Adenovirus E1A orchestrates the urokinase-plasminogen activator system and upregulates PAI-2 expression, supporting a tumor suppressor effect

V. FERNáNDEZ-SORIA[1], M.E. LLEONART[1], M. DIAZ-FUERTES[1], R. VILLUENDAS[2], R. SáNCHEZ-PRIETO[3], A. FABRA[4] and S. RAMÓN Y CAJAL[1]

[1]Department of Pathology, Hospital Vall d'Hebron, Passeig Vall d'Hebron 119-129, 08035 Barcelona; [2]Centro Nacional de Investigaciones Oncológicas (CNIO), c/ Melchor Fernandez Almagro 3, 28029 Madrid; [3]Laboratorio de Oncología Molecular, CRIB, Facultad de Medicina, Avda. Almansa s/n, 02071 Albacete; [4]Departament de Cancer & Metastasis, Institut de Recerca Oncologica (IRO), Gran Via, Km 2.7, 08907 L'Hospitalet de Llobregat, Barcelona, Spain

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Abstract. Invasiveness and metastatic potential are the two most important properties defining malignancy. The adenovirus E1A (Ad-E1A) gene has a dual effect as a proliferative gene and as a tumor-suppressor gene, decreasing tumor growth and the metastatic potential of malignant cells. In order to study genes related with the antimetastatic effect of Ad-E1A in human cells, we performed a microarray analysis using OncoChip™. In three independent experiments, NIH3T3, IMR90 and MDA MB 435 cells were infected with pLPC retroviruses carrying the adenovirus 12S E1A gene or the GFP gene. We analyzed cDNA expression by using the CNIO OncoChipTM, a cDNA microarray containing a total of 6386 genes represented by 7237 clones. uPA, uPAr, tPA, PAI-1 and PAI-2 were also studied at RNA and protein levels. Microarrays of cDNA expression, RT-PCR and Western blot performed in IMR90 E1A-expressing cells showed downregulation of uPA, uPAr, tPA, PAI-1 and upregulation of PAI-2. These results were confirmed in NIH3T3 and MDA MB 435 breast carcinoma cells, with PAI-2 upregulation by RT-PCR and Western blot. In addition, zymographic analysis demonstrated that E1A expression greatly reduced the gelatinase activity of the pro-MMP2 and -MMP9 proteins. We propose that adenovirus E1A may orchestrate the expression of most members of the urokinase-plasminogen activation system, downregulating potentially invasive genes and upregulating PAI-2, which is associated with a better prognosis in human tumors.

Correspondence to: Dr Santiago Ramón y Cajal, Department of Pathology, Hospital Vall d'Hebron, Pg. Vall d'Hebron 119-129, 08035 Barcelona, Spain
E-mail: sramon@vhebron.net

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Introduction

Early region E1A of human adenovirus type 5 (ad5) encodes two major proteins of 289R and 243R that exert a myriad of cellular effects. Most of the effects are modulated by binding to different cellular proteins (1-4). Interestingly, the E1A gene appears to have a dual and paradoxical effect on different kinds of cells. The E1A protein can activate DNA synthesis and cell proliferation, or promote apoptosis in serum-free medium or after DNA-damage (5,6). The pro-apoptotic effect of E1A has been shown in several malignant human tumors and has been reported to be independent of the p53 protein (7). A previous report by our group associated E1A expression with inhibition of the PI3K/AKT proliferative pathway (8). Importantly, adenovirus E1A exerts a clear antitumoral effect by constitutive E1A expression after injection of either retrovirus E1A producer cells (7,9) or E1A liposomes into the tumor. Moreover, E1A-expressing adenovirus has been associated with a decrease in the malignant potential of human cancers, preventing metastatic disease in human breast carcinoma xenografts (10).

To produce metastasis, malignant cells must detach from the primary tumor and then invade and transverse the basement membrane and extracellular matrix. Adenovirus E1A has been shown to up-regulate E-cadherin, which increases cell-cell adhesion, and to repress expression of matrix-degrading proteins, such as stromelysin-1 (MMP-3), interstitial collagen (MMP-1), MMP-9 and urokinase-type plasminogen activator, uPA (11,12). The 289R E1A protein also increases the expression of TIMP-2, which leads to a further decrease in MMP activity, downregulates the adhesion molecule, CD44, and upregulates the NM23 protein (13).

