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The Interaction of Adenovirus E1A With p300 Family Members Modulates Cellular Gene Expression to Reduce Tumorigenicity

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Abstract The use of adenovirus serotype 2 or 5 (Ad2/5) E1A as therapy for human malignancy requires an understanding of the mechanisms involved in E1A-induced tumor suppression. The prevailing use of E1A in the treatment of human malignancy stresses the non-immunologically mediated, anti-tumorigenic activities of E1A. However, the capacity of E1A to elicit a NK-cell and T-cell anti-tumor immune response and to sensitize tumor cells to lysis by immune effector molecules utilized by NK cells and T cells is also an important component of the anti-tumorigenic activity of E1A. This immune-mediated anti-tumorigenic activity of E1A is not shared by functionally similar viral oncoproteins such as the human papillomavirus type 16 (HPV16) E7 oncoprotein and is dependent on the capacity of E1A to interact with transcriptional coadapter, p300. To further define the molecular mechanisms whereby E1A reduces tumorigenicity, we compared total cellular gene expression in H4 cells, a human fibrosarcoma cell line, to gene expression in H4 cells stably expressing E1A, E7, or mutant forms of E1A that do not bind p300. The expression of E1A, but not E7, in H4 cells modulated the expression of cellular genes that may promote apoptosis, enhance immunogenicity and reduce tumor cell metastasis. The difference in the ability of E1A and E7 to modulate the expression of cellular genes that may influence tumorigenicity was largely attributable to distinct interactions of E1A and E7 with p300. Results of this study will be useful in designing novel strategies to augment the anti-tumorigenic activities of E1A. J. Cell. Biochem. 100: 929–940, 2007.
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Key words: adenovirus; E1A; papillomavirus; E7; p300; tumor immunity; microarray

The adenovirus (Ad) *E1A* gene is currently being used in clinical trials for a variety of tumors either through delivery of E1A in liposome vectors or expressed in conditionally replicative adenovirus (CRAd) [Madhusudan et al., 2004; Yamamoto, 2004]. In HER2/neu-expressing tumors, E1A downregulates HER2/neu through direct inhibition of transcriptional coadapter p300/CBP. However, E1A also reduces tumorigenicity independent of HER2/neu, through complex mechanisms such as sensitizing cells to the induction of apoptosis, maintenance of an epithelial phenotype, inhibition of angiogenesis and metastasis, and interaction with transcriptional corepressor, CtBP [Chinnadurai, 2004; Frisch, 2004].

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The capacity of E1A to elicit an NK-cell and T-cell anti-tumor immune response is an important component of the anti-tumorigenic activity of E1A. Animal studies demonstrate that tumor cells that express E1A are over 1,000-fold less tumorigenic than parental fibrosarcoma cells [Cook et al., 1996; Routes et al., 2000b]. The ability of E1A to reduce primary tumor formation is mediated in large part by a vigorous immune response (T cells, NK cells, and macrophages) directed against tumor cells that express E1A [Cook et al., 1996; Routes et al., 2000b; Miura et al., 2004]. E1A also sensitizes tumor cells to lysis by macrophages and NK cells and to the cytolytic effector molecules used by these killer cells, including TNF-α, TRAIL, Fas-L, and nitric oxide [Routes and Ryan, 1995; Routes et al., 2000a; Cook et al., 2003; Miura et al., 2003, 2004], thereby enhancing the effectiveness of the cellular anti-tumor immune response. The capacity of E1A to elicit an anti-tumor immune response and sensitize tumor cells to cytolytic effector molecules is dependent on the capacity of E1A to bind the transcriptional co-adaptors p300/CBP [Cook et al., 1996; Miura et al., 2004].

In contrast to reducing tumorigenicity, the E1A oncoprotein can also immortalize primary cells, including human cells. The E1A oncoproteins from all serotypes of Ad contain two highly homologous conserved regions (CR1, CR2) that are essential for the ability of E1A immortalize cells ([Berk, 2005], and references therein). The E7 oncoprotein expressed by high-risk human papillomaviruses also expresses conserved regions (CR1, CR2) that are highly homologous to the CR of E1A. The CR of E1A and E7 bind a common set of cellular proteins (e.g., pRb, p300) and are interchangeable for immortalization function [Brokaw et al., 1994; Munger et al., 2004]. However, HPV16 E7 neither elicits an NK-cell and T-cell anti-tumor immune response when stably expressed in tumor cells nor does E7 sensitize tumor cells to lysis by macrophages and NK cells and to the cytolytic effector molecules used by these killer cells. Thus, while E1A and E7 alter the expression of a common set of cellular genes necessary for cellular immortalization, E1A and E7 must differentially regulate the expression of cellular genes that regulate sensitivity to immune-mediated rejection and reduced tumorigenicity.

To define the molecular mechanisms whereby E1A reduces tumorigenicity by immune- and non-immune-mediated mechanisms, we compared total cellular gene expression in H4 cells, a human fibrosarcoma cell line, to gene expression in H4 cells stably expressing Ad5 E1A, HPV16 E7, or two mutant forms of E1A that do not bind p300 (E1A-RG2, E1A-dl1104). These cell lines were chosen because they have been extensively characterized with respect to their sensitivity to NK cells, macrophages and immune effector molecules as well as their tumorigenicity in nude mice. H4-E1A is more sensitive to lysis by NK cells and macrophages and is less tumorigenic than H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cells. These cellular phenotypes are stable over prolonged tissue culture passage [Routes and Cook, 1995; Routes and Ryan, 1995; Routes et al., 1996, 2000a; Cook et al., 1999, 2003; Miura et al., 2003, 2004]. The cDNA microarray analysis identified several cellular genes that were differentially regulated by E1A that may reduce tumorigenicity through immune- and non-immune-mediated mechanisms. These data also demonstrated that the interactions of E1A and E7 with cellular p300 have very different effects on tumor cell gene expression.

