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Delivery of pharmacologically active dexamethasone into activated endothelial cells by dexamethasone–anti-E-selectin immunoconjugate

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Abstract

To deliver selectively anti-inflammatory agents into activated endothelial cells, drug-targeting conjugates were developed. Dexamethasone (Dexa) was covalently linked to a monoclonal antibody specifically recognizing E-selectin, which is strongly upregulated in endothelial cells at inflammatory sites. In the present study, the pharmacological effects of this Dexa–mouse antihuman E-selectin antibody (H18/7) (Ab_{hEsel}) conjugate were investigated and compared to the effects obtained by free Dexa in human umbilical vein endothelial cells. Flow cytometry and ELISA were performed to analyze the levels of cell adhesion molecules (ICAM-1 and VCAM-1) and secreted cytokines (IL-6 and IL-8). The studies were extended by analysis of a complex gene expression pattern, using a cDNA expression array containing 268 genes encoding human cytokines/cytokine-receptors. Fifty genes and 28 genes were upregulated (ratio ≥ 2) upon incubation of human umbilical vein endothelial cells with TNFα for 6 and 24 hr, respectively. This gene expression profile was markedly altered when cells were activated with TNFα in the presence of Dexa (100 nM) or Dexa–Ab_{hEsel} conjugate (10 μg/mL conjugate corresponding to 100 nM Dexa). Relative and competitive RT–PCR analysis verified downregulation of TNFα-mediated expression of *CD40L* and *IL-8* by Dexa and Dexa–Ab_{hEsel}, respectively. These results indicated a successful internalization and processing of Dexa–Ab_{hEsel} in activated endothelial cells, allowing the intracellularly delivered Dexa to exert its pleiotropic anti-inflammatory activity.

Keywords: Endothelial cells; Inflammation; Drug-targeting; Cytokines; Dexamethasone; cDNA expression array

1. Introduction

During chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease, the endothelium plays an active role in leukocyte recruitment and infiltration [1]. These disorders are characterized by a continuously increased expression of inflammatory genes by a variety of cell types. Activated endothelial cells express adhesion molecules on their surface and secrete various cytokines leading to a multi-step cascade of leukocyte adhesion and subsequent transmigration of leukocytes into the inflamed tissue. Hence, manipulating the processes of endothelial cell activation may be advantageous for therapeutic outcome of these diseases.

Glucocorticoids are commonly used in anti-inflammatory therapy [2]. Once inside the cytoplasm of the cell, the glucocorticoid binds to a GR (Fig. 1). As a result, the receptor translocates into the nucleus where it can bind to glucocorticoid responsive elements found in promoters of various genes, thereby inducing their transcription [3]. The anti-inflammatory effects of glucocorticoids may partly be caused by enhanced expression of genes encoding anti-inflammatory proteins [4]. However, the most prominent

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Abbreviations: Dexa, dexamethasone; Ab_{hEsel}, mouse antihuman E-selectin antibody (H18/7); HUVEC, human umbilical vein endothelial cells; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; CD40L, CD40 ligand; ICAM-1, Intercellular Adhesion Molecule-1; MCP1, monocyte chemoattractant protein 1; Mip2-α, macrophage inflammatory protein 2 alpha; VCAM-1, Vascular Cell Adhesion Molecule-1.

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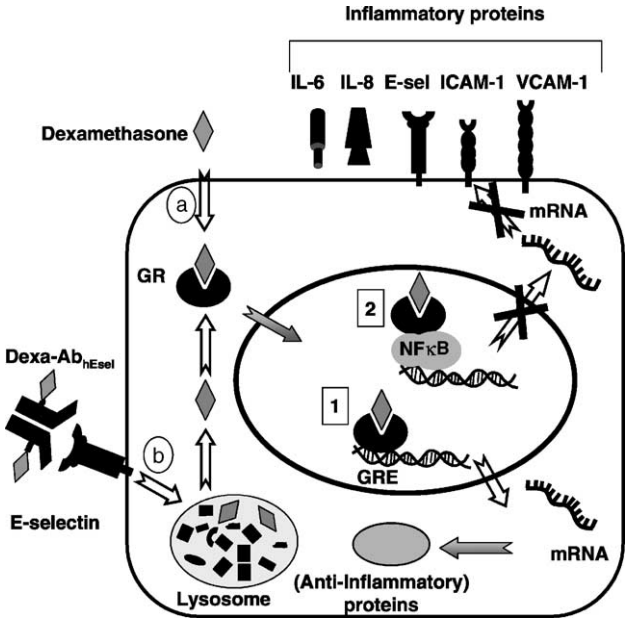

Fig. 1. Schematic representation of how (targeted) Dexa modulates gene expression in endothelial cells. In contrast to free Dexa, which enters the cell *via* passive diffusion (a), the immunoconjugate Dexa–Ab$_{hEsel}$ is intracellularly delivered *via* specific binding to E-selectin expressed on the surface of activated endothelium (b). After internalization, the protein part of the conjugate is degraded in the lysosomal compartment, thus releasing free Dexa. Upon diffusion into the cytoplasm, Dexa is recognized by the GR, which then translocates to the nucleus where it can modulate gene expression in different ways: (1) GR binds to glucocorticoid responsive elements in promoters of genes, thereby inducing their expression. These genes may encode anti-inflammatory proteins; (2) GR blocks the transcription of pro-inflammatory genes that are under regulation of NFκB or AP-1 (not shown), either by interacting directly with these transcription factors or by competing for common co-activators (not shown).

pharmacological effect of glucocorticoids is inhibition of expression of inflammatory genes encoding cytokines, receptors, and cell adhesion molecules. This repression of gene activity is predominantly caused by an inhibitory interaction of GR with other transcription factors, such as nuclear factor kappa B (NFκB) and activator protein 1 (AP1) [2,5]. Competition for common co-activators has also been proposed as a mechanism for this phenomenon [6,7]. Similarly, induction of IκB expression by glucocorticoids, the natural inhibitor of NFκB, was reported for lymphocytes and monocytes [8] but this was not observed in endothelial cells [9,10].

For cell-specific drug delivery, endothelial cells are attractive targets due to their direct contact with the blood. To deliver glucocorticoids into activated endothelial cells at the inflammatory site, Dexa was conjugated to a monoclonal antibody recognizing E-selectin, a cell adhesion molecule strongly upregulated by activated endothelium [11,12]. Previously, it was demonstrated that this Dexa–Ab$_{hEsel}$ conjugate was internalized by activated endothelium, not resting endothelium, *via* the lysosomal pathway [13]. According to the chemical conjugation strategy, the linkage between Dexa and the protein part of the construct will be degraded within the lysosomal compartment, resulting in a release of the free drug into the cytoplasm (Fig. 1). Here, the pharmacological effects of the intracellularly delivered conjugate in HUVEC are reported and compared to the effects mediated by free Dexa, which enters the cell *via* passive diffusion. Analyses of gene expression levels were performed by cDNA expression array studies and RT–PCR.

