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Activated Ras induces cytoplasmic vacuolation and non-apoptotic death in glioblastoma cells via novel effector pathways

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Abstract

Expression of activated H-Ras induces a unique form of non-apoptotic cell death in human glioblastoma cells and other specific tumor cell lines. The major cytopathological features of this form of death are the accumulation of large phase-lucent, LAMP1-positive, cytoplasmic vacuoles. In this study we sought to determine if induction of cytoplasmic vacuolation a) depends on Ras farnesylation, b) is specific to H-Ras, and c) is mediated by signaling through the major known Ras effector pathways. We find that the unusual effects of activated H-Ras depend on farnesylation and membrane association of the GTPase. Both H-Ras(G12V) and K-Ras4B(G12V) stimulate vacuolation, but activated forms of Cdc42 and RhoA do not. Amino acid substitutions in the Ras effector domain, which are known to selectively impair its interactions with Raf kinase, class-I phosphatidylinositide 3-kinase (PI3K), or Ral nucleotide exchange factors, initially pointed to Raf as a possible mediator of cell vacuolation. However, the MEK inhibitor, PD98059, did not block the induction of vacuoles, and constitutively active Raf-C*aax* did not mimic the effects of Ras(G12V). Introduction of normal PTEN together with H-Ras(G12V) into U251 glioblastoma cells reduced the PI3K-dependent activation of Akt, but had no effect on vacuolation. Finally, co-expression of H-Ras(G12V) with a dominant-negative form of RalA did not suppress vacuolation. Taken together, the observations indicate that Ras activates non-conventional and perhaps unique effector pathways to induce cytoplasmic vacuolation in glioblastoma cells. Identification of the relevant signaling pathways may uncover specific molecular targets that can be manipulated to activate non-apoptotic cell death in this type of cancer.

Keywords: Ras; Non-apoptotic cell death; Vacuoles; Glioblastoma; Raf; PI 3-Kinase; Farnesylation; Autophagy

1. Introduction

Oncogenic mutations in *ras* genes occur in approximately 30% of human malignancies [1]. In their active GTP-bound conformation, Ras proteins interact with a variety of downstream effectors that regulate cell proliferation, differentiation, and survival [2,3]. Signals are normally terminated when GTP is hydrolyzed and Ras reverts to the inactive GDP state. The mutant forms of Ras typically found in tumors harbor amino acid substitutions (e.g., G12→V) that reduce their GTPase activity [4,5]. This results in sustained stimulation of Ras pathways that promote cell proliferation and survival [6]. However, in some types of cells activated Ras can trigger cellular senescence [7], apoptosis [8] or non-apoptotic cell death [9]. The most notable example of the latter has been observed in human glioblastoma, gastric carcinoma, and neuroblastoma cells, where introduction of H-Ras(G12V) triggers accumulation of cytoplasmic vacuoles and cell death without caspase activation or DNA fragmentation.[9–11]. This form of cell death was originally described as Type-II programmed cell death [10], a term often used synonymously with autophagic cell death [12,13]. Type-II cell death has received increasing attention as a significant alternative pathway for cell death in cancer [14–18]. However, to date there have been no follow-up studies aimed at defining the signaling pathways through which activated Ras can trigger the initial cytoplasmic vacuolation that ultimately leads to loss of cellular integrity and non-apoptotic death.

In the present study we set out to determine a) if the induction of vacuolation in glioblastoma cells requires H-Ras farnesylation and membrane association, b) if the effect is specific to H-Ras,

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and c) if vacuolation is linked to activation of the major known Ras effector pathways. We find that the induction of vacuolation requires Ras farnesylation and is sensitive to effector domain mutations, but it does not depend on activation of the Raf, phosphatidylinositide 3-kinase (PI3K), or RalGDS signaling pathways. These observations raise the prospect that Ras may be acting through an atypical or perhaps unique effector pathway to induce vacuolation and non-apoptotic death in glioblastoma cells.

2. Materials and methods

2.1. Cell culture

U251 glioblastoma cells were obtained from the DCT Tumor Repository (National Cancer Institute, Frederick, MD, USA). Cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Phase contrast images of the live cells were obtained using an Olympus IX70 inverted microscope equipped with a digital camera, using SPOT imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.2. Generation of expression constructs for nucleofection

H-Ras(*G12V*) cDNA was purchased from Clontech (Palo Alto, CA, USA). The cDNA was subcloned into a pCMV5 vector that had been modified by PCR to encode either an in-frame myc epitope tag (MEQKLISEEDL) [19] or a FLAG epitope tag (DYKDDDDKG) [20]. Amino acid substitutions (C186S, Y40C, D38E and E37G) were created by PCR modification of the *myc-H-Ras*(*G12V*) cDNA using Pfu polymerase (Stratagene, La Jolla, CA, USA) and appropriate oligonucleotide primers. pcDNA3.1 containing the G12V mutant form of 2x myc-tagged *K-Ras2* (*i.e.*, *K-Ras4B*) was purchased from the UMR cDNA Resource Center (http://www.cdna.org Rolla, MO, USA). PRK5 vectors encoding myc-Cdc42(G12V) and myc-RhoA(G14V) were provided by A.L. Wilson-Delfosse, Case Western University, Cleveland, OH. Human *PTEN* in pCMV6-XL5 was purchased from Origene Technologies Inc., Rockville, MD, USA. The pCMV-*RafCaax* construct was purchased from Clontech. The insert was subcloned into the pCMV5 vector with the myc-tag sequence described above. Human RalA (GenBank accession number NM_005402) was modified by overlap extension PCR to generate the S28N point mutation. The resulting PCR product was subcloned into the aforementioned pCMV5 vector with an in-frame 5′ myc-tag sequence. All of the expression vectors were introduced into the U251 cells by nucleofection, using the Nucleofector II system from Amaxa Biosystems (Koeln, Germany) following the manufacturer's protocol with Nucleofector Solution T in combination with program T-30. For each reaction, 1–5 μg of plasmid DNA was introduced into 3×10^6 cells that had been harvested in RPMI 1640 medium. Following nucleofection, the cells were plated in 60 mm dishes and analyzed 18–24 h later.