MATERIALS AND METHODS

Cell Lines

H4, a subclone of fibrosarcoma line HT1080, and H4-E1A (P2AHT2A) were provided by Dr. S. Frisch, The Burnham Institute, La Jolla, CA. H4-E7 expresses the HPV16 E7 oncoprotein [Routes et al., 2000b]. H4-E1A-RG2 and H4-E1A-dl1104 express mutated E1A proteins that do not bind p300 [Routes et al., 1996; Cook et al., 1999; Miura et al., 2004]. E1A-RG2 has a point mutation, (R to G at residue 2) and E1A-dl1104 has a deletion of 12 amino acids in CR1 [Egan et al., 1988; Wang et al., 1993]. The 13S and 12S mRNA are the two major transcripts encoded by *E1A*. These E1A transcripts translate proteins that differ only by a unique internal stretch of 46 amino acids (CR3) present in the larger form of E1A. Expression of either the 13S or 12S forms of E1A sensitizes tumor cells to immune-mediated killing [Cook et al., 1996]. H4-E1A and H4-E1A-dl1104 cell lines express both the 12S and 13S forms of E1A while the H4-E1A-RG2 cell line expresses only the 12S RNA form of E1A. The C57/B6-derived, methylcholanthrene-induced sarcoma cell lines MCA102 and MCA205 were provided by Dr. N. Restifo

(National Institutes of Health, Bethesda, MD). MCA102-E1A and MCA205-E1A express both the 12S and 13S forms of Ad5 E1A [Routes et al., 2000b, 2005b]. Cells were maintained in DMEM with 5% calf serum, 15 mM glucose, and antibiotics.

cDNA Microarray Hybridizations

Three cDNA microarray experiments were performed, each using total cellular RNA from the H4, H4-E1A, and H4-E7 cell lines. Two cDNA microarray experiments were performed using cells that express the p300-binding mutants, H4-E1A-RG2 and H4-E1A-dl1104. Total cellular RNA was isolated from 10^7 cells using the RNeasy kit (Qiagen, Valencia, CA). cDNA was prepared from 10 μg total RNA using T7-$(dT)_{24}$ primer and SuperScript II (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA was synthesized from the cDNA using the ENZO BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY). The biotin-labeled cRNA was fragmented and hybridized to hu6800 arrays (Affymetrix, Santa Clara, CA) at 45°C, overnight. Arrays were washed and scanned following protocols provided by Affymetrix.

cDNA Microarray Data Analysis

Microarray fluorescence intensities were analyzed using GeneChip Operating Software (Affymetrix). Non-biological variations in signal intensities were corrected by applying a linear regression-scaling factor to the fluorescence data from each hybridization [Schadt et al., 2001]. This allowed direct comparison between microarrays without bias towards increased or decreased mRNA abundance based on nonspecific differences in average microarray intensity. The relative abundance of mRNA (signal log ratio) was calculated using the one-step Tukey's biweight method based on the average of the differences in fluorescence intensities between the labeled cellular RNA binding to perfectly matched probes as compared to corresponding probes containing a single mismatched nucleotide. Signal log ratios are expressed as $\log_2$. A difference in mRNA abundance of at least twofold (signal log ratio of 1) was considered significant.

Northern Analysis

Total cellular RNA was isolated using RNAzol B reagent, according to the manufacturer's protocol (Tel-Test Inc., Friendswood, TX) and was quantitated by spectrophotometry. Ten micrograms of RNA was electrophoresed through a MOPS-formaldehyde gel and was transferred to a positively charged Magna-Graph nylon membrane (ISC BioExpress, Kaysville, UT) by upward capillary transfer. Secreted protein acidic and rich in cysteine (SPARC; BM-40/osteonectin) and human homologue of yeast Atx1 (HAH1) RNAs were detected using probes representing the full length cDNA sequences [Hung et al., 1998; Schiemann et al., 2003]. SPARC cDNA (774 bp) was provided by Dr. W. Schiemann, National Jewish Medical and Research Center, Denver CO. HAH1 cDNA (500 bp) was provided by Dr. J. Gitlin, Washington University, St. Louis, MO. cDNA probes were labeled with ^{32}P-dCTP by random priming, using the RadPrime kit according to the manufacturer's recommendations (Invitrogen). ^{32}P-labeled probes were denatured, then hybridized to the northern membranes at 50°C overnight, in buffer containing 5× Denhardt's solution, 5× SSC, 0.5 mg/ml salmon sperm DNA, 50 mM $NaPO_4$, and 0.2% SDS. Membranes were washed extensively in 2× SSC/0.1% SDS and 0.2× SSC/0.1% SDS, then exposed to X-ray film for autoradiographic analysis. X-ray film was scanned and densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA).