2. Material and methods

2.1. Preparation and characterization of Dexa–Ab$_{hEsel}$ conjugate

Dexa was conjugated as described previously [13] *via* a succinate linker to Ab$_{hEsel}$ (kindly provided by Dr. M. Gimbrone Jr., Harvard Medical School). Briefly, Dexa–hemisuccinate was synthesized and its identity was confirmed by mass spectrometry. Then Dexa–Ab$_{hEsel}$ conjugate was prepared by coupling the carboxylic acid group of Dexa–hemisuccinate to primary amino-groups of the protein. The final product was purified by dialysis against PBS at 4°, filtered through a 0.2 μm filter and stored at −20°. The Dexa–Ab$_{hEsel}$ conjugate was analyzed for protein content [14], Dexa content (HPLC and anti-Dexa Western blotting), molecular size (SDS–PAGE), and reactivity with E-selectin (BIAcore and immonohistochemical staining of activated endothelial cells) as described [13].

2.2. Endothelial cells and culture conditions

HUVEC were obtained from the Endothelial Cell Facility RuG/AZG, and were isolated and cultured as described previously [15]. Primary isolates were cultured on 1% gelatin-precoated plastic tissue culture plates or flasks (Costar Europe) at 37° under 5% CO_2/95% air. The culture medium consisted of RPMI 1640 (BioWittaker) supplemented with 20% heat-inactivated fetal calf serum (Integro), 2 mM L-glutamine (GIBCO-BRL), 5 U/mL heparin (Leo Pharmaceutical Products BV), 100 U/mL penicillin (Yamanouchi Pharma BV), 100 μg/mL streptomycin (Radiumfarma-Fisiopharma), and 20 μg/mL endothelial cell growth factor extracted from bovine brain (Roche Diagnostics). After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/mL in PBS) and split at a 1:3 ratio. For the experiments presented here, HUVEC were used up to passage three. Where indicated HUVEC were stimulated with 100 ng/mL recombinant human TNFα (Roche Diagnostics).

To determine endothelial cell responses to inhibitors of cell activation, the effects on adhesion molecule expression of the following test compounds were examined: Dexa (Genfarma), the NFκB-inhibitor parthenolide (Biomol), the p38 mitogen-activated protein kinase (MAPK) inhibitor

SB203580 (Alexis Biochemicals), and the phoshatidylinositol 3-kinase (PI3K) inhibitor wortmannin (Alexis Biochemicals), all dissolved in dimethyl sulfoxide (DMSO; Mallinckrodt Baker) in a concentration of 10 mM. Compounds were added to HUVEC in a final concentration of 10 μM, 1 hr prior to stimulation with TNFα. In the absence of test compounds, TNFα-activated HUVEC were incubated in medium containing 0.1% DMSO. This concentration of organic solvent did not interfere with the cellular responses studied, as tested in control experiments. After 24 hr, cells were processed for analysis of adhesion molecule expression by flow cytometry.

When analyzing the secretion of IL-6 and IL-8, cells were stimulated for 24 hr with TNFα. Dexa (100 nM and 10 μM) was added 1 hr prior to TNFα and 10 μg/mL Dexa–Ab_{hEsel} conjugate (corresponding to 100 nM Dexa) was added at the start of TNFα treatment.

For cDNA array analysis, HUVEC were stimulated for 6 or 24 hr with TNFα. Where indicated, Dexa (final concentration 100 nM) was added to the cells 1 hr before TNFα addition, whereas Dexa–Ab_{hEsel} conjugate was added at the start of TNFα treatment at a final concentration of 10 μg/mL.

2.3. Binding and uptake studies

The capacity of activated HUVEC to bind and internalize the Dexa–Ab_{hEsel} conjugate was investigated using a chloramine-T ^{125}I-radiolabeled conjugate [16]. The radiolabeled conjugate was purified by gel filtration on a PD10 column (Amersham Pharmacia Biotech) and contained less than 5% free ^{125}I, as determined by precipitation with 10% trichloroacetic acid. ^{125}I-radioactivity was counted in an LKB multichannel counter (LKB). Binding/uptake of Dexa was studied using ^{3}H-Dexa (Amersham Pharmacia Biotech). ^{3}H-radioactivity was counted in a calibrated scintillation counter (Packard Instruments) after addition of 3.5 mL scintillation liquid (Ultima Gold XR, Packard Biosciences) and vigorous mixing for 1 hr.

Radioactive binding experiments were performed with confluent HUVEC monolayers in 12-well culture plates (Costar). Viability of the cells was monitored by microscopic evaluation and consistently found to be unchanged throughout the experiments. To analyze the uptake of conjugate and free Dexa in resting and TNFα-activated (100 ng/mL) HUVEC, cells were incubated (6 hr, 37°) with ^{125}I-Dexa–Ab_{hEsel} or ^{3}H-Dexa. At the end of the incubation period, medium was removed and wells were washed with ice-cold 1% BSA/PBS. Surface-bound ^{125}I-Dexa–Ab_{hEsel} was released by incubating the cells for 10 min with 0.1 N HCl at 4°. Subsequently, cells were lyzed with 1 N NaOH (30 min at 37°) to retrieve internalized conjugate. Total uptake (membrane associated plus internalized) of ^{3}H-Dexa was determined by lysing the cells with 1 N NaOH (30 min at 37°). The amount of Dexa in the cell-associated fractions was calculated using the added concentrations, the specific activities (^{125}I-Dexa–Ab_{hEsel}: 10 μg/mL, specific activity 800 Bq/μg and ^{3}H-Dexa: 100 nM, specific activity 24 Bq/pmol) and 2:1 Dexa:antibody molar ratio, in the case of the ^{125}I-Dexa–Ab_{hEsel} conjugate.

2.4. Flow cytometric analysis of cell adhesion molecule expression

Cells were trypsinized and incubated with the following mouse antihuman antigen antibodies: hu5/3 (anti-ICAM-1) and E1/6 (anti-VCAM-1), both kindly provided by Dr. M. A. Gimbrone Jr., JC/70A (anti-CD31; DAKO), and a control antibody OX8 that does not bind to human antigen (mouse antirat CD8; MRC). Primary antibodies were subsequently detected by FITC-labeled rabbit antimouse $F(ab')_2$ (DAKO) and samples were analyzed using a Coulter Epics-Elite flow cytometer (Coulter Electronics). Data were analyzed using WinList software (version 3D, Verity Software House). The observed levels of cell adhesion molecules were related to those in the presence of TNFα but in the absence of inhibitors after correction for binding of OX8 control antibody.