In order to explain the antimetastatic effect of Ad-E1A in human cells, we performed a microarray analysis using the Central Nacional de Investigacion Oncológicas (CNIO)

OncoChip™ . We focused on the modulation of genes related with invasiveness and metastasis and we demonstrated that E1A decreases mRNA expression of tPA, uPA and uPAR. In contrast, E1A up-regulates the expression of PAI-2 mRNA, a member of the urokinase plasminogen activator system that is associated with a more favorable prognosis in most human tumors (14,15).

Materials and methods

Cell lines and retroviral infection. GP293 cells were purchased from Clontech (Clontech, CA), NIH 3T3 and IMR90 human fibroblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and a previously described human breast carcinoma cell line (MDA-MB-435) (10) was used. Cell lines were grown in DMEM medium (Gibco BRL) containing 10% FBS (BioWhittaker Europe), at 37˚C and 5% CO_2. Cell lines were infected with retroviruses, as previously described (8); pLPC E1A (IMR90-E1A and MDA-MB-435-E1A, NIH 3T3-E1A), or pLPC GFP (IMR90-GFP and MDA-MB-435-GFP, NIH 3T3-GFP). Polybrene was used at a final concentration of 10 μg/ml and puromycin at 4 μg/ml (Sigma, St. Louis, MO).

Construction and analysis of cDNA microarray. The CNIO OncoChip, is a cDNA microarray containing a total of 6386 genes represented by 7237 clones. Human cDNA clones were purchased from Research Genetics (Huntsville, AL). The set consists of 7237 sequence-validated IMAGE clones, including 5253 clones representing known genes and the remaining 1984 clones representing expressed sequence tags (ESTs). Cancer-related clones (2489) were printed twice. The list of genes on the array can be found at the following website: http://bioinfo.cnio.es/data/oncochip.

cDNA microarray target preparation. Target RNA (1-3 μg) was amplified using a T-7-based *in vitro* transcription system as described previously (16). RNA (5 μg) from IMR90-E1A was directly labeled with cyanine 5 (Cy5)-conjugated deoxyuracil triphosphate (dUTP), and 5 μg of RNA from the IMR90-GFP was labeled with cyanine 3 (Cy3)-conjugated dUTP as reference. Additionally, a dye-swap experiment was carried out. The CNIO OncoChip was used for all the microarray studies and hybridizations were performed as previously described (16). Scanning was performed with a Scanarray 5000 XL (GSI Lumonics, Kanata, ON, Canada) and images were analyzed with GenePix 4.0 Pro Software (Axon Instruments, Union City, CA).

Data analysis and normalization. Fluorescence intensity measurements were subjected to automatic background subtraction, and Cy3:Cy5 ratio values were normalized to the median ratio value of all spots in the array. The sum of the median background for each channel was calculated, and spots with total intensity values below the calculated sum of median backgrounds were discarded. All ratio values were log transformed (base 2). Inconsistent duplicates were discarded, consistent duplicate spots and genes were averaged, and genes with <80% of available data were excluded from further analysis (17).

RNA extraction and reverse transcription. Total cellular RNA from cultured cells was extracted with TriReagent™ (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Samples were then treated with RQ1 RNAse-free DNAse (Promega, Madison, WI) according to the manufacturer's instructions.

Total RNA (1 μg) was reverse transcribed using ImProm-II Reverse Transcriptase (Promega) in a total volume of 20 μl. Briefly, RNA samples were preincubated with oligo-dT primer for 5 min at 70˚C and held on ice for 5 min. Samples were then added to the reaction mix and incubated for 1 h at 37˚C, followed by a 15-min incubation at 70˚C to denature the enzyme.