RT-PCR

Total cellular RNA was isolated from H4, H4-E1A, H4-E7, and H4-E1A-RG2 cell lines and quantitated as described above. Reverse transcription (RT) was performed using an oligo dT primer and SuperScript III enzyme (Invitrogen). Polymerase chain reaction (PCR) was performed on 2 μl of the RT-generated cDNA with VEGF-C and actin-specific primers using Taq DNA polymerase (Fisher Scientific, Pittsburg, PA). PCR reactions were heated to 95°C for 2 min, then underwent 25 cycles of 95°C/30 s, 50°C/30 s, and 72°C/1 min, followed by a 10 min extension at 72°C. The VEGF-C primers amplified a 583 bp product. (Sense: CCAGTGTAGATGAACTCATG; Antisense: AGCCAGGCATCTGCAGATGTG). The commercially available actin primers (Applied Biosystems, Foster City, CA) generated a 320 bp product. PCR products were visualized by electrophoresis of 10 μl through a 1% agarose gel stained with ethidium bromide.

Flow Cytometry

The presence of CD9 on the surface of cells was analyzed by flow cytometry. Cells were labeled using monoclonal antibody ALB6 [Boucheix et al., 1983] (Provided by E. Rubinstein, Institut Andre-Lwoff, Universite Paris) and a fluorescein-conjugated anti-mouse secondary antibody (Cappel, West Chester, PA). Fluorescently labeled cells were analyzed using a FACScan cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

ELISA

The secretion of TGF-β1 by cells in culture was measured by ELISA, using a kit according to the manufacturer's recommendations (ELISA Tech, Aurora, CO). H4, H4-E1A, MCA102, MCA102-E1A, MCA205, and MCA205-E1A cell lines were cultured under serum-free conditions for 18 h, then medium was collected from the cells, treated with HCl to release bound TGF-β1, and assayed by capture ELISA using antibodies specific for TGF-β1 (ELISA Tech). The amount of TGF-β1 produced by each E1A-expressing cell line was normalized to the amount produced by the E1A(−) parental cell line.

RESULTS

cDNA Microarray Analysis

Total cellular gene expression was compared between the cell lines using data generated from hybridization of cellular mRNA to Affymetrix microarrays. Table I contains a list of all genes whose RNA abundance was changed significantly when comparing the H4-E1A cell line to H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines in three independent microarray experiments. E1A-RG2 has a single amino acid change in the amino terminal region and E1A-dl1104 has a 12 amino acid deletion in conserved region 1, both changes abolish p300-binding by E1A [Egan et al., 1988; Wang et al., 1993]. mRNAs that encode products that promote apoptosis (IEX-1, myeloperoxidase, mitochondrial creatine kinase) were more abundant in the H4-E1A, compared to the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines. Furthermore, mRNAs encoding products that protect against apoptosis (ceramide glucosyltransferase, anti-oxidants) were decreased in H4-E1A. The H4-E1A cell line expressed decreased levels of mRNAs encoding products with anti-oxidant functions including nicotinamide N-methyltransferase (NNMT), ferritin light chain, HAH1, and hexokinase. The levels of expression of stress-response genes, including heat shock protein 70 (Hsp70) and S100A13, were also greater in the H4-E1A cell line.

The expression patterns of different TGF-β superfamily members were markedly altered in the H4-E1A cell line compared to expression patterns in the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines. Expression of Smad 7, an inhibitor of the TGF-β signaling pathway, was increased, whereas expression of TGF-β and TGF-β receptor II were decreased in the H4-E1A cell line. Furthermore, expression of TGF-β-induced genes (SPARC, transglutaminase, and BIGH3) was decreased in H4-E1A compared to expression in the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines.

Several genes known to promote metastasis or associated with metastatic tumors including SPARC, collagenase IV, Mac25, aminopeptidase N, VEGF-C, calcyclin, HMGIC, prointerleukin-1β, and uPA were expressed at lower levels in H4-E1A cells as compared to the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines. Additionally, expression of CD9, which inhibits metastasis, was increased in the H4-E1A cell line.

Genes whose expression was significantly changed in three independent experiments comparing H4-E7 and H4 are listed in Table II. Only one gene (α-tubulin) had increased and two genes (BDP-1 and stearoyl-coenzyme desaturase) had decreased mRNA abundance in H4-E7, compared to the H4 cell line. The similarity in cDNA microarray data from the H4-E7 and H4 cell lines (Table II) contrasts with the large number of gene expression differences between the H4-E1A and parental H4 cells or either cell line that express an E1A mutant that does not bind p300 (Table I).

Confirmation of cDNA Microarray Data

Expression of genes with mRNA levels that were slightly (HAH1), moderately (SPARC, VEGF-C), or considerably (CD9) different between the H4-E1A and H4-E7 cell lines was analyzed further to confirm the microarray data.