2.5. Analysis of secreted IL-6 and IL-8

Collected culture medium was centrifuged to remove cellular debris (400 *g*, 10 min, 4°) and stored at −20° pending analysis. IL-6 and IL-8 concentrations were analyzed using sandwich ELISA as previously described [17,18]. In short, either mouse antihuman IL-6 (CLB.MIL6/16; Central Laboratory of Blood Transfusion Services) or mouse antihuman IL-8 (6217.111; R&D Systems) were used as capture antibodies, followed by incubation of serially diluted culture medium. Detection of cytokines was performed by either polyclonal biotinylated antihuman IL-6 (CLB.SIL6-D; Central Laboratory of Blood Transfusion Services) or biotinylated antihuman IL-8 (BAF208; R&D Systems), followed by poly-HRP-labeled streptavidin (Central Laboratory of Blood Transfusion Services) and 3,3′,5,5′-tetramethylbenzidine (Brunschwick Chemie) for color development. Absorbance was read using a microplate reader and analyzed using SOFTmax Pro software, version 2.4.1 (both Molecular Devices).

2.6. Total RNA isolation

Twenty-five square centimeters confluent monolayers of HUVEC were scraped from the flask surface and total cellular RNA was extracted using Trizol reagent (Life Technologies). DNase treatment was carried out with the DNAfree kit (Ambion). RNA yield was determined spectrophotometrically by measuring optical density at 260 and 280 nm. Integrity of RNA samples was checked by electrophoresis on a 1% agarose/formaldehyde gel and

the absence of DNA from the samples was verified by PCR with GAPDH primers, omitting the reverse transcriptase (RT) step. RNA was stored at −80° until used for ^{32}P-labeling and RT–PCR analysis.

2.7. cDNA expression array analysis

^{32}P-radioactive cDNA was synthesized from 3 μg RNA using Powerscript RT-enzyme (BD Biosciences Clontech) and hybridized to Atlas expression array (BD Biosciences Clontech) according to manufacturers protocol. This array contained 268 genes encoding human cytokines/cytokine-receptors (http://www.clontech.com/atlas/genelists/index.shtml). The obtained signals were quantified by Storm 860 PhosphorImager (Amersham Pharmacia Biotech) and analyzed using Atlas Image 1.01 software (BD Biosciences Clontech). Hybridization signals on each filter were normalized for the expression of nine housekeeping genes present on the array.

2.8. RT–PCR

One microgram RNA was heated at 70° for 10 min, immediately cooled on ice and applied for reverse transcription in a total volume of 20 μL using 1 μL Powerscript RT-enzyme (BD Biosciences Clontech), 0.5 μg oligo dT_{15}-primers (Promega), 1 mM dNTP, 10 mM DTT, and 1× First Strand Buffer (BD Biosciences Clontech) at 42° for 90 min. After cDNA synthesis, the solution was diluted to 50 μL with nuclease free water. PCR for GAPDH and the first round of nested PCR for CD40L was performed with 1 μL cDNA and a final concentration of 200 nM for each gene-specific primer, 1.5 mM $MgCl_2$ (Eurogentec), 200 μM dNTP (MBI Fermentas), 1× PCR-buffer and 1 U SilverStar DNA polymerase (Eurogentec) in a total volume of 50 μL. cDNA was amplified using the following primers: GAPDH (forward primer 5′-CCATCACCATCTTCCAGGAG-3′; reverse primer 5′-CCTGCTTCACCACCTTCTTG-3′; PCR product 576 bp), $CD40L_1$ (forward $primer_1$ 5′-GGGAGTCTTCATAATACAGCACAGCGGTTAAG-3′; reverse $primer_1$ 5′-CATAGGAACCCAGAGTCAACCATAACTGAATG-3′; PCR product 414 bp). Second round of nested PCR for CD40L was carried out as described above except using 1 μL PCR product from the first round as a template and internal $CD40L_2$ primers (forward $primer_2$ 5′-CACCCCCTGTTAACTGCCTA-3′; reverse $primer_2$ 5′-AGACACTGCACGCAAAGAAA-3′; PCR product 283 bp). GAPDH PCR cycle conditions were 5 min 95°, followed by 25 cycles 95° for 30 s, 58° for 30 s and 72° for 30 s, and finally 72° for 5 min. $CD40L_1$ and $CD40L_2$ PCR cycle conditions were 5 min 95°, followed by, respectively, 35 and 14 cycles: 95° for 30 s, 50° for 45 s and 72° for 45 s, and finally 72° for 5 min. All PCRs were carried out in duplicate and 10 μL PCR products were analyzed on 2% agarose-gels stained with ethidium bromide. The signals from different samples were normalized for the expression of *GAPDH* and quantified using the QuantityOne quantification software (BioRad Laboratories).

Competitive RT–PCR for *IL-8* was carried out using primers and conditions provided with the competitive hIL-8 quantitative RT–PCR kit (Ambion).

3. Results

3.1. Uptake of Dexa–Ab_{hEsel} conjugate in HUVEC

The conjugate Dexa–Ab_{hEsel} used throughout this study contained an average of two Dexa molecules per molecule Ab_{hEsel}. Characterization of the conjugate and determination of the Dexa:antibody ratio was described previously [13]. The conjugate Dexa–Ab_{hEsel} showed specific binding to activated HUVEC, which was mediated by E-selectin, reaching a maximum after 4 hr and saturation at 10 μg/mL, a concentration corresponding to 100 nM Dexa [13,19]. Since Dexa has to be released intracellularly before becoming pharmacologically active, analyses of the binding and effects of selectively delivered Dexa were performed 2 hr after maximum binding, i.e. 6 hr after start of incubation. From the specific activity of the radioactive conjugate and previously determined drug loading (2:1 Dexa:antibody molar ratio), the amount of surface bound and intracellularly delivered Dexa at 6-hr incubation was calculated to be 1600 and 1250 fmol, respectively. In comparison, incubation of activated HUVEC with 100 nM unconjugated ^{3}H-Dexa resulted in an uptake of 100 fmol of the drug (Fig. 2A). Uptake of ^{125}I-Dexa–Ab_{hEsel} was observed by activated endothelium only, whereas the uptake of unconjugated Dexa was similar for resting and TNFα-activated HUVEC (Fig. 2B).

