Identification of drugs as regulators on the activity of Egr-1 promoter in human fibroblasts transduced with AdΔegr-1-Luc7

Identificación de fármacos reguladores de la actividad del promotor Egr-1 en fibroblastos humanos transducidos con AdΔegr-1-Luc7

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Abstract

Introduction: The early growth response protein (EGR-1) is a transcription factor involved in cell differentiation and proliferation, whose expression is regulated by its promoter in response to various physical, chemical and drug factors. Hereby, we describe some of the main effects of steroid drugs and EGF-1 on promoter activity, through a reporter system transduced by AdΔegr-1-Luc7 in human primary fibroblasts (HPF). **Methods:** Human primary fibroblasts transduced with AdΔegr-1-Luc7 were exposed to betamethasone, hydrocortisone, dexamethasone, testosterone, beta-estradiol, and EGF-1 during 1, 3 and 6 h. Reporter assay was quantified by luminometry. **Results:** The activity of the promoter in presence of betamethasone, hydrocortisone, dexamethasone, testosterone and beta-estradiol were similar to the basal activity of the promoter at 1, 3 and 6 h. The positive control showed an activity 17.8 folds higher ($p \leq 0.05$) at 6 h. EGF-1 showed activity of 22.07 folds greater than cells without drug. **Conclusion:** The activity of the EGR-1 promoter in human fibroblasts is negatively regulated by steroid drugs and positively by the EGF-1.

KEY WORDS: EGR-1 promoter. Steroids. Adenovirus. Human fibroblasts. EGF-1. Gene therapy.

Resumen

Introducción: La proteína de respuesta temprana a crecimiento 1 (EGR-1) es un factor de transcripción involucrado en la diferenciación y la proliferación celulares, cuya expresión es regulada por su promotor en respuesta a diversos factores físicos y químicos, y a fármacos. Aquí se describen algunos de los principales efectos de los fármacos esteroides y del factor de crecimiento epitelial 1 (EGF-1) sobre la actividad del promotor, mediante un sistema reportero transducido por el adenovirus AdΔegr-1-Luc7 en fibroblastos primarios humanos. **Método:** Los fibroblastos primarios humanos fueron cultivados en pase 5, transducidos con AdΔegr-1-Luc7 y expuestos a betametasona, hidrocortisona, dexametasona, testosterona, beta-estradiol y EGF-1 durante 1, 3 y 6 horas. La actividad de reportero fue cuantificada por luminometría y ajustada a la concentración de proteínas totales. **Resultados:** La actividad del promotor en presencia de betametasona, hidrocortisona, dexametasona, testosterona y beta-estradiol fue similar a la actividad basal del promotor a las 1, 3 y 6 horas. El control positivo mostró una actividad 17.8 veces mayor a las 6 horas ($p \leq 0.05$). De manera similar, las células expuestas a EGF-1 mostraron una actividad 22.07 veces mayor que las células sin fármaco. **Conclusión:** La actividad del promotor Egr-1 en fibroblastos humanos es regulada negativamente por los fármacos esteroides y positivamente por el EGF-1.

PALABRAS CLAVE: Promotor Egr-1. Esteroides. Adenovirus. Fibroblastos humanos. EGF-1. Terapia génica.

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Introduction

The early growth response protein (EGR-1) is a transcription factor involved in the activation of mitogenic pathways and cell differentiation in embryonic tissues, in the lung, joint tissue, skin fibroblasts and glioma and leukemia tumor cells¹⁻⁵. EGR-1 expression is essentially regulated by the gene promoter region in response to different inducers, including factors such as temperature, osmotic and osmotic concentration changes, pressure, hypoxia, ultraviolet (UV) light, gamma radiation, and stimulation of tumor growth factor beta transcriptional activity and of proteins involved in the pathway of collagen synthesis⁶⁻¹⁰.

The specific mechanism for the regulation of transcriptional activity of the promoter is mediated by cyclic AMP-responsive binding sequences (CRE), serum response elements or through EGR-1 protein binding sites (EBS) as a negative self-regulation mechanism and that are found in the first 600 base pairs upstream of the transcription start site (TATA box)¹¹⁻¹⁵.