By microarray analysis, HAH1 mRNA abundance was decreased slightly (signal log ratio

TABLE I. Genes Whose Expression Was Changed Significantly by Microarray Analysis in the H4-E1A Versus H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 Cell Lines

		Signal Log Ratio[a] of E1A vs.			
Accession#	Description	H4[b]	E7[b]	RG2[c]	dl1104[c]
Gene expression					
X51345	junB	3.8	3.0	2.2	3.0
U20734	junB	3.4	4.4	2.9	4.2
M62831	Transcription factor ETR101	1.8	2.2	1.5	2.1
X13293	B-myb	1.3	1.3	1.3	2.3
X52541	Early growth response protein 1	4.0	5.0	1.5	3.4
X70940	Elongation factor 1 alpha-2	−3.2	−2.9	−4.2	−3.3
U28749	High-mobility group isoform I-C	−2.2	−2.6	−2.1	−2.9
M63838	Interferon-gamma induced protein 16	−2.3	−2.8	−2.3	−3.0
U59831	Forkhead related activator 4	−1.4	−1.9	−1.6	−1.1
Z46629	SOX9	−2.4	−2.0	−2.6	−2.5
M88163	hSNF2-like	−2.3	−2.3	−2.5	−2.2
Inflammation					
M11717	Heat shock protein (hsp 70)	4.3	3.7	3.8	2.9
M59830	HSP70-2 (MHC III)	4.7	3.6	2.7	2.3
AB000584	TGF-beta superfamily protein	−3.5	−2.7	−2.1	−3.8
D50683	TGF-betaIIR alpha	−1.9	−1.6	−1.3	−2.2
J03040	SPARC/osteonectin	−2.5	−2.0	−1.2	−1.9
M55153	Transglutaminase	−4.9	−4.0	−3.3	−4.3
M77349	BIGH3	−3.5	−2.5	−1.3	−3.0
AF010193	Smad7	2.3	2.3	1.6	2.0
M93056	Mononcyte/neutrophil elastase inhib	1.8	1.9	2.4	2.4
X82200	Staf50	−3.0	−3.9	−3.0	−3.9
X04500	Prointerleukin 1 beta	−6.2	−4.3	−3.0	−6.2
U04636	Cyclooxygenase-2	2.8	3.7	4.0	5.7
Apoptosis					
M19507	Myeloperoxidase	4.2	3.4	2.2	4.3
U36922	FKHR	0.8	1.2	1.0	1.5
S81914	IEX-1	1.3	1.5	1.5	1.8
X57351	1-8D gene from interferon-inducible	−2.5	−2.5	−2.4	−2.3
D50840	Ceramide glucosyltransferase	−2.0	−1.6	−1.2	−1.2
Cell Surface					
L13210	Mac-2 binding protein	−6.0	−5.2	−4.7	−6.2
U59289	H-cadherin	−3.9	−4.3	−3.3	−4.1
X02419	uPA	−6.0	−5.3	−5.0	−5.6
U19147	GAGE-6	−2.8	−3.1	−3.1	−3.9
X52022	Type VI collagen alpha3 chain	−4.2	−4.1	−5.3	−5.3
M59911	Integrin alpha-3 chain	−1.3	−1.4	−1.6	−2.0
M22324	Aminopeptidase N/CD13	−2.4	−2.0	−2.3	−2.5
M23197	Differentiation antigen (CD33)	−3.0	−3.2	−2.9	−2.9
U43142	VEGF-C	−2.7	−2.8	−2.6	−1.2
M28249	Very late antigen-2 (VLA-2)	−2.8	−2.2	−2.1	−3.3
HG987	Mac25	−3.5	−3.9	−4.5	−5.4
M55267	EV12 protein	−3.6	−3.7	−3.5	−2.5
J03779	CALLA	−2.2	−2.6	−1.7	−2.5
M33653	Alpha-2 type IV collagen	−2.6	−2.3	−2.2	−2.0
M55593	Collagenase IV	−2.4	−1.8	−2.4	−3.4
U03877	Extracellular protein (S1-5)	−3.8	−3.9	−2.9	−5.2
X56253	Mannose 6-phosphate receptor	−1.5	−1.3	−1.3	−1.8
HG174	Desmoplakin I	4.0	3.2	3.1	4.6
M38690	CD9 antigen	4.7	5.2	4.0	7.1
Metabolism/Cellular Homeostasis					
J04469	Mitochondrial creatine kinase	2.1	2.7	3.2	4.3
X59834	Glutamine synthase	3.1	2.3	2.9	5.1
Y00339	Carbonic anhydrase II	3.1	2.6	1.6	3.5
X97065	Sec23B	1.6	1.4	1.5	1.6
M75126	Hexokinase 1	−1.2	−1.0	−1.2	−0.9
M10942	Metallothionein-Ie	−3.0	−3.3	−2.1	−1.3
X95190	Branched chain acyl-CoA oxidase	−3.7	−3.6	−3.3	−2.0
U08021	Nicotinamide N-methyltransferase	−3.2	−3.4	−3.2	−3.1
U51010	Nicotinamide N-methyltransferase	−2.3	−2.8	−2.7	−2.2
U01317	Epsilon-globin gene	−5.6	−5.8	−4.3	−5.5
L11005	Aldehyde oxidase (hoax)	−2.2	−3.2	−2.9	−1.4
M11147	Ferritin L chain	−1.4	−0.8	−1.2	−1.6
U70660	HAH1	−1.1	−1.1	−1.0	−0.7
L35546	Gamma-glutamylcysteine synthetase	2.0	1.8	2.2	1.3
Transport/Cytoskeleton					
U33632	Two P-domain K+ channel TWIK-1	2.4	2.6	2.1	2.2
X58528	Peroxisomal membrane protein 70	2.2	1.6	1.9	1.7
J02923	L-plastin/p65	2.5	3.0	3.3	2.7

(*Continued*)

TABLE I. (*Continued*)