3.2. Inhibitory effects on ICAM-1 and VCAM-1 expressions

First the question to what extent Dexa affected the expression of cell adhesion molecules that mediate recruitment of leukocytes into inflamed tissue was addressed. ICAM-1 and VCAM-1 were abundantly present on the cell surface of HUVEC after 24 hr activation by TNFα (Fig. 3A). Dexa (10 μM) did not reduce TNFα-induced expression of these adhesion molecules. Likewise, no effect was observed when the cells were activated with TNFα in the presence of 100 nM Dexa or 10 μg/mL Dexa–Ab_{hEsel} conjugate (data not shown). On the other hand, both ICAM-1 and VCAM-1 were downregulated by the NFκB inhibitor parthenolide. In addition, incubation with, respectively, the P38-MAPK inhibitor SB203580 and the PI3K inhibitor wortmannin, resulted in a considerable repression of VCAM-1. Expression of the endothelial marker CD31 was unaffected under all circumstances tested (Fig. 3A).

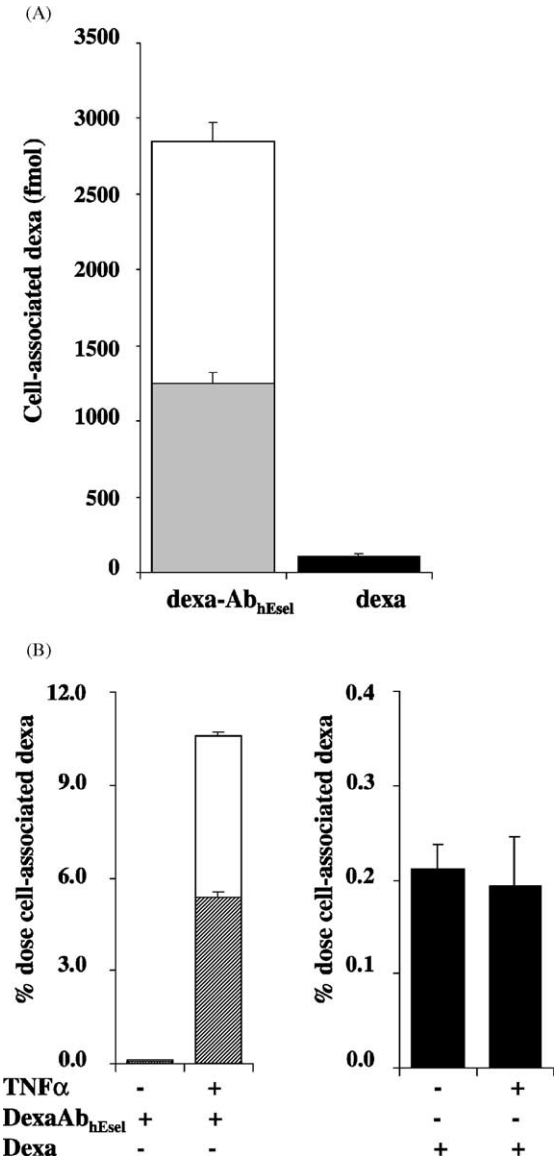

Fig. 2. Binding, uptake, and internalization of ^{3}H-Dexa and ^{125}I-Dexa–Ab$_{hEsel}$ in HUVEC. (A) Cell-associated Dexa in TNFα-activated HUVEC after incubation (6 hr at 37°) with ^{125}I-Dexa–Ab$_{hEsel}$ (10 μg/mL) or ^{3}H-Dexa (100 nM). Corresponding amounts of cell-associated Dexa were calculated by using the specific activities of the radioactive compounds and, in the case of ^{125}I-Dexa–Ab$_{hEsel}$, the average Dexa content of 2 moles Dexa per mole antibody. (B) Comparison of cell associated Dexa–Ab$_{hEsel}$ and Dexa in resting and TNFα-activated HUVEC. White bar, surface-bound; hatched bar, intracellular; and black bar, total cell-associated. Values represent the average (±SD) of three experiments.

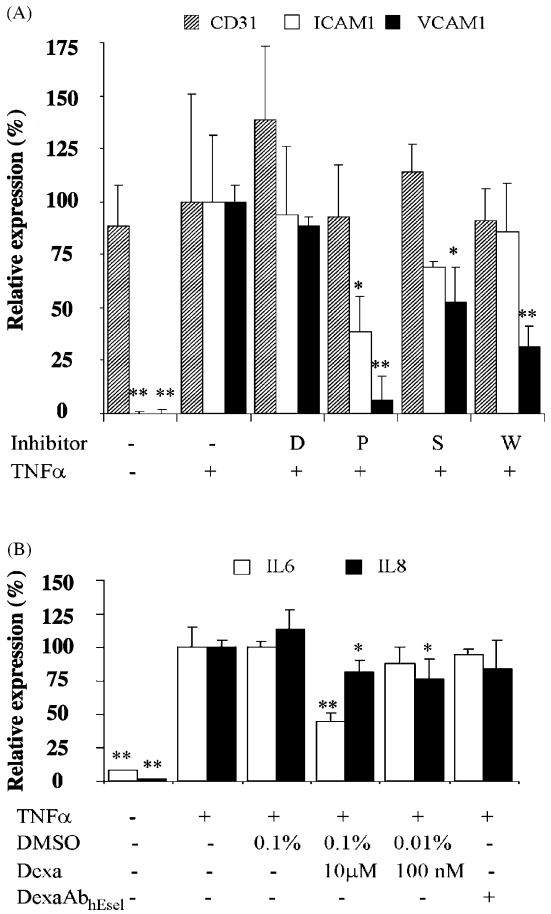

Fig. 3. Inhibition of surface expression of cell adhesion molecules (A) and secretion of cytokines (B). Cells were activated with 100 ng/mL TNFα for 24 hr. (A) Where indicated, the inhibitors (10 μM) were added 1 hr prior to TNFα. D, Dexa; P, parthenolide; S, SB203580; and W, wortmannin. (B) Dexa was added 1 hr prior to TNFα and the conjugate at $t = 0$. Values represent the average (±SD) of three experiments. Statistically significantly difference of relative expression compared to HUVEC activated with TNFα were determined by unpaired t-test ($^{*}P < 0.05$; $^{**}P < 0.001$).