EGR-1 protein mitogenic effect is mediated by its folding into zinc fingers that recognize consensus sequences (CCGG/TGGGCCG) in the promoter of several genes and that, in addition, confers a self-regulation mechanism through the binding site on its own promoter (EBS)^{16,17}.

Natural and synthetic glucocorticoids are molecules with pleiotropic activity, whose mechanism of action is mediated by cytoplasmic receptors that translocate the nucleus and activate glucocorticoid-responsive sequences¹⁸. Once in the nucleus, the drug-receptor complex binds to sequences (5'AGAACAN-NNTGTTCT-3') of promoters that regulate the activity of protein kinases or proteins involved in mitogenic signaling pathways (Fos, Jun and Egr-1), as well as of mitogen-activating kinases (MAP-kinases, ERK and ELK)^{19,20}.

Steroid drugs represent one of the essential therapeutic elements to regulate systemic and local inflammatory processes. However, their effects on promoter activity are not fully known, as in the case of EGR-1 in skin fibroblasts²¹⁻²³.

Herein, we report the effects of steroid-derived drugs (dexamethasone, hydrocortisone, betamethasone), steroid hormones such as beta-estradiol and testosterone, and epithelial growth factor 1 (EGF-1), with the purpose to identify pharmacological regulators (negative or positive) on the EGR-1 protein promoter.

Method

Obtainment of primary human fibroblasts

The experimental assays described in this article are derived from the research protocol with registration number INR-CII-14B-2011, authorized by Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra Internal Research Committee. The primary human fibroblasts (PHF) were obtained from the Skin and Tissue Bank of the same Institute, in accordance with the standardized protocol (PR-DQ17- ISO-9001- 2008). Briefly, the PHFs were isolated from medium-thickness dermal allografts obtained from multi-organ donors and subjected to enzymatic cleavage with 1% collagenase in modified Dulbecco's phosphate buffered solution (D-PBS) for 1 hour. They were immediately centrifuged at 1000 G for 10 minutes at 4 °C and washed with modified Dulbecco's minimum essential medium (D-MEM) supplemented with antibiotics and heat-inactivated fetal bovine serum (FBS) (Gibco-Invitrogen Corp.). Subsequently, the obtained cells were seeded in 10-cm diameter boxes in the presence of D-MEM medium supplemented with 10% FBS and antifungal antibiotic at standardized concentrations (penicillin G, streptomycin and amphotericin B).

Culture of primary human fibroblasts

The PHF cells were cultured in D-MEM medium with 10% FBS and 5 mM ascorbic acid in an environment with 5% CO₂, 20% O₂ and 100% humidity at 37 °C, until 80% confluence was reached in collagen-covered T-75 culture boxes (GrainerBioOne, Corp., Germany). The cells were disaggregated and reseeded until passage 5. Subsequently, they were cryopreserved in liquid nitrogen until the drug induction assays were performed.

Drug-induction experimental model

The system for evaluation and analysis of the pharmacological induction effect on the promoter is based on a reporter system, transduced by a third-generation non-replicating adenovirus (AdΔ3). The cells were cultured and infected with a non-replicating recombinant adenovirus that transduces the Egr-1 promoter cassette, to direct the expression of the luciferase gene cloned in *Ad-egr-1-Luc7* (Fig. 1). Luciferase is an enzyme expressed in lampyridae (*Insecta-Coleoptera*) that hydrolyzes luciferin in the presence of ATP and

Figure 1. Structure of the Ad-Egr1-Luc7 non-replicating recombinant adenovirus. Diagram illustrating reporter cassette incorporation into the non-replicating adenoviral vector. The 600 bp of EGR-1 promoter were cloned upstream of the luciferase gene sequence as reporters.

whose light emission can be quantified in a luminometer. The light counts are dependent on the concentration of the protein in a total cellular extract of exposed cells. In turn, the increase or decrease in luciferase concentration results from negative or positive regulation of the promoter in the presence of different drugs (Fig. 1).