Accession#	Description	Signal Log Ratio[a] of E1A vs. H4[b]	E7[b]	RG2[c]	dl1104[c]
X05608	Neurofilament subunit NF-L	−5.0	−5.0	−5.3	−4.9
X81420	hHKb1	−6.0	−5.9	−6.4	−6.4
U11717	Ca activated K+ channel (hslo)	−4.0	−4.3	−4.8	−5.8
Signal transduction					
X99920	S100 calcium-binding protein A13	1.9	1.8	1.4	1.8
Z29067	nek3 protein kinase	3.7	4.2	3.4	4.2
U60808	CDP-diacylglycerol synthase	4.1	3.8	2.8	3.1
X95632	Arg protein tyrosine kinase-binding	1.9	2.1	1.4	1.8
U19523	GTP cyclohydrolase I	1.4	1.5	1.5	2.1
U42412	5-AMP-activated protein kinase	−1.6	−1.6	−1.4	−1.8
U48959	Myosin light chain kinase	−3.9	−3.6	−3.7	−4.6
HG2788	Calcyclin	−1.9	−1.9	−1.5	−1.5
X05908	Lipocortin	−4.4	−4.2	−3.3	−2.2
U25997	Stanniocalcin precursor	−6.4	−6.6	−6.5	−6.4
Cell division					
U68485	BIN-1	−2.5	−1.9	−1.9	−2.0
U31875	Hep27 protein	−2.6	−2.2	−2.7	−1.8
X59798	PRAD1 cyclin	−2.4	−2.8	−2.7	−2.7
M94856	Fatty acid-binding protein homologue	−3.9	−5.1	−4.2	−3.9
U18300	Damage-specific DNA-binding protein	−1.7	−1.9	−1.5	−1.2
M94250	Retinoic acid inducible factor (MK)	2.9	3.6	3.1	3.2
M97815	Retinoic acid-binding protein II	1.7	1.7	2.6	3.3
U02082	Guanine nucleotide regulatory protein	2.4	2.2	3.5	3.0
Miscellaneous					
U15552	Acidic 82 kDa protein	1.9	1.3	1.6	1.5
U16954	AF1q	2.0	1.9	2.2	1.5
S78771	NAT = CpG island-associated gene	3.0	3.0	2.4	3.2
X57348	Clone 9112	3.8	4.7	3.8	5.4
Z84497	Cosmid O14 on chromosome 6	3.1	3.0	2.2	3.6
X76029	Neuromedin U	2.1	2.2	1.4	4.0
D42040	KIAA9001	3.0	2.8	2.3	3.1
D79990	KIAA0168	2.8	4.5	4.1	4.4
D80010	KIAA0188	−2.2	−2.2	−1.6	−2.0
M31166	Tumor necrosis factor-inducible 14	−3.1	−2.7	−2.5	−3.1
M22960	Protective protein	−1.7	−1.7	−1.6	−2.0
D28589	KIAA00167	−2.6	−2.5	−1.4	−1.9
D29810	Unknown product	−3.9	−2.6	−2.2	−2.3
X73608	Testican	−2.0	−2.2	−3.3	−3.1
X16663	Hematopoietic lineage cell specific	−3.2	−2.6	−3.5	−1.1
X17042	Hematopoietic proteoglycan core	−1.7	−1.8	−1.3	−1.6

[a]Signal log ratio is the relative abundance of mRNA expressed as log base 2. A positive value indicates an increase in abundance in the H4-E1A cell line, while a negative value indicates a decrease in abundance in the H4-E1A cell line.
[b]Values represent the mean signal log ratio calculated from three independent experiments.
[c]Values represent the mean signal log ratio calculated from two independent experiments.

−0.7 to −1.1) in the H4-E1A cell line as compared to the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cell lines (Table I). Levels of SPARC mRNA were moderately decreased (signal log ratio of −1.2 to −2.5) in the H4-E1A cell line (Table I). Northern analysis confirmed that the levels of HAH1 mRNA were slightly reduced in H4-E1A cells compared to H4 cells, and significantly reduced in H4-E1A cells compared to H4-E7 and H4-E1A-RG2 cells

TABLE II. Genes Whose Expression Was Changed Significantly by Microarray Analysis in the H4-E7 Versus H4 Cell Line

Accession#	Description	Signal log ratio[a] of E7 vs. H4: Experiment 1	Experiment 2	Experiment 3
D16227	BDP-1 protein	−1.2	−1.3	−1.6
HG3930-HT4200	Stearoyl-coenzyme A desaturase	−1.5	−1.7	−3.5
X06956	Alpha-tubulin	1.6	1.7	1.9

[a]Signal log ratio is the relative abundance of mRNA expressed as log base 2. A positive value indicates an increase in abundance in the H4-E1A cell line, while a negative value indicates a decrease in abundance in the H4-E1A cell line.

(Fig. 1). Northern analysis also confirmed that the levels of SPARC RNA were lower in H4-E1A cells, compared to H4, H4-E7, and H4-E1A-RG2 cell lines (Fig. 1).