3.3. Effects of (targeted) Dexa on the secretion of IL-6 and IL-8

The effects of Dexa (10 μM and 100 nM) and the conjugate Dexa–Ab$_{hEsel}$ (10 μg/mL) on the TNFα-induced secretion of the cytokines IL-6 and IL-8 were analyzed (Fig. 3B). Secretion of IL-6 and IL-8 was, respectively, 13- and 57-fold higher by TNFα-activated than by resting HUVEC. The secretion of IL-6 was reduced by more than 50% when cells were activated with TNFα in the presence of 10 μM Dexa. Neither 100 nM Dexa nor Dexa–Ab$_{hEsel}$ inhibited the secretion of IL-6. The secretion of IL-8 was only slightly repressed by Dexa at both concentrations tested. Incubation with Dexa–Ab$_{hEsel}$ conjugate did not result in inhibition of IL-8 secretion.

3.4. Gene expression profiling

cDNA expression analysis was applied to study the pharmacological activity of Dexa–Ab$_{hEsel}$ and unconjugated Dexa in activated HUVEC with respect to their effect on gene expression. In total 268 genes encoding human cytokine/cytokine-receptors present on the array were studied.

3.4.1. TNFα-modulated gene expression

After 6 hr of activation with TNFα, the expression of 50 genes was considerably increased in HUVEC. Nineteen genes were upregulated 2–5-fold compared to resting cells. Twenty-two genes had a 5–10-fold increased expression and nine genes were expressed at ≥ 10-fold levels com-

pared to resting HUVEC. At 24 hr, twenty-eight genes were markedly upregulated by TNFα (ratio ≥ 2 above resting HUVEC) of which eight genes exhibited a 5–10-fold induction and another eight genes an elevated expression of $\geq$ 10-fold. Table 1 includes those genes exhibiting at least 5-fold-increased expression after 6 or 24 hr TNFα stimulation. Apparently, TNFα did not markedly down-regulate the expression of the cytokine/cytokine-receptor genes present on the array. In fact, most of the genes displayed a low expression level prior to TNFα incubation. *Mip2-α*, *Jagged 1*, *IL-8*, and *MCP1* demonstrated a moderate basal expression in resting HUVEC. However, their expression was extensively enhanced after TNFα induction at both time points. *IL-6* expression was slightly modulated at $t = 6$ hr but had increased to a larger extent after 24 hr TNFα stimulation (Table 1).

3.4.2. (Targeted) Dexa-modulated gene expression

The modulation of TNFα-induced gene expression by Dexa and Dexa–Ab_{hEsel} was analyzed. First, inhibition by

Table 1
Gene expression profiling of TNFα-activated HUVEC with cDNA expression array

Accession no.	Gene name	Abbreviation	Resting	TNFα ($t = 6$ hr)		TNFα ($t = 24$ hr)	
			Adjusted intensity[a]	Adjusted intensity[a]	Fold induction	Adjusted intensity[a]	Fold induction
M11220	Granulocyte-macrophage colony stimulating factor	GM-CSF	1	1	1.0	40	40.0
X03438	Granulocyte colony-stimulating factor	G-CSF	1	1	1.0	48	48.0
X53655; M37763	Neurotrophin-3	NT-3	1	2	2.0	9	9.0
M18391	Ephrin type-A receptor 1; tyrosine-protein kinase receptor eph	Eph	1	2	2.0	5	5.0
X78686	Granulocyte chemotactic protein 2	GCP 2	1	4	4.0	32	32.0
L12260; L12261 + U02326 + M94165	Glial growth factor 2	GGFHPP2	1	5	5.0	2	2.0
L07414	Death receptor 5; cytotoxic TRAIL receptor 2	DR5	1	5	5.0	6	6.0
X72304	Corticotropin releasing factor receptor 1	CRF-R	1	5	5.0	8	8.0
U05875	Interferon-gamma receptor beta subunit	IFN-gamma-R beta	1	5	5.0	9	9.0
X77722	Interferon-alpha/beta receptor beta subunit	IFN-alpha-R	1	6	6.0	5	5.0
X14454	Interferon regulatory factor 1	IRF1	1	6	6.0	10	10.0
M18391	Glia-derived neurite-promoting factor	GDNPF	1	7	7.0	1	1.0
U95299	Notch4	Notch4	1	7	7.0	1	1.0
U94354	Lunatic fringe	Lunatic fringe	1	7	7.0	1	1.0
A03911	Neurotrophin-4	NT-4	1	9	9.0	0	0.0
X14445	Fibroblast growth factor 3	FGF3	1	9	9.0	0	0.0
K02770	Interleukin-2	IL-2	1	10	10.0	1	1.0
D10925	C–C chemokine receptor type 1	CC CKR1	1	11	11.0	1	1.0
L38518	Sonic hedgehog	SHH	1	11	11.0	1	1.0
L09753	CD40 ligand	CD40-L	1	14	14.0	0	0.0
M29366; M34309	ERBB-3 receptor protein-tyrosine kinase	ERBB-3	2	11	5.5	3	1.5
X01057; X01058; X01402	Interleukin-2 receptor alpha subunit	IL2RA	2	12	6.0	4	2.0
M19154; M22045; M22046; Y00083	Erythroid differentiation protein	EDF	2	12	6.0	4	2.0
D13365; M93311	Growth inhibitory factor; metallothionein-III	MT-III	2	13	6.5	33	16.5
M65290	Interleukin-12 beta subunit	IL-12B	2	15	7.5	2	1.0
M86528	CD30 ligand	CD30L	2	15	7.5	1	0.5
X68203; X69878; U43143	Vascular endothelial growth factor receptor 3	VEGFR3	2	15	7.5	1	0.5
S59184	Related to receptor tyrosine kinase	RYK	2	16	8.0	2	1.0
J04156	Interleukin-7	IL-7	2	18	9.0	2	1.0
X02811; X02744; M12783; M16288	Transforming growth factor beta 2 precursor	TGF-beta2	2	19	9.5	1	0.5
M28622	Interferon-beta	IFN-beta	2	20	10.0	2	1.0
AF016268	Platelet-derived growth factor B subunit	PDGFB	2	21	10.5	3	1.5
M86492	Glia maturation factor beta	GMF-beta	4	20	5.0	4	2.0
K02770	Interleukin-1 beta	IL-1beta	4	22	5.5	16	4.0
X53799	Macrophage inflammatory protein 2 alpha	Mip2-α	5	37	7.4	496	99.2
AF028593	Jagged 1	HJ1	5	62	12.4	31	6.2
X04602; M14584	Interleukin-6	IL-6	10	18	1.8	56	5.6
Y00787	Interleukin-8	IL-8	17	338	19.9	591	34.8
M24545	Monocyte chemotactic protein 1	MCP1	50	1045	20.9	1327	26.5

Genes with at least 5-fold increased expression levels after 6 or 24 hr TNFα stimulation compared to resting HUVECs are shown. Cells were stimulated with 100 ng/mL TNFα. Genes are listed in order of the adjusted intensity of the resting control.