Large-scale production of the Ad-Egr1/Luc7 recombinant adenovirus and AdCMV-hGFP

Inocula of each virus were packaged in HEK-293 cells (Invitrogen Corporation-AATC, USA) and purified on a large scale for in vivo applications from 250 million HEK-293 cells, in D-MEM/F12 medium (50:50) with 5 mM L-glutamine and 10% heat-inactivated and filtration-sterilized BFS (Thermo-Hyclone Corp., USA). At the end of the infection time, the packaging cells were maintained under usual culture conditions for virion maturation and were monitored by optical microscopy until the appearance of 90% of cytopathic effect, which occurred 48 hours after infection (Fig. 2).

Subsequently, the cells were collected, concentrated and lysed by three cycles of thermal shock in liquid nitrogen alternating with a temperature of 37 °C for 3 minutes, respectively, in a class II-AB laminar flow hood, for purification of the recombinant virions.

Non-replicating adenoviral vectors purification

In order to separate mature from immature virions, as well as from cellular debris, total lysates were kept cold and purified by ultracentrifugation in cesium chloride gradients at 28,000 G, at 4 °C for 12 hours (WX-100 Thermo Corp., USA). Subsequently, the virions

were isolated by sterile puncture and dialyzed (in the presence of magnesium and 5 mM HEPES at 4 °C for 12 h) in a 10K-MWCO membrane cassette (Pierce Bioscience, USA). The purified recombinant virions were diluted in viral preservation solution (1% albumin and 5 mM HEPES and Mg^{++}) and stored at -70 °C. The infectivity efficiency and titration of the infecting viral particles were determined with a plate assay in HEK-293 cell monolayers in serial dilutions (10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11}), as well as by reading the dialysate optical density at 260 nm.

Infection and determination of multiplicity of infection with Ad-CMV-hGFP adenovirus in primary human fibroblasts

To determine the optimal quantity of recombinant adenovirus necessary for PHF efficient transduction, an AdCMV-hGFP adenovirus that expresses the humanized green fluorescent protein (GFP) was used. For this, 3×10^5 cells were seeded in six-well boxes (Costar, Madison, USA). After 24 hours, the cells were infected at 25, 50, 75, 100 and 200 multiplicity of infection (MOI) ratios with the Ad-CMV-hGFP reporter adenovirus in serum-reduced medium. After 2-hour exposure, the infectious medium was replaced with fresh medium and the transduced cells were maintained on culture for 24 hours. To document infection efficiency, the GFP signal was sought using an Axioplan 2 equipment (Carl Zeiss, Germany). GFP emitted an excitable light signal at 488 nm in Ad-CMV-hGFP adenovirus-infected cells.

Identification of the EGR-1 protein in human fibroblasts

The co-localization of the EGR-1 protein was carried out by means of immunofluorescence and was

Figure 2. Ad CMV-hGFP virions maturation. The HEK-293 cells were transfected and subsequently infected for large-scale production in order to determine the maturation process of the different virions generated at 48 hours and documented by confocal laser microscopy at 488 nm.

documented by confocal laser microscopy. For this assay, 1×10^7 PHFs were seeded in a culture chamber for microscopy purposes (Slide-chamber Nunc Corp., USA) in D-MEM medium with 2% FBS. The cells were exposed to UV light for 30 seconds, fixed in PBS and 1% glutaraldehyde for 10 minutes at 4 °C. The slides were permeabilized with phosphate buffered saline with 1% Tween 20 (TBS) for 15 minutes, followed by 4 hours of blockage at 4 °C with 100 mM glycine in TBS. Incubation with the primary antibody was performed in 0.5% TBS and 1% albumin with a 1:100 dilution of the human anti-EGR-1 IgG antibody developed in rabbits (Santacruz, Biotech). A secondary anti-rabbit antibody developed in mice and coupled to PE-Cy7 was used at the dilution recommended by the manufacturer and incubated for 2 hours at 4 °C. The slides were washed and covered with Vecta Shield mounting medium (Santacruz, Biotech) for analysis at 630 nm by confocal laser microscopy on a LSM-100 Meta equipment (Carl Zeiss, Germany).

