because CD40/CD40L interaction favors the binding of leukocytes to ECs, the present study evaluated the effects of estrogens on CD40 and CD40L expression by PAEC and neutrophil adhesion. Our results demonstrate that 17βE down modulates the impact of IFN-γ on CD40 and CD40L expression both at the mRNA and protein levels in PAEC. Furthermore, 17βE mediates its effects through ERα activation and significantly reduces neutrophil adhesion to PAEC.

CD40 was originally discovered in immunohistochemical studies on the surface of B lymphocytes and its expression level varied with the B cell activation state. Activation of B lymphocytes required direct contact with CD40L expressed on T helper cells. The biological function of this receptor/ligand dyad established CD40/CD40L interactions as a crucial process in T cell-dependent B cell activation and differentiation. Further studies demonstrated that both CD40 and CD40L are also expressed on non-lymphoid cells, such as dendritic cells, monocytes, macrophages, NK cells, epithelial cells, platelets, SMC, fibroblasts, and ECs providing autocrine and paracrine effects between circulating and vascular cells [24]. It has been proposed that the disruption of CD40–CD40L dyad could have therapeutic applications in a number of pathophysiological diseases such as the formation of inflammatory lesions and plaque instability [6]. Therefore, previous studies showed minimal expression of CD40 and CD40L on unstimulated human ECs but upregulated expression following IFN-γ stimulation [3,25]. However, IFN-γ was observed to reduce expression of CD40L by fibroblasts [26]. The current study demonstrated that a treatment of ECs with IFN-γ increased CD40 and CD40L mRNA and total protein expression, an effect that can be blocked by a pretreatment with 17βE. By itself, 17βE did not modulate CD40 and CD40L mRNA and protein expression or the IFNγR level on unstimulated PAEC (data not shown). Our results are in agreement with previous studies in which a pretreatment with 17βE decreased CD40 and CD40L surface expression in platelets of ovariectomyzed female pigs and in LPS-treated microglial cells [13,15]. In addition to the downregulation of ICAM-1, VCAM-1 and E-selectin in human ECs, the inhibition of IFN-γ-induced CD40 and CD40L expression by 17βE may represent an additional possible mechanism by which estrogens attenuate inflammatory effects.

The implication and specific roles of each ER on the estrogens anti-inflammatory effects are not completely understood. Therefore, in apoE-deficient female mice treated with 17βE, ERα was considered the major mediator in the reduction of atherosclerosis [27]. In a previous work, we showed the capacity of 17βE to increase PAEC migration, proliferation and activation of mitogen-activated protein kinases through ERα stimulation [17]. In this current study, we used an antisense therapy approach to assess the role of each ERs on CD40/CD40L regulation on ECs. We demonstrated that the blockage of ERα mRNA translation, but not ERβ mRNA, prevented the inhibitory effects of 17βE on CD40 and CD40L protein expression induced by IFN-γ. Thus, these results suggested that ERα is mostly responsible for the prevention by 17βE of IFN-γ mediated effects on ECs.

It is well documented that the induction of the JAK–STAT signalling pathway represents one potential mechanism by which IFN-γ may influence gene expression [5]. In human ECs, the up-regulation of CD40 expression at the transcriptional level by IFN-γ is known to involve the Stat1

transcription factor [25]. Previous studies have also shown that short and long term exposure to IFN-γ are capable to increase Stat1 phosphorylation [28]. As expected, we observed that 24 h stimulation with INF-γ activated Jak1, Jak2 and Stat1 phosphorylation. Treatment of ECs with 17βE did not influence the ability of IFN-γ to activate Jak1 and Jak2. However, 17βE acting via ERα reduced Stat1 phosphorylation induced by IFN-γ on ECs. Relationship between Stat1 and CD40 expression in infiltrated-cells was also reported in a study using a single application of decoy oligonucleotide specific for Stat1 [29]. However, at this point, the exact mechanism by which 17βE-ERα diminished Stat1 phosphorylation is unknown. Moreover, because the exposure time with 17βE (24 h), we cannot exclude that long-term stimulation may transiently influence Jak1, Jak2 and Stat1 activity.

The ability of adhesion molecules to influence leukocyte–EC interactions and therefore to regulate neutrophil recruitment play a pivotal role in the modulation of the inflammatory and immune responses. Neutrophil binding and infiltration into the vascular wall are critical cellular steps of the inflammatory response during the early stage of restenosis formation. In response to endoluminal injury, various CAMs are rapidly induced on neutrophils and ECs such as β2 integrin, ICAM-1, VCAM-1 and P-selectin. It is now well recognized that the induction of CD40 and CD40L expression at the surface of ECs also contributes to neutrophil adhesion on the endothelium [30]. Recently, it was demonstrated that blockage of CD40–CD40L interaction by silencing human CD40 expression on ECs led to a reduction of leukocyte adherence on these cells [31]. Since both CD40 and CD40L are present on ECs and can bind with their counter receptors expressed on neutrophils, we used an in vitro adhesion and myeloperoxidase assay to assess the relative importance of CD40 and CD40L on neutrophil adhesion to ECs. Our data showed that blocking either CD40 or CD40L on PAEC with their representative antibody partially reduce the number of adhered neutrophils onto activated ECs. However, when PAEC were treated with both blocking antibodies, additive inhibition of neutrophil adhesion onto PAEC was achieved. Thus, both CD40 and CD40L on ECs can modulate the adhesion of neutrophils induced by IFN-γ in a static condition. However, these finding needs to be evaluated in vivo where shear stress and blood flow could modulate successive adhesion molecules expression implicated in the interaction of circulating leukocytes with the activated endothelium.

A number of other CAMs were shown to be required for neutrophil–EC interaction [32]. Previous study observed that IFN-γ stimulation increase ICAM-1 and VCAM-1 but not P-selectin and E-selectin expression in human EC [33]. However, because the counter receptor of VCAM-1 (VLA-4) was not expressed by neutrophils, the induction of ICAM-1 by proinflammatory cytokines was the main regulator of neutrophil migration and localisation in this specific system [34,35]. Indeed, previous studies demonstrated that ligation of CD40 on human EC upregulates various CAMs such as ICAM-1 [36,37]. However, this effect can be drastically reduced in the presence of an anti-CD40 antibody in presence of proinflammatory molecule TNF-α [38]. Therefore, using a blocking antibody against ICAM-1, our results indicate only minor abrogation (less than 15%) of neutrophil adhesion to ECs (Fig. 5). Interestingly, some authors reported differences in IFN-γ effects on ICAM-1 levels between two distinct human EC even if both cell types exhibited a similar number of IFN-γ receptors [39]. Moreover, previous study reported that recombinant porcine IFN-γ only weakly stimulated ICAM-1 expression when incubated with PAEC [40]. Indeed, our findings are in agreement with others, supporting the hypothesis that alternative adhesion pathway(s) independent of P-selectin and ICAM-1 participate in neutrophil adhesion [41,42].

In conclusion, as compared to previous work on estrogen mediated down regulation of CD40 and CD40L in platelets, our study explored the role of estrogen receptors and the signalling pathway involved in the inhibition by 17βE of INF-γ-induced expression of CD40 and CD40L in PAEC. Therefore, the present study demonstrated that 17βE, through ERα-activation, diminished Stat1 phosphorylation induced by IFN-γ, abrogated the upregulation of CD40 and CD40L protein expression by PAEC and subsequently reduced the adhesion of neutrophils. Our results provide new insights to the understanding of the specific contribution of ERs on the control of inflammatory marker expression by PAEC.

Acknowledgements

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