Quantitative real-time RT-PCR. The PCR product from GAPDH amplification was cloned and serial dilutions from 10^8 to 10^2 were made to perform a standard curve. LightCycler-FastStart DNA Master Sybr-Green I (Roche, Grenzacherstrasse, Switzerland) was used to amplify 1 μl of cDNA samples (50 ng RNA) and 1 μl of one plasmid dilution from the external standard. Procedures were performed following the manufacturer's indications on a LightCycler instrument (Roche). Reaction conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 sec, 67˚C for 5 sec, and 72˚C for 13 sec. The second derivative maximum method was used to analyze the data. Expression from each sample was normalized with respect to its GAPDH expression running in parallel. The primers used were: PAI-1-F, 5'-GGCCATTACTACGACATCCTGGAACTGC-3', and PAI-1-R, 5'-TGGAGAGGCTCTTGGTCTGAAAGACTCG-3'; PAI-2-F, 5'-TGTTCTTGTTGCTTCCAGATGAAATTGCCG-3', and PAI-2-R, 5'-CATTCCTCTCCGACATCCCTGAGAAATTGG-3'; uPA-F, 5'-GACCATCTGCCTGCCCTCGATGTATAACG-3', and uPA-R, 5'-CAGCTCACAATTCCAGTCAAAGTCATGCG-3'; uPAR-5, 5'-CCCATGAATCAATGTCTGGTAGCCACCGGC-3', and uPAR-3, 5'-ACAGTCTGGCAGTCATTAGCAGGGTGATGG-3'; GAPDH-5, 5'-GGCAAGGTCATCCCTGAGCTGAACG-3', and GAPDH-3, 5'-CACCCTGTTGCTGTAGCCAAATTCG-3'.

Western blot. Cell extracts were collected in RIPA lysis buffer. Medium from overnight serum-starved cells was concentrated 20x using a VivaSpin concentrator (VivaScience, Hannover, Germany) with a 10,000 MW cut-off. Protein quantification was performed by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein extract (50 μg) was subjected to 10% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked by washing in methanol, allowed to dry, and probed against PAI-2 (dilution 1:300), PAI-1 (1:100), uPA (1:100), uPAR (1:200) or E1A (1:300), followed by incubation with HRP-conjugated secondary antibody. Antibody detection was achieved by enhanced chemiluminescence (Amersham, Buckinghamshire, UK), according to the manufacturer's protocol. Monoclonal antibodies against E1A (M73 Clone) and uPA (Ab-1) were from Oncogene Science (Boston, MA). The anti-PAI-2 goat polyclonal antibody (A-19) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). uPAR (goat polyclonal, ref. AF807) and PAI-1 (clone TJA6, ref: NCL-PAI-1) were purchased from Novocastra Laboratories Ltd. (Newcastle, UK).

Table I. Microarray analysis of several downregulated and upregulated genes after E1A infection in normal human IMR90 cells.[a]

Name	IMR90-E1A/IMR90	SD
PLAT	0.378	0.08
PLAUR	0.240	0.08
PLAU	0.106	0.13
PAI-1	0.325	0.12
PAI-2	2.657	0.42
MMP1	0.423	0.10
MMP2	0.125	0.06
COL1A2	0.086	0.03
COL3A1	0.129	0.06
COL6A3	0.046	0.01
PCNA	2.784	0.31
CDC2	3.026	0.67

[a]Note the positive control of the microarray represented by the PCNA mRNA upregulation by E1A. Mean results of triplicate experiments from IMR90-E1A cells are shown. IMR90-GFP were considered as control levels. Genes were deemed to be upregulated or downregulated if the difference ratio was at least 2-fold. SD, standard deviation from at least three points in different experiments.

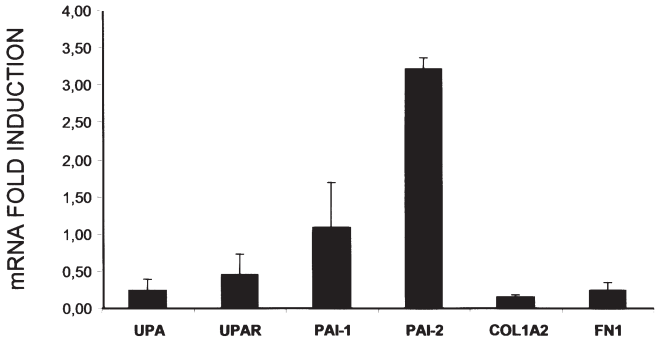

Figure 1. Quantitative real-time RT-PCR of IMR90 cells infected with E1A is shown. Results were normalized respect GADPH mRNA.