Drug-induced luciferase reporter assay

3×10^5 PHF were seeded in a six-well box under standard culture conditions until their adherence. After

12 hours, the medium was replaced with 1% FBS to reduce endogenous EGR-1 promoter activity to baseline values during the following 24 hours. At the end of this period, the medium was replaced by D-MEM medium (Gibco-Invitrogen Corp., USA) with drugs at the following concentrations: testosterone 25 ng/ml, beta-estradiol 50 ng/ml, hydrocortisone 50 ng/ml, betamethasone 50 ng/ml and by EGF-1 at a concentration of 50 ng/ml, all for 1, 3 and 6 hours. At the end of each period, the cells were lysed for protein extraction and luminometry analysis.

Total protein extraction

Total protein extraction from cells exposed to different drugs was carried out as follows: at the end of the exposure time (1, 3 and 6 h) the cells were washed with PBS (with Ca^{++} and Mg^{+}) (Invitrogen Corp.) at 37 °C and lysed with 300 μl of lysis solution (Glo-Cell lysis Buffer, Promega Corp., USA) at room temperature for 5 minutes. The lysate was collected in a sterile microtube, stirred for 1 minute and immediately centrifuged at 5000 G at 4 °C. The supernatant was recovered and stored at -20 °C until its reading in a luminometry quantifier.

Figure 3. EGR-1 protein co-localization. Human anri-EGR-1 antibody-coupled rhodamine showed ultraviolet light-exposed fibroblasts cytoplasmic and nuclear localization.

Luciferase activity quantification

Luminometry assays were carried out with 50 μ l of the cell lysate, at a 1:1 ratio of Glo-Luciferase substrate volumes (Promega, USA). The mixture was incubated for 10 minutes at 23 °C and the reaction was immediately quantified in a luminometer (DTX-800 Beckman Coulter). The luciferase present in the protein extract hydrolyzes the luciferin in the presence of ATP, which generates a luminous signal that is identified by the reader and expressed in light counts per second (LC/s). LC/s were adjusted to the protein concentration in order to obtain Egr-1 promoter-controlled luciferase activity actual data. The LC/s analysis was performed with the Multimode Analysis Software program (Beckman Coulter, USA).

Results

The MOI required to efficiently transduce PHFs was determined with the *Ad-CMV-hGFP* reporter adenovirus. The data obtained indicated that the use of a MOI of 75 is sufficient to efficiently transduce the cassette of interest to the PHFs in an episomal form, without producing a cytopathic effect (data not shown).

Previously, typing of the cells obtained and cultured in vitro was carried out with immunolocalization of marker proteins such as actin 1 and collagen I as surface markers (data not shown). This process is systematically carried out to characterize the cells obtained from skin allograft donors.

To determine EGR-1 protein endogenous expression, induction assays were performed in order to

overexpress the protein. These assays were carried out by exposing the PHF cells to UV light for 30 seconds and processing them for immunolocalization, with cytoplasmic and nuclear localization of the EGR-1 protein being shown (Fig. 3).

Effect of steroid drugs on the Egr-1 promoter in primary human fibroblasts

PHFs transduced with *Ad-Egr-1Luc7+* showed a baseline luciferase activity with 1478.33, 1340.66 and 1333.36 LC/s at 1, 3 and 6 hours, respectively. As expected, luminous activity was not found in non-transduced cells, since luciferase is not expressed in human cells (Fig. 4).

The effects of betamethasone, hydrocortisone, testosterone and beta-estradiol were low in comparison with control cells (PHF with 2% FBS), which showed an activity of 175 LC/s per microgram of total protein.

Hydrocortisone had a negative effect on the promoter activity. The luminous counts obtained were 781.66, 1433.66 and 1782.66 LC/s at 1, 3 and 6 hours, respectively. Betamethasone showed an activity of 1254.33, 1572 and 4419.33 LC/s at 1, 3 and 6 hours, respectively; and beta-estradiol, 2189.66, 2434.66 and 3925.33 LC/s at the same time-points.