[a] Adjusted intensity is hybridization-signal value after subtraction of the background signal.

Table 2
Inhibition of gene expression by free and conjugated Dexa

Gene	TNFα	TNFα + Dexa		TNFα + Dexa–Ab_{Esel}	
	Adjusted intensity	Adjusted intensity	Inhibition (%)	Adjusted intensity	Inhibition (%)
$t = 6$ hr					
SHH	11	0	100	3	73
FGF3	9	0	100	3	67
CD40-L	14	0	100	6	57
GMF-beta	20	0	100	10	50
CD30L	15	0	100	8	47
RYK	16	0	100	9	44
IL-12B	15	0	100	9	40
IFN-beta	20	0	100	12	40
IL-7	18	1	94	13	28
PDGFB	21	2	90	11	48
TGF-beta2	19	2	89	9	53
NT-4	9	1	89	4	56
CC CKR1; Mip1-α R	11	2	82	4	64
ERBB-3	11	2	82	5	55
VEGFR3	15	3	80	7	53
IL-2	10	2	80	6	40
IL-1	22	5	77	18	18
L2RA	12	3	75	5	58
Notch4	7	2	71	2	71
IFN-alpha-R	6	2	67	4	33
IL-8	338	140	59	190	44
Lunatic fringe	7	3	57	1	86
GDNPF	7	3	57	2	71
HJ1	62	30	52	32	48
$t = 24$ hr					
MT-III	33	6	82	48	<0

Listed genes showed at least 5-fold increased expression after TNFα stimulation and 50% Dexa-mediated reduction in expression level. For nomenclature of genes, see Table 1. Genes are listed in order of the level of inhibition caused by Dexa. Cells were stimulated with 100 ng/mL TNFα and co-incubated with 100 nM Dexa or 10 μg/mL Dexa–Ab_{Esel}.

Dexa of TNFα-mediated gene expression was studied. Genes that showed ≥ 5-fold induction with TNFα alone (listed in Table 1) were selected for this analysis. Table 2 demonstrates that at 6 hr after start of TNFα incubation, twenty-four genes were downregulated by 50% or more by treatment with free Dexa. In all cases, analogous effects were obtained by treatment with the Dexa–Ab_{hEsel} conjugate. At 24 hr, free Dexa reduced the expression of only one gene, metallothionein-III, more than 50%. No downregulation of metallothionein-III expression was observed when treating the cells with Dexa–Ab_{hEsel}.

Upregulation of gene expression by Dexa was also detected at both time points (data shown on http://www.farm.rug.nl/ffk/unyphar/biochempharm/asgeirsdottir3.html). This implies that, whereas Dexa represses the TNFα-induced expression of several genes, it can also induce the expression of genes to levels above those of TNFα stimulation alone. This differential regulation by Dexa of the expression of three genes with abundant transcripts in activated HUVEC is demonstrated in Fig. 4. IL-8 mRNA level was clearly downregulated by Dexa, whereas the expression of *Mip2-α* was increased compared to incubation with TNFα alone. On the other hand, Dexa did not alter the expression levels of *MCP1*. In these cases, but also in general, Dexa–Ab_{hEsel} demonstrated identical regulation patterns as unconjugated Dexa at both time points analyzed.

3.5. RT–PCR

The results of the cDNA expression array after 6 hr of TNFα stimulation were confirmed by RT–PCR analysis for two genes: *IL-8* and *CD40L*. Both genes were expressed in activated HUVEC but to a different extent (Table 1). Whereas *IL-8* was highly transcribed, CD40L mRNA can be classified as a rare transcript in the endothelium. RT–PCR of CD40L applying 35 cycles of amplification was not sufficient to visualize a PCR product on an ethidium-bromide stained agarose gel. Therefore, nested PCR using internal gene-specific primers with additional 14 amplification cycles was carried out as described in Section 2. Nested-PCR with 12 and 16 cycles resulted in, respectively, 1/4 and 4 times as much PCR products, indicating that the analysis was carried out within the linear range of the amplification. The signals were normalized for *GAPDH* expression and quantified (Fig. 5A and B). These data corroborate the regulation patterns observed by cDNA expression array analysis. Almost three times as