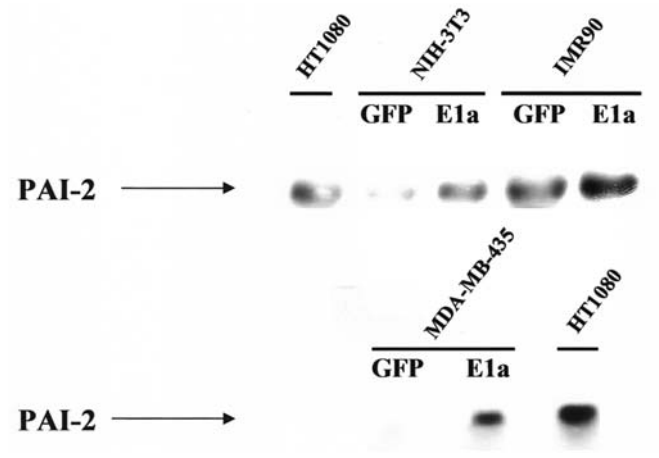

Figure 2. Western blot of PAI-2 in IMR90, NIH-3T3 and MDA-MB-435. HT1080 cells were used as a positive control of PAI-2 expression. PAI-2 was detected in the supernatant of cultured cells.

Zymogram. To analyze MMP2 and MMP9 activity in IMR90 and MDA-MB-435 cell-culture supernatants, $1x10^5$ MDA-MB-435 and IMR90 cells were plated onto a 24-well tissue-culture plate and allowed to adhere. The medium was then replaced with serum-free medium. Cell-free supernatant (30 μl) was mixed with 10 μl of nondenaturing sample buffer and proteins were subjected to electrophoresis on 10% Zymogram gelatin gel (Invitrogen, Carlsbad, CA) in SDS running buffer. The gel was developed and stained with Coomasie blue following the manufacturer's protocols.

Results

Microarray findings. Data analysis of hybridization signals from three independent experiments, including dye-swap replicates, enabled us to identify several up- and down-regulated genes in IMR90-E1A cells after normalizing the data to IMR90-GFP control cells. In Table I, detailed data are shown for the urokinase plasminogen activation system, uPA, uPAR, PAI-1 and PAI-2, and other genes implicated in extracellular matrix degradation, such as MMP1, MMP2 and collagen. To confirm and extend the array data, we investigated the expression of these genes at the mRNA and/or protein levels throughout the quantitative real-time RT-PCR, Western blot and enzymatic activity assays.

Confirmation of PAI-2 upregulation in IMR90-E1A infected cells. Several genes identified by microarray screen were further examined for differential expression by quantitative real-time PCR (RT-PCR). RNA was extracted from three independent E1A and control (pLPC alone or GFP) infections for each cell line, including those used in the microarrays. We confirmed downregulation of mRNA coding for uPA, uPAR, PAI-1, COL1A2 and FN1 and upregulation of coding for PAI-2 (Fig. 1). Value 1 refers to the basal levels shown by control cells (GFP-infected). In order to determine whether mRNA expression was reflected in protein levels, we performed Western blot of PAI-2 in IMR90 cells. PAI-2 protein was upregulated in the E1A-expressing cell lines (Fig. 2). E1A expression was confirmed by RT-PCR and Western blot.

PAI-2 upregulation in MDA-MB-435 cells. MDA-MB-435-E1A and MDA-MB-435-GFP cell lines were grown in culture for RNA extraction. RNA was retrotranscribed to cDNA and PAI-2 semiquantitative and real-time quantitative RT-PCR were performed. The results from MDA-MB-435 cells are shown in Fig. 3. Cell extracts were collected from MDA-MB-435 cells expressing E1A or GFP. In the PAI-2 Western blot, protein upregulation was shown in E1A-expressing cells. Similar results were obtained with NIH 3T3 mouse cells infected with E1A. PAI-2 was highly upregulated in E1A-expressing cell lines (Fig. 2). Expression of other components of the uPA system (uPA, uPAR, PAI-1) was assessed by RT-PCR and Western blot. No significant differences were observed between GFP- and E1A-infected cells when analyzing other components of the uPA system (data not shown). E1A

