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Original Article

E1a is an exogenous *in vivo* tumour suppressor

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ABSTRACT

The E1a gene from adenovirus has become a major tool in cancer research. Since the discovery of E1a, it has been proposed to be an oncogene, becoming a key element in the model of cooperation between oncogenes. However, E1a's *in vivo* behaviour is consistent with a tumour suppressor gene, due to the block/delay observed in different xenograft models. To clarify this interesting controversy, we have evaluated the effect of the E1a 13s isoform from adenovirus 5 *in vivo*. Initially, a conventional xenograft approach was performed using previously unreported HCT116 and B16-F10 cells, showing a clear anti-tumour effect regardless of the mouse's immunological background (immunosuppressed/immunocompetent). Next, we engineered a transgenic mouse model in which inducible E1a 13s expression was under the control of cytokeratin 5 to avoid side effects during embryonic development. Our results show that E1a is able to block chemical skin carcinogenesis, showing an anti-tumour effect. The present report demonstrates the *in vivo* anti-tumour effect of E1a, showing that the *in vitro* oncogenic role of E1a cannot be extrapolated *in vivo*, supporting its future use in gene therapy approaches.

Introduction

The E1a gene from adenovirus has become a potent tool in cancer research. In this regard, during the 1990s, E1a was used as a model to study cooperation with well-established oncogenes as *v-H-Ras* [1]. Indeed, the effect of E1a as a blocker of the tumour suppressor pRB [2] led to its acceptance as an oncogene for most of the scientific community. However, a growing body of experimental evidence showed an unexpected behaviour of E1a as a tumour suppressor gene. On one hand, E1a was able to block tumour growth in xenograft models [3] and showed a surprising ability to promote chemo/radio sensitivity in different experimental models [4]. All these evidence led to the consideration of E1a as a therapeutic gene which could be used in gene therapy approaches. Indeed, several types of tumours have been proposed as potential targets for E1a-based therapy including breast, ovarian, etc. [5]. Furthermore, some attempts have been performed to use E1a as a therapeutic agent in clinical trials [6,7].

The mechanisms proposed to explain E1a associated transformation has related to cell cycle alteration, escape from oncogenic-induced senescence, blockage of tumour suppressor genes, etc. (for a review see Ref. [8]). In the case of the anti-tumour behaviour of E1a, several possibilities have been proposed. For

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example, the effect of E1a on certain oncogenic proteins as Her2/neu or EGFR have been considered as major mechanisms [9,10]. However, this type of mechanism does not seem to be universal [11]. Indeed, recent evidence supports the existence of more complex mechanisms that could account for the anti-tumour activity of E1a, such as the deregulation of miRNA 520 h [12]. Nonetheless, the molecular basis of E1a functions in transformation or in tumour suppression is an intriguing question. For example, both properties seem to share the same region in terms of binding to cellular proteins, mainly through the CR2 domain [13]. For instance, the presence of a CR2 domain seems to be mandatory to avoid senescence induced by *v-H-Ras* in normal cells as a preliminary step for transformation [14], but it has also been shown that this region is strictly required for the anti-tumour effect of E1a in murine carcinoma derived cell lines [15]. Nonetheless, most, if not all, of the evidence of the properties of E1a in terms of transformation/tumour suppression are based on cell culture and xenograft approaches in immunocompromised mice which, although useful tools in cancer research, are quite far from the context of real tumours.

In an attempt to fully clarify this interesting discrepancy of E1a as an oncogene or a putative tumour suppressor, we decided to use immunosuppressed and immunocompetent animal models, and to develop an inducible, transgenic mouse model to study the oncogenic or anti-tumour properties of E1a.

Our results show that expression of E1a 13s is related to tumour suppression in animals. Specifically, E1a expression was related to the blockage of tumour growth of either tumours produced by injection of tumour cells in animals, or those induced by 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoyl-phorbol-13-acetate (DMBA/TPA) in a skin carcinogenesis assay.

Materials and methods

Cell lines

Human colon cancer cell line HCT116 and mouse melanoma B16-F10 cells (ATCC) were maintained in 5% CO_2 and 37 °C. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 1% glutamine plus 1% antibiotics (Sigma Aldrich, Tres Cantos, Madrid, Spain).

Transfections and infections

Lentiviral production and infection was performed as previously described [16]. Host cells were infected with lentivirus expressing empty vector (E.V.) or E1a 13s and 48 h later, infected cells were selected using puromycin (Sigma–Aldrich) at 2 μg/ml for HCT116 and 1.5 μg/ml for B16 cells. Infected cells were routinely maintained at the appropriate concentrations of puromycin.

Western blotting

Cell collection, lysis and western blotting were developed as previously described [16]. Antibodies against E1a (sc-25) and tubulin (sc-32293) were purchased from Santa Cruz Biotechnology (Quimigen, Madrid, Spain). Antibody detection was achieved by enhanced chemoluminescence (Amersham, GE Health Care, Barcelona, Spain) in a LAS-3000 system (FujiFilm, Tokyo, Japan). The results show a representative blot out of three with nearly identical results. Tubulin was used as a loading control.

Viability assays

Viability was evaluated by MTT assay [17]. Briefly, the MTT assays were performed using 2×10^4 cells/well plated in 24-well plates up to 96 h. The absorbance at 570 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). Data are the average of at least 3 independent experiments performed in triplicate.

Animal studies

Animal studies were carried out according to the NIH-Intramural Animal Guide for the Care and Use of Laboratory Animals and approved by the Ethics in Animal Care Committee of the University of Castilla-La Mancha.