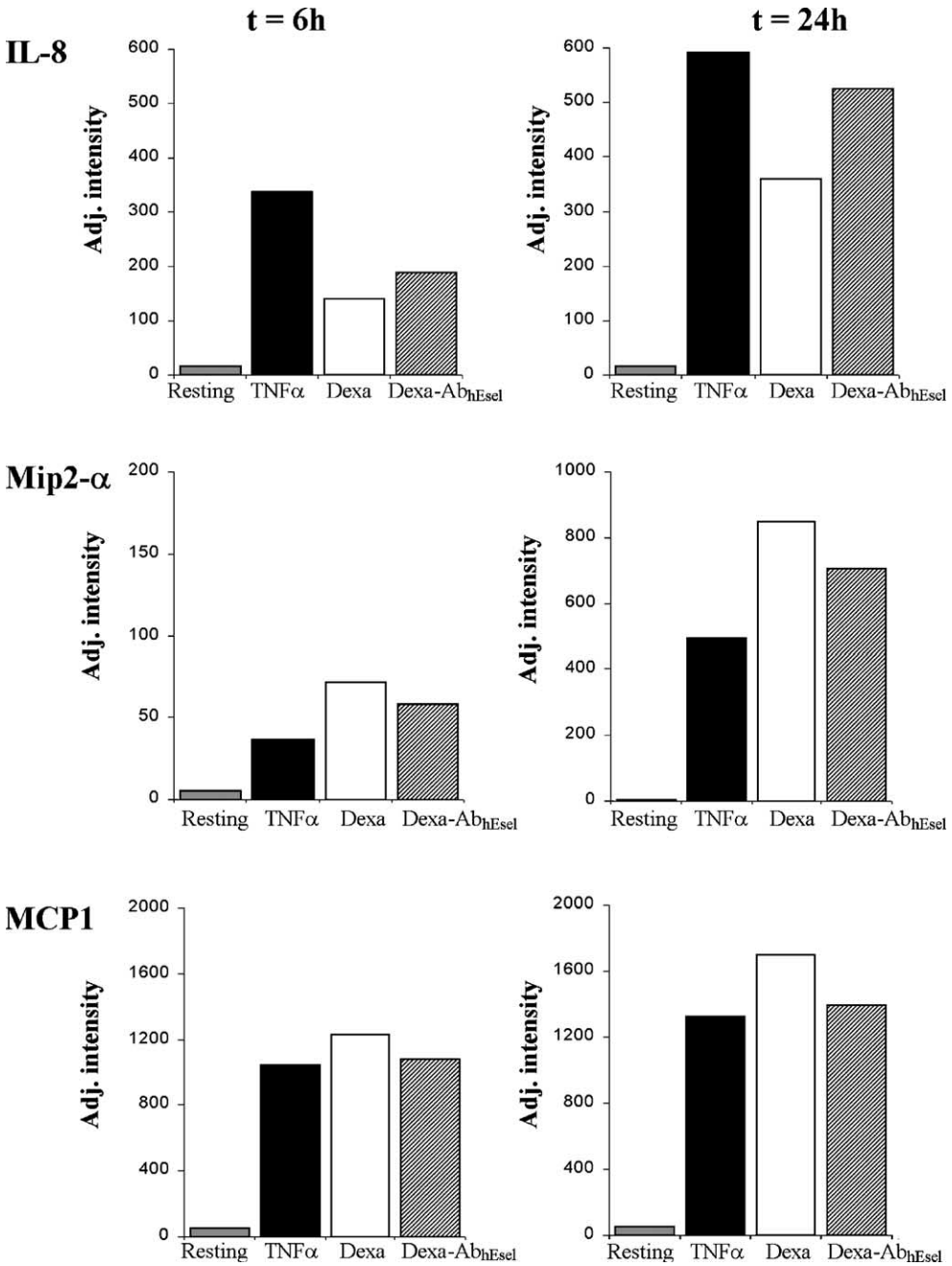

Fig. 4. Differential regulation of gene expression in HUVEC by Dexa and Dexa–Ab$_{hEsel}$. *IL-8*, *Mip2-α*, and *MCP1* were highly expressed after stimulation with 100 ng/mL TNFα for 6 hr (left-hand panel) and 24 hr (right-hand panel). Co-incubation with free Dexa (100 nM) or Dexa–Ab$_{hEsel}$ (10 μg/mL) resulted in downregulation of *IL-8* and upregulation of *Mip2-α* but had little or no effect on the expression of *MCP1*, as assessed by cDNA expression array. Adj. intensity is adjusted intensity of the hybridization signal after subtraction of the background value.

much PCR product was obtained from TNFα-stimulated cells compared to resting cells. No PCR product was detected from activated cells co-incubated with Dexa. Dexa–Ab$_{hEsel}$ conjugate caused a partial downregulation of *CD40L* expression in activated HUVEC.

To assay modulation of *IL-8* expression, competitive RT–PCR was used. This method is considered to be more quantitative than relative RT–PCR, allowing for a more accurate assessment of differences in gene expression levels. Amplification of an externally added IL8-specific competitor resulted in a PCR product that was 10% smaller than the corresponding endogenous target. As illustrated in Fig. 5C, the expression level of *IL-8* was estimated by comparing the staining intensity of the endogenous IL-8 PCR product to the intensity of the PCR product of different amounts of competitor added. Thus, in the case of resting HUVEC, 1.25×10^5 copies of competitor was sufficient for equal product intensity. Equal amount of cDNA derived from TNFα-stimulated cells contained 20 times as many copies of the IL-8 transcript (2.5×10^6).

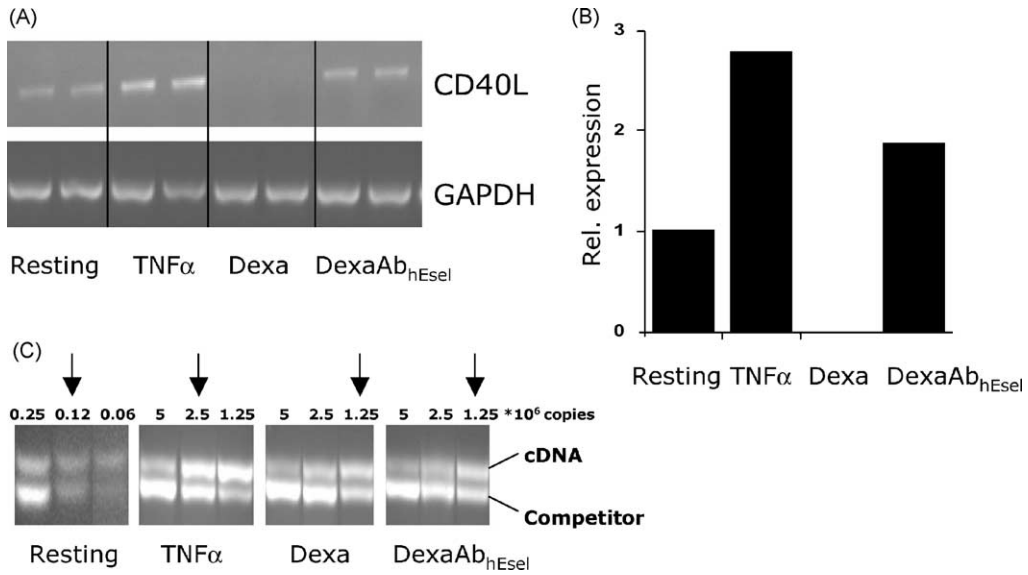

Fig. 5. Relative and competitive RT–PCR verified the results obtained by array hybridization. (A) Nested RT–PCR was carried out in duplicate for CD40L, as described in Section 2. For comparison, GAPDH was amplified from the same samples. (B) PCR-product levels for CD40L were normalized against GAPDH signals. Bars indicate the average of the duplicate lanes. (C) Competitive RT–PCR for IL-8 was carried out as described in Section 2. The upper signal was derived from the endogenous cDNA, the lower signal from the competitor. Arrows indicate the copy number of the competitor equal to the concentration of IL-8 cDNA in the sample.

Activated cells treated with either Dexa or Dexa–Ab$_{hEsel}$ contained 1.25×10^6 copies IL-8 mRNA, indicating a 50% reduction in *IL-8* expression compared to cells stimulated with TNFα in the absence of drugs. These data confirmed the ratios detected in the cDNA expression array analysis (Table 2).