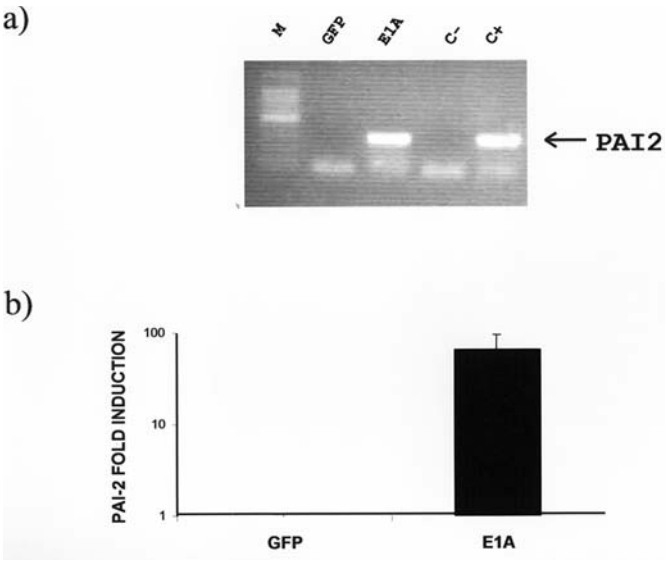

Figure 3. Qualitative (a) and quantitative (b) RT-PCR of PAI-2 is shown in MDA-MB-435 cells. PAI-2 upregulation is shown. M, markers; C- and C+, negative and positive control of PAI-2-expressing HT1080 cells is shown.

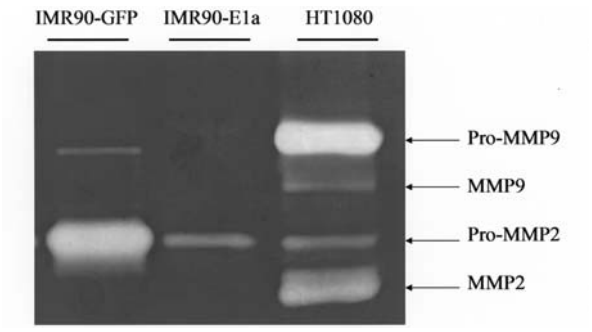

Figure 4. Zymogram study of IMR90 cells infected with GFP and E1A. Lower activity of pro-MMP9 and pro-MMP2 can be observed in E1A-expressing cells. Positive control of HT1080 cells shows pro- and full activity MMP2 and MMP9. The full activity of both MMPs (pro and mature form) is shown after TPA treatment in HT1080 cells to validate a positive control of the immature MMP form. This figure represents the results of one of at least three independent experiments.

expression was confirmed by qualitative and quantitative real-time RT-PCR and Western blotting.

MMP activity in E1A-infected cells. Zymographic analysis was performed to determine whether gelatinase activity varied according to E1A- versus GFP-expressing IMR90 cells (Fig. 4) or MDA-MB-435 cells (data not shown). Zymographic analysis demonstrated that E1A expression greatly reduced the gelatinase activity of pro-MMP2 (highly expressed in fibroblasts) and pro-MMP9 in IMR90 cells, thereby confirming the microarray results. Weak differences in gelatinase activity between GFP and E1A expression were observed in MDA-MB-435 cells.

Discussion

In this study, we show that adenovirus E1A can modulate the expression of several members of the urokinase-plasminogen activation system in normal and malignant human cells.