Xenograft assays

For xenograft assays, 5×10^6 HCT116 cells and 1×10^6 B16-F10 cells expressing E1A or E.V. were injected in 5–6-week-old BALB/c-Nude or C57BL/6 female mice, respectively. Tumour size was measured by a calliper and calculated using the formula $V = D \times d^2/2$.

Animal transgenesis and genotyping

The cK5-rtTA/tet-E1a transgenic mouse model was generated by crossing cK5-rtTA and Tet-E1A transgenic mice derived from the FVB/N mouse strain. Transgenic cK5-rtTA and wild type FVB/N mice have been previously described [18]. For the generation of Tet-E1a transgenic FVB/N mice, E1a 13s coding sequences were cloned between BamHI and NotI downstream of the seventh Tet-responsive element (Tet-O7) in a modified pBSRV vector by PCR using pLSIP-13s as a template, which contains E1a 13s sequence from adenovirus 5 [16,19]. Briefly, E1a 13s was amplified by conventional PCR from pLESIP-13s vector by using the following primers to add BamHI and NotI cloning sequences: forward sequence 5′-GGGGGGATCCACCATGAGACATATTATCTGCCACGGAGG-3′, reverse sequence 5′-GGGGGCGGCCGCTTATGGCCTGGGGCGTTTAC-3′. The PCR amplification was performed starting with an initial denaturation step (94 °C for 10 min) followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min, and a final elongation step (72 °C for 10 min). The amplified E1a 13s was run in a 2% agarose gel and purified by using a DNA Purification Kit (Promega). After purification, E1a 13s PCR product and the modified pBSRV vector were digested using BamHI and NotI restriction enzymes (Fermentas), then cloned with TAKARA ligation kit. Five positive colonies were tested by PCR, BamHI and NotI digestion, and sequenced. The sequences were aligned by using the ClustalW software and all five clones exhibited 100% homology regarding the E1a 13s sequence (data not shown). Response to doxycycline induction was evaluated by western blot (data not shown) and the most inducible clone was selected for microinjection. The DNA fragment containing the expression cassette was isolated from vector by PmeI digestion, and purified for microinjection into FVB/N mouse fertilised oocytes. Transgenic mice were identified for the presence of transgenes by screening genomic DNA from tail biopsies by PCR using the following primers for E1a: forward sequence 5′-GCAGGAAGGGATTGACTTACTC-3′, reverse sequence 5′-CAAACTCCTCACCCTCTTCATC-3′; and for rtTA: forward sequence 5′-CCGGATCCACCATGCCTAAGAGCCCACG-3′, reverse sequence 5′-ATCTGAATGTACTTTTGCTCCATTGCGAT-3′. PCR was performed under the following conditions: 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final cycle of 5 min at 72 °C. The identification protocol for the presence of the transgenes is the same throughout the present work. For E1a induction in transgenic mice, Doxycycline was provided after birth in grain-based food pellets (Test-Diet Ltd.) at 6 g/kg. No randomisation was used and all experiments were conducted using littermate controls.

Tumour induction

Mice were shaved in the back and tumours were initiated by topical treatment with a single dose of DMBA (0.5 μg/μl in acetone) and followed 15 days later by the tumour promotion phase in which mice were treated twice a week with TPA (0.06 μg/μl in acetone) for 24 weeks.

RNA isolation, reverse transcription and real-time quantitative PCR

Expression of E1a in mice was analysed by SYBR Green quantitative real-time PCR using a $2^{-\Delta\Delta Ct}$ method and referred to the lowest positive expressing cK5-rtTA-E1a + Doxy animal. Total RNA was obtained using the RNeasy Fibrous Tissue Mini Kit (Cat. 74704, Qiagen) following the instructions provided by the manufacturer, and reverse transcription was performed from 1 μg of RNA following the instructions for RevertAid First Strand cDNA Synthesis Kit (Cat. K1621, ThermoFisher). The amount of cDNA was quantified by SYBR Green quantitative real-time PCR using an ABIPrism 7500 FAST Sequence Detection System (Applied Biosystems). cDNA was amplified using SYBR1 Green PCR Master Mix (Applied Biosystems) in the presence of specific oligonucleotides. Primers for all target sequences were designed using the Primer Express software provided with the 7500 Sequence Detection System (Applied Biosystems). Oligonucleotides used for E1a amplification were the following: forward sequence 5′-TACCCGCCGTCCTAAAATGG-3′, reverse sequence 5′-AAGGACCGGAGTCACAGCTA-3′. As an endogenous control, mouse ribosomal P0 mRNA levels were evaluated using the following primers: forward sequence 5′-AAGCGCGTCCTGGCATTGTCT-3′, reverse sequence 5′-CCGCAGGGGCAGCAGTGGT-3′. The PCR conditions and quantification was performed as previously described [17].

Tissue preparation, histology, and immunohistochemistry

Histological images were obtained from fixed sections of skin samples. All tissue samples were fixed in zinc formalin fixative buffer (Sigma–Aldrich) overnight and then transferred to 70% ethanol. Fixed tissues were embedded in paraffin and sectioned to a thickness of 4 μm. For immunohistochemistry, paraffin sections were automatically de-paraffinised and treated with cell conditioning 1 solution (pH 8) for antigen retrieval (Ventana Medical Systems, Tucson, AZ, USA). Staining was performed with an automated immunostainer (Beckmarck XT, Ventana Medical