2.3. Generation of Ras constructs for retroviral expression

Myc-tagged H-Ras constructs, (G12V and S17N) were subcloned into the EcoR1-BamH1 sites of the retroviral expression vector, pFBneo (Stratagene, La Jolla, CA, USA). Retrovirus was produced in HEK293-GPG packaging cells and glioblastoma cells were infected as described previously [21]. Non-infected cells were eliminated by incubation for 2 days post-infection in medium containing 2.0 mg/ml G418.

2.4. Immunofluorescence microscopy

Cells were grown on laminin-coated glass coverslips, washed with Hank's balanced salt solution (HBSS), fixed in ice-cold methanol for 10 min, and blocked with 10% goat serum in PBS. For detection of myc-H-Ras(G12V, C186S) and 2x-myc-K-Ras2(G12V) the procedure was modified slightly to include a preliminary fixation with 3% paraformaldehyde prior to the methanol step. Myc-tagged proteins were detected by incubation for 1 h with an anti-myc monoclonal antibody (Calbiochem, San Diego, CA, USA) diluted in PBS + 10% goat serum, followed by a 1-h incubation with goat anti-mouse IgG conjugated with Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA). Antibodies and procedures used for immunofluorescence localization of LAMP1 have been described previously [22]. For experiments where myc-tagged RalA was co-expressed with FLAG-tagged Ras, we used an anti-FLAG monoclonal antibody (Sigma, St. Louis, MO, USA) followed by goat anti-mouse IgG-Alexa Fluor 568, combined with rabbit anti-myc IgG conjugated directly to FITC (ICL Labs, Newberg, OR, USA). Photomicrographs were taken with a Nikon Eclipse 800 fluorescent microscope equipped with a digital camera and ImagePro software (Media Cybernetics, Silver Spring, MD, USA).

2.5. Western blot analysis

Antibodies used for western blotting were obtained from the following sources: p44/42 MAP Kinase (ERK1/2), phospho-p44/42 MAP Kinase (ERK1/2) (Thr202/Tyr204), phospho-Akt (Ser473), and total Akt (Cell Signaling Technology, Danvers, MA, USA); myc epitope tag (EMD Biosciences, San Diego, CA, USA); Raf-1 and PTEN (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein determinations, SDS-PAGE, and western blot analyses were performed as described previously [19]. Chemiluminescent immunoblot signals were quantified using a Kodak 440CF Image Station.

3. Results

3.1. Induction of cytoplasmic vacuolation in human glioblastoma cells by activated H-Ras(G12V) requires Ras farnesylation

In their initial study Chi et al. [10] demonstrated that expression of oncogenic H-Ras(G12V), but not GDP-locked H-Ras(S17N), causes massive accumulation of phase-lucent cytoplasmic vacuoles in U251 glioblastoma cells and gastric carcinoma cells. The vacuolated cells round up, detach from the substrate and disintegrate. This appears to be a form of non-apoptotic programmed cell death, since it occurs without substantial mitochondrial swelling, caspase activation or chromosomal DNA fragmentation [10]. We have observed a similar effect of activated H-Ras in a broad spectrum of human glioblastoma cell lines, but not in other commonly studied transformed cell lines (Overmeyer et al., unpublished).

H-Ras normally undergoes a series of sequential modifications commencing with the attachment of a farnesyl moiety to cys-186, followed by proteolytic removal of the three distal amino acids, and finally, carboxyl-methylation of the terminal cysteine [23]. In H-Ras, palmitylation of two upstream cysteines also occurs. In the absence of farnesylation, H-Ras remains predominantly in the cytosol, and subsequent modifications by membrane-associated enzymes do not occur [24]. Therefore, to evaluate the importance of membrane localization of H-Ras(G12V) for induction of the vacuolar phenotype, we introduced an amino acid substitution that prevents farnesylation; *i.e.*, C186→S. The cysteine mutation eliminated the ability of activated H-Ras to induce phase-lucent cytoplasmic vacuoles (Fig. 1a) and the cells continued to proliferate without detaching from the dish (not shown).

Many of the Ras-induced vacuoles contain the lysosomal membrane protein, LAMP1 (ref. [10] and Fig. 4 in this report), suggesting that they arise from lysosomal compartments. However, these structures have not yet been characterized in sufficient detail rule to out other origins (e.g., late endosomes,