Our microarray results show downregulation by the adenovirus E1A gene of several members of the plasminogen family, uPA, uPAr, tPA, and PAI-1, and clear up-regulation of the inhibitory factor, PAI-2, at both the mRNA and protein levels in IMR90 fibroblasts. PAI-2 is a member of the serpin superfamily of proteins (serin protease inhibitors) and is an efficient inhibitor of uPA and tPA. PAI-2 constitutive expression is observed in monocytes, macrophages, placenta, fibroblasts, fibroblast-like cells, differentiated keratinocytes, and stratified squamous epithelia of the esophagus, vagina, oral mucosa and tongue (18). Interestingly, PAI-2 upregulation has been associated with a better prognosis in human tumors (14). PAI-2 has been proposed to exert two apparently unrelated functions. First, when PAI-2 is secreted, it modulates the activity of the urokinase plasminogen activator. A decrease in fibrinolysis can regulate cell migration; in addition, over-expression of PAI-2 in human melanoma cells inhibits spontaneous metastasis in severe combined immunodeficient (SCID) mice (19). This inhibition of extracellular proteolysis may explain the association of PAI-2 with a favorable prognosis in human tumors and inhibition of metastasis. The second role of the PAI-2 protein is related with inhibition of apoptosis (20,21).

The cellular mechanisms and pathways that regulate PAI-2 transcription are still not completely defined. The PAI-2 gene is located at chromosome 18q21.3 and contains 8 exons. Two regulatory AP-1 like elements and one CRE-like element have been identified in the PAI-2 promoter (18,22). An association has been described between the AP-1 family of transcription factors and E1A (23,24).

Several proteins have been tested to modulate PAI-2 expression in macrophages and other cell types. In this regard, IL-1, IL-2, INF-γ, phorbol ester and TNF-A, are inducers of PAI-2 expression, whereas TGF-ß downregulates PAI-2. At the transcription level, gastrin stimulates CREB and AP-1 via activation of PKC and Rho A and then stimulates PAI-2 expression via both CREB and AP-1. Interestingly, the tumor suppressor gene, menin, is able to inhibit PAI-2 expression via binding to junD, which is a member of the AP-1 family of transcription factors (22). A recent observation has related PAI-2 expression to stabilization of the retinoblastoma protein. Because of the interaction between PAI-2 and pRb, PAI-2 may enhance Rb's tumor supressor activity, suggesting a potential therapeutical role for PAI-2 (25). This novel association could explain the correlation of PAI-2 with a better prognosis in many human tumors (26-29).

E1A can activate and repress the transcription of a large number of genes (30) through the CR1 region (31). Adenovirus E1A can prevent the interaction between CBP and the c-fos component of the AP-1 complex by repressing the AP-1 promoter. Repression of AP-2 has also been described for the E1A gene, as well as downregulation of some metalloproteinase genes, such as MMP-1, MMP-3, MMP-9 and uPA. The fact that microarray screening found downregulated genes by E1A expression related to extracellular matrix alterations, such as collagen 1A2, col 3A, col 6A3, MMP1, MMP2 and fibronectin, reinforces the observed antimetastatic role of E1A previously shown *in vivo*. In order to confirm the involvement of the E1A gene in MMP2 and MMP9 inhibition, we

performed zymographic analysis in IMR90 cells infected with E1A. Fig. 4 clearly shows the downregulation of mature and premature concentrations of MMP2 and MMP9 proteins in E1A-infected cells; thereby, confirming the microarray results.

Adenovirus E1A modulates the transcription of hundreds of genes by affecting coactivator proteins such as p300/CBP, and downregulating AP1 and AP2, but also activates the expression of many other genes through the CR1, CR2 or CR3 regions (32). Transcription can be regulated by two general mechanisms: interaction and activation with multiprotein coactivator complexes that modify chromatin structure, or promoting the assembly of a preinitiation complex with the general transcription factors and pol II on promoter regions in DNA. Adenovirus E1A can act through the first mechanism, modifying the chromatin structure (33,34), and there is evidence that the N-terminal region of the E1A protein contains a transcriptional activator domain, which has the capacity to activate target genes directly. Moreover, the CR3 region can activate transcription by interacting with the Sur2 subunit of mediator complexes (35,36).

From our microarray data, we conclude that adenovirus E1A protein expression in human cells downregulates genes related with invasion and metastasis, such as fibronectin, collagenases and metalloproteinases, as well as most of the genes linked to the urokinase-plasminogen activator system, and upregulates the PAI-2 inhibitor. This complex and orchestrated regulation of adenovirus E1A suggests a novel mechanism by which E1A can exert an antimetastatic effect in human tumors.

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