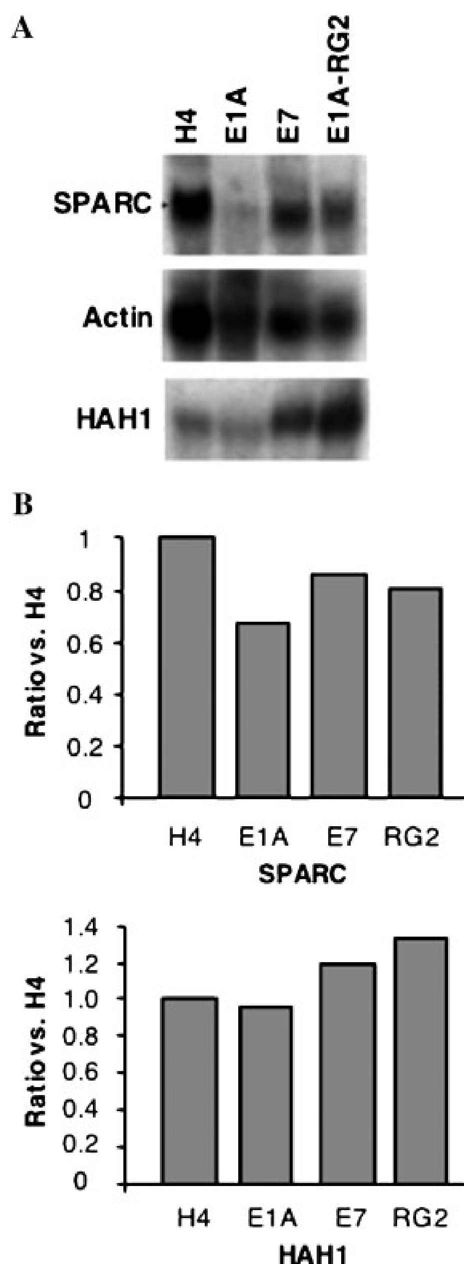

Fig. 1. H4-E1A cells express decreased SPARC and HAH1 RNA. **A**: Northern blot analysis of RNA from H4, H4-E1A, H4-E7, and H4-E1A-RG2 cell lines using a ^{32}P-DNA probe specific for SPARC, HAH1, or actin. **B**: Northern in (A) were quantitated using Quantity One software (Bio-Rad). Band intensity data for SPARC and HAH1 were normalized to the corresponding lane on the actin blot, then graphed as a ratio of normalized expression versus the H4 cell line.

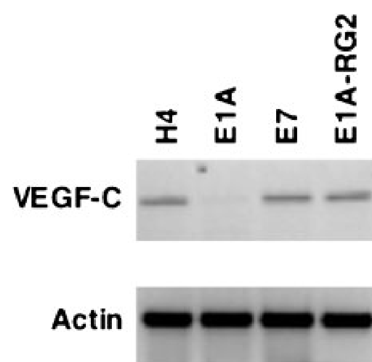

Fig. 2. H4-E1A cells express decreased VEGF-C RNA. RT-PCR analysis of RNA from H4, H4-E1A, H4-E7, and H4-E1A-RG2 cell lines using primers specific for VEGF-C or actin.

mRNA levels of VEGF-C were also moderately decreased (signal log ratio of -1.2 to -2.6) in the H4-E1A cell line (Table I). RT-PCR was used to detect VEGF-C or actin-specific RNA in the H4, H4-E1A, H4-E7, and H4-E1A-RG2 cell lines (Fig. 2). Consistent with the microarray data, VEGF-C RNA was not amplified from H4-E1A cells while it was amplified from H4, H4-E7, and H4-E1A-RG2 cell lines. Actin mRNA was amplified from all of the cell lines.

The microarray analysis indicated that CD9 mRNA levels were considerably higher (signal log ratio ≥ 4.0) in H4-E1A cells than in the H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104 cells (Table I). By FACS analysis, CD9 was highly expressed on the surface of H4-E1A cells, but was not detected on H4, H4-E7, or H4-E1A-RG2 cells (Fig. 3).

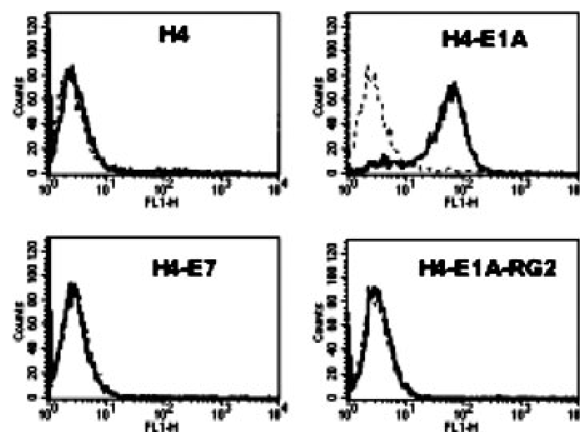

Fig. 3. H4-E1A cells have increased expression of CD9. FACS analysis of cell surface expression of CD9 on H4, H4-E1A, H4-E7, and H4-E1A-RG2 cell lines. Solid lines indicate incubation with an anti-CD9 antibody and FITC-conjugated secondary antibody. Dotted lines indicate incubation with FITC-conjugated secondary antibody alone.

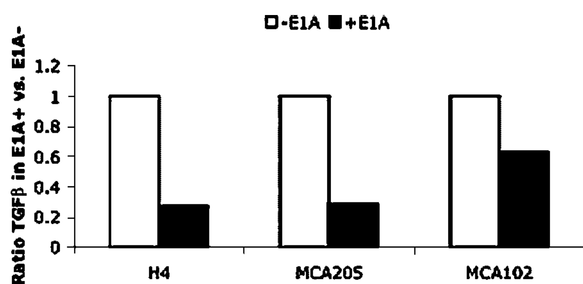

Fig. 4. Expression of E1A in human and murine tumor cell lines decreases secretion of TGF-β1 from these cells. TGF-β1 was measured by ELISA on medium from E1A+ and E1A− tumor cell lines of human (H4) and murine (MCA102 and MCA205) origin. Data are represented as the ratio of TGF-β1 from cells expressing E1A versus the E1A− control.