4. Discussion

In the present study, the pharmacological activity of an immunoconjugate selectively delivering Dexa to activated endothelial cells was demonstrated. It is generally accepted that the anti-inflammatory effects of glucocorticosteroids are primarily based on repression of proinflammatory genes. However, different cell-types can respond differently to the inhibitory action of glucocorticoids, possibly caused by differences in relative abundance of transcription factors [4]. Conflicting results concerning the effect of Dexa on the expression of cell adhesion molecules in cultured HUVEC have been published [20,21]. The present data showed no decrease in the levels of ICAM-1 and VCAM-1 after 24 hr stimulation with TNFα in the presence of 10 μM Dexa, whereas several inhibitors of signal transduction pathways were fully capable of downregulating the surface expression of these cell adhesion molecules (Fig. 3A). The amount of IL-6 and IL-8 in the culture medium was quantified by ELISA. Whereas the secreted level of IL-6 was reduced by 10 μM Dexa, no reduction was detected when HUVEC were activated in the presence of 100 nM Dexa or 10 μg/mL Dexa–Ab$_{hEsel}$. Only a minor down-regulatory effect was detected on IL-8 secretion under all experimental conditions. This result is in line with the analysis of Krishnaswamy *et al.* [22], who detected an inhibitory effect of 1 μM Dexa on TNFα-mediated IL-8 production in three endothelial cell types, including HUVEC, although the inhibition was statistically significant in human pulmonary artery endothelial cells only.

The present study showed that Dexa can modulate complex endogenous gene expression profiles in HUVEC. Furthermore, the pharmacological activity of an immunoconjugate directed at E-selectin was demonstrated. Free Dexa was generally a stronger modulator of gene expression than the conjugate. Possibly, the effectiveness of the conjugate is limited by its uptake *via* E-selectin. However, internalization studies demonstrated that at the concentration analyzed, more than a 10-fold higher amount of Dexa was delivered into activated HUVEC *via* receptor-mediated uptake of the conjugate compared to free Dexa (Fig. 2A). Therefore, it was concluded that Dexa-mediated effects of the conjugate were not limited by uptake but more likely by intracellular processing of the conjugate. Previously, internalization of Dexa–Ab$_{hEsel}$ into activated HUVEC was studied by confocal laser scanning microscopy [13]. Noteworthy, staining intensity of the conjugate decreased in time, indicating its intracellular degradation. Yet, little is known about the efficiency of conjugate processing and cellular redistribution kinetics of the drug after degradation of the conjugate in the lysosomes. Similarly, little is known about (intra)cellular disposition kinetics of freely administered Dexa. Consequently, at present, no direct correlation between intracellular concentration of free drug and glucocorticoid receptor activation can be put forward.

The expression profiles of cytokine genes were studied by applying cDNA array analysis. In general, more genes

were upregulated after 6 hr of TNFα incubation than after 24 hr, although some mRNAs levels were considerably increased at the later time point (Table 1). A simple explanation for diverse gene-expression profiles cannot be given easily. TNFα stimulation acts on gene transcription *via* complex signaling cascades and networks, resulting in the activation of the transcription factors NFκB and AP-1 [23]. Other regulatory factors, as well as the basal transcriptional machinery, contribute to transcriptional activity. Different cofactors may be required for the transcription of different genes and in some cases repressor proteins can (temporarily) occupy promoters. Besides, differences in mRNA stability may play a role as well.

Dexa and Dexa–Ab_{hEsel} conjugate downregulated the majority of genes that showed enhanced expression after 6 hr of TNFα-stimulation (Table 2). However, in some cases, treatment with Dexa and Dexa–Ab_{hEsel} increased TNFα-induced gene expression. This different regulatory pattern was illustrated specifically for *MCP1*, *IL-8*, and *Mip2-α*. These three genes were highly expressed after both 6- and 24-hr incubation with TNFα but were modified by Dexa in a different manner (Fig. 4). MCP1, a member of the C-C chemokine family, is a secreted 14-kDa glycoprotein, pivotal for transendothelial migration of monocytes. The hybridization signal of MCP1 was the strongest on the cDNA array (Table 1). Its intensity was not considerably altered when cells were activated in the presence of free or conjugated Dexa. Similarly, Kakizaki *et al.* [24] showed that Dexa had no effect on TNFα-induced MCP1 mRNA expression in bovine glomerular endothelial cells. IL-8 mRNA expression was downregulated by Dexa as well as by the conjugate Dexa–Ab_{hEsel}. This inhibitory effect, as measured by cDNA array analysis, was verified by competitive RT–PCR demonstrating a 50% reduction in the accumulation of IL8 mRNA. Taken together, Dexa and Dexa–Ab_{hEsel}, are able to decrease, though not completely block, *IL-8* expression in HUVEC. Whereas downregulation of *IL-8* expression by Dexa in endothelial cells is an established phenomenon [4,25], to our knowledge, no reports on the regulation of *Mip2-α* by Dexa in HUVEC have been published. Mip2-α, like IL-8, is a member of the C-X-C chemokine family. Both proteins have proinflammatory activities *in vivo* and participate in recruitment and transmigration of inflammatory cells. Somewhat surprisingly, the accumulation of Mip2-α mRNA apparently increased when HUVEC were activated with TNFα in the presence of Dexa or Dexa–Ab_{hEsel}. Although effects of Dexa on TNFα-induced secretion of the murine homologue Mip2 protein has been described, the same paper detected no Dexa-mediated effect on *Mip2* gene expression by RT–PCR [26]. Further studies are required to elucidate the effect of Dexa on the protein level of Mip2-α in human endothelial cells.

Dexa did prevent the accumulation of several mRNAs to levels below the detection limit. These genes were in general expressed at relative low levels after TNFα stimulation (Table 2). We attempted to measure the concentration of four rare mRNAs (CD40L, VEGFR3, FGF3, and CCCKR1) by conventional RT–PCR but were unsuccessful, due to no detectable PCR product or loss of linear amplification when increasing the number of cycles. However, by using nested RT–PCR, the expression pattern observed in the gene expression array in the case of *CD40L*, a member of the TNFα ligand superfamily, was confirmed (Fig. 5A and B).

In conclusion, anti-E-selectin-immunoconjugate-mediated delivery of Dexa resulted in pleiotropic pharmacological effects in HUVEC. The regulation pattern of gene expression was analogous to that observed with the free drug, although the effects of the conjugate were generally less profound. Our results indicate that endothelial cells are able to respond to Dexa in a way that can contribute to the anti-inflammatory effects of the drug, although care must be taken when projecting *in vitro* data to the *in vivo* situation.

One can hypothesize that the therapeutic consequence of Dexa lies in a combinatory effect of subtle modulation of the expression of a variety of genes rather than a complete inhibition of few abundantly expressed genes. We now have, with cDNA expression array analysis, the tools to investigate this phenomenon in more detail. Without a doubt, the challenge of future investigations will be to determine, in animal models of disease, whether such an immunoconjugate can benefit from its targeting property for an increased therapeutic effect and decreased toxicity as compared to systemic administration of the free drug.

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