Steroid hormones effect on the primary human fibroblasts promoter

PHFs were exposed to testosterone and beta-estradiol in order to explore the effect of steroid hormones

Figure 4. Endogenous promoter baseline activity in primary human fibroblasts (PHF). To determine the effect of inducers on protein expression, the presence of the EGR-1 protein in PHFs was documented, and its expression as a platform for the reporter system action was determined. FBS: fetal bovine serum.

on the EGR-1 promoter in transduced fibroblasts. Testosterone showed 1353, 2720.66 and 2070.33 LC/s values at 1, 3 and 6 hours, respectively. Beta-estradiol showed 2189.662, 1434.66 and 3925.33 LC/s values at 1, 3 and 6 hours, respectively.

Effect of EGF-1 on the promoter in primary human fibroblasts

A positive regulatory effect was observed in cells exposed to EGF-1. The cell extracts showed an average of 29,511.33 LC/s at 6 hours, with similar values to the positive control (PHFs transduced with Ad-Egr-1/Luc7+ and exposed to 10% FBS showed a reading of 23,915.33 LC/s).

Discussion and conclusions

Cell replication is an indispensable and essential element in tissue repair processes. In the case of patients with large tissue losses, such as heavily burned subjects, cell proliferation is essential for the granulation and healing of injured tissues. The EGR-1 protein is essential for inducing the expression of growth and differentiation factors, as well as activation of mitogenic pathways that promote tissue recruitment, differentiation and repair. However, the initial triggering of this expression mechanism is abolished in the autocrine and paracrine regulatory pathways between keratinocytes, fibroblasts and hair

follicle cells, which are absent in heavily burned individuals. The use of drugs that allow maintaining this mechanism stimulated, or identifying negative regulatory effects, is essential to determine the most efficient pharmacological therapeutics for the patient in acute state.

The results obtained suggest that the use of steroids such as betamethasone and hydrocortisone has an inhibitory effect on gene regulation through the EGR-1 promoter, indicating that hydrocortisone has the most potent inhibitory effect of all tested drugs. These effects cannot be concluded as being direct, but they allow determining the promoter biological effect. Testosterone and beta-estradiol also showed an inhibitory growth pattern, with similar values to those of cells in basal conditions (PHF in Ad-egr-1+ with 2% FBS) (Fig. 5).

EGF-1 positively regulates the EGR-1 promoter

Interestingly, EGF-1 positively regulates the promoter activity. The increase in luciferase activity shows a similar pattern in relation to the positive control of cells cultured under normal conditions (Fig. 5). This finding allows continuing with further studies to locally use recombinant EGF-1 in heavily burned patients and accelerate the process of clinical recovery, while controlling for negative regulators that withhold this effect after completing the process of tissue repair (Fig. 6).

Figure 5. Comparative effect of steroid drugs on the EGR-1 promoter in human fibroblasts. The quantitative analysis of protein extracts of transduced cells exposed to the different drugs is summarized in this graph. The presence of fetal bovine serum (FBS, 10%) as EGFR-1 activator was used as promoter induction positive control. The graph shows a higher epithelial growth factor-1 stimulation pattern than that of FBS. The other steroidal drugs tested showed lower or similar activity to the baseline expression activity in cells subjected to FBS depletion (2%).

Figure 6. EGR-1 promoter pharmacological regulation model. Ad-Egr-1 Luc adenovirus-infected cells are exposed to ultraviolet light and then are subjected to a medium with 50 ng of betamethasone. The cells are lysed to determine reporter gene activation by light activity quantification.

Even when these drugs have the advantage of being approved for clinical use, further studies are needed to explore their effect on cell proliferation, as well as a study of the mitogenic pathways involved, in order to explore their interaction and clinical utility.

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Ethical responsibilities

Protection of people and animals. The authors declare that no experiments have been conducted on humans or animals for this research.

Confidentiality of data. The authors declare having followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare there are no conflicts of interest.

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