By Western analysis, we previously demonstrated that the expression of Hsp70 was increased in H4-E1A cells, compared to H4 or H4-E7 cells [Miura et al., 2003]. This result is consistent with the microarray data on Hsp70 gene expression in these lines (Table I).

The expression of a number of genes in the TGF-β superfamily was altered in the H4-E1A cell line. We therefore compared secretion of TGF-β in three different E1A-expressing cell lines versus their parental cell lines. In addition to the H4 and H4-E1A cell lines two murine tumor cell lines (MCA102 and MCA205) were included in this analysis to determine if these findings will be applicable to a murine model of tumorigenesis. Expression of E1A in H4, MCA102, and MCA205 cell lines resulted in decreased secretion of TGF-β1 into the culture medium (Fig. 4). These data confirmed the microarray data on TGF-β expression (Table I) in the H4-E1A cell line, and demonstrated that TGF-β is also decreased by E1A expression in two murine tumor cell lines.

DISCUSSION

Our studies provide insight on the molecular mechanisms whereby the expression of E1A reduces tumorigenicity, which is dependent upon binding to p300. We demonstrated that E1A, but not E1A-RG2, E1A-dl1104, or E7, increased the expression of genes that would be predicted to promote an innate and adaptive cell-mediated anti-tumor immune response (e.g., Hsp70) while at the same time inhibited the expression of genes that have potent immunosuppressive effects (e.g., TGF-β). E1A also altered the expression of cellular genes that would be predicted to reduce the cellular defense against immune-mediated apoptosis. This additional activity of E1A may further enhance the immune-mediated rejection of tumor cells that express E1A.

TGF-β promotes tumorigenicity through its potent immunosuppressive activity, including inhibition of macrophage effector functions and antigen presentation, T-cell differentiation and proliferation, and the production of inflammatory cytokines and chemokines by T cells, NK cells, and macrophages [Takeuchi et al., 1997; Gorelik and Flavell, 2001]. In several tumor model systems, blocking TGF-β enhances development of tumor-specific cellular immune responses [Gorelik and Flavell, 2001]. E1A inhibits the biological activities of TGF-β at several levels. Prior studies showed that E1A inhibits cell signaling initiated by TGF-β by repressing TGF-β-RII expression and by interrupting Smad association with p300/CBP [Missero et al., 1991; Kim et al., 1997; Nishihara et al., 1999]. Consistent with these studies, we showed that E1A repressed TGF-β-RII mRNA expression in H4 cells. We also found that E1A may inhibit signaling through the TGF-β receptor by increasing the expression of inhibitor Smad7 (Table I). E1A also repressed the expression of TGF-β and other TGF-β family members (Table I and Fig. 4). TGF-β-induced genes such as BIGH3, transglutaminase, and SPARC were also inhibited by E1A (Table I), a finding consistent with reduced TGF-β production and signaling through the TGF-β receptor. Our finding that expression of E1A in human tumor cells decreases secretion of TGF-β1 was also extended to two murine tumor cell lines, MCA102 and MCA205.

Our microarray data showed that E1A upregulated the expression of Hsp70 and other stress-response genes in H4 tumor cells (Table I). We have previously shown that Hsp70 protein expression is increased by E1A in H4 cells as well as the murine tumor cell line, MCA102 [Miura et al., 2003]. Hsp70 exhibits several pro-immunogenic functions that may enhance the anti-tumorigenic activity of E1A. Hsp70 increases the production of inflammatory cytokines and activates NK cells, macrophages, and dendritic cells. Through its ability to chaperone tumor antigens to dendritic

cells, Hsp70 augments antigen-specific, T-cell-mediated, anti-tumor immune responses ([Panjwani et al., 2002; Srivastava, 2002], and references therein). Hsp70 is also expressed on the surface of cells and triggers NK-cell-mediated degranulation and sensitizes cells to apoptosis by granzymes [Multhoff, 2002; Gross et al., 2003a,b]. As a result of these many pro-immunogenic activities, Hsp70 is being utilized to augment antigen-specific immune responses in a number of vaccines [Srivastava, 2002].

We hypothesize that the ability of E1A to inhibit expression of TGF-β and increase expression of Hsp70 would act synergistically to promote a vigorous innate and adaptive immune response to tumor cells, resulting in tumor rejection. The demonstration that the data obtained on the H4 tumor cells lines was applicable to murine tumor cells (MCA102 and MCA205) is an important first step in establishing the biological relevance of these findings. Studies are in progress to determine if modulation of the expression of Hsp70, TGF-β or Hsp70 and TGF-β in MCA102 or MCA205 cells contributes to E1A-mediated tumor suppression *in vivo*.

The studies reported here also provide insight into the molecular mechanisms whereby E1A sensitizes cells to lysis by NK cells and macrophages and the killing mechanisms used by these effector cells. Macrophages kill cells that express E1A predominantly by the secretion of nitric oxide and to a lesser extent TNF-α [Miura et al., 2003; Routes et al., 2005a]. Nitric oxide-induced cell death is regulated by the balance of cellular defense mechanisms that protect cells against oxidant-induced injury and mechanisms that promote cell death following oxidant exposure. Our microarray data suggested that E1A affects both of these pathways in a manner that promotes cell death. In comparison to H4, H4-E7, H4-E1A-RG2, and H4-E1A-dl1104, the expression of mRNA for HAH1, hexokinase, NNMT, and ferritin light chain was decreased in H4-E1A cells (Table I). Previous studies demonstrated that these proteins are involved cellular defense against oxidative injury [Hung et al., 1998; Bryson et al., 2002; Kim and Kim, 2002]. There was also increased expression in H4-E1A cells relative to H4 cells of myeloperoxidase, IEX-1, FKHR, and mitochondrial creatine kinase, gene products that promote cell death upon oxidative injury.

Ceramide has been implicated as an important mediator of apoptosis induced by Fas-L, TRAIL, and TNF-α [Gulbins, 2003; Watanabe et al., 2004]. Ceramide glucosyltransferase catalyzes the glycosylation of ceramide thereby eliminating its ability to mediate TNF-induced apoptosis. The expression of ceramide glucosyltransferase was decreased in cells that express wild-type E1A, but not in H4 cells or those that express E7, E1A-RG2, or E1A-dl1104 (Table I). Thus, E1A-induced repression of ceramide glucosyltransferase may increase the sensitivity of tumor cells to lysis by Fas-L, TRAIL, and TNF-α.

Our studies also provide insight on the molecular mechanisms that E1A utilizes to reduce tumorigenicity by non-immunological mechanisms. Prior studies demonstrated that the expression of E1A inhibits tumor cell metastasis [Pozzatti et al., 1988; Steeg et al., 1988; Frisch et al., 1990; Bernhard et al., 1995]. We found several genes altered by E1A that may contribute to the anti-metastatic activity of E1A. The expression of E1A, but not E1A-RG2, E1A-dl1104, or E7, inhibited the expression of the pro-metastatic genes uPA, type IV collagenase, memD/ALCAM, SPARC, aminopeptidase N, VEGF-C, HMGIC, prointerleukin-1β, and calcyclin (Table I). E1A also increased the expression of CD9 on the surface of H4 cells (Table I, Fig. 3). Decreased amounts of CD9 on the surface of tumor cells correlates with increased tumor metastasis in a variety of human malignancies [Miyake et al., 1995; Cajot et al., 1997; Funakoshi et al., 2003; Mhawech et al., 2003; Sauer et al., 2003]. Overexpression of CD9 on tumor cells appears to inhibit metastasis by reducing tumor cell motility [Ikeyama et al., 1993; Miyake et al., 2000]. In addition to inhibiting metastasis, overexpression of CD9 increases the susceptibility of target cells to killing by NK cells [Shallal and Kornbluth, 2000].

Our microarray data are consistent with our prior observations that indicate that the transcriptional effects of E1A-p300 and E7-p300 binding are qualitatively different [Miura et al., 2004]. E1A binds the C/H3 domain of p300 through amino acid residues in the N-terminus and conserved region 1, whereas E7 binds the C/H1 domain of p300 through amino acid residues in conserved region 2 [Bernat et al., 2003]. E7 does not have a region homologous to the N-terminal p300-binding domain of E1A. We have demonstrated that these differences in p300 binding are important in the capacity of E1A,

but not E7, to sensitize cells, including H4 cells, to lysis by macrophages [Miura et al., 2004]. We found that many of the differences in gene expression induced by E1A may be attributed to the unique p300-binding region present in E1A but absent in E7. H4 cells expressing either of two different mutant forms of E1A that do not bind p300 (E1A-RG2 or E1A-dl1104) exhibited a pattern of gene expression more similar to that induced by E7 than that induced by wild-type E1A. Unlike E1A, expression of E7 resulted in very few changes in gene expression in the fibrosarcoma tumor model (Table II). These data are in agreement with a previous microarray analysis of E7-expressing cells that found that expression of E7 in rapidly dividing cells resulted in very few changes as compared to the parental cells [Nees et al., 2001].

The parental and oncogene-expressing human H4 cell lines used in this study have been extensively characterized with respect to their sensitivity to NK cells, macrophages, and immune effector molecules (TRAIL, Fas-L, TNF-α, nitric oxide) and their tumorigenicities in nude mice. We have reported that the ability of E1A, but not E7, to sensitize tumor cells to lysis by NK cells and macrophages and to reduce tumorigenicity is a consistent finding among different rodent and human cell lines, including human keratinocytes [Routes and Ryan, 1995; Cook et al., 1996, 2003; Routes et al., 2000b, 2005a; Miura et al., 2003, 2004]. Therefore, we hypothesize that our findings demonstrating differential patterns of cellular gene expression induced by E1A compared to E7 will also be valid in other tumor model systems, including keratinocyte-derived tumor cells.

In summary, this study demonstrates that the expression of Ad5 E1A or HPV16 E7 oncoproteins in a human tumor cell line induces distinct transcriptional programs that likely account for the increased immunogenicity and decreased tumorigenicity of E1A-expressing cells, contrasted with E7-expressing cells. This distinct transcriptional response delineates a difference in the interaction of E1A and E7 with cellular p300. E1A, but not E7, increased expression of genes that are pro-apoptotic or pro-immunogenic and decreased expression of genes known to suppress cellular immunity or promote metastasis. The considerable impact that E1A has on tumor cell gene expression suggests that interplay of complex mechanisms is involved in E1A-mediated tumor suppression. Understanding the effects of E1A on tumor cell gene expression will be important in optimizing the use of E1A for cancer therapy.

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