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Effects of HRAS Oncogene on Cell Cycle Progression in a Cervical Cancer-Derived Cell Line

Emilio Córdova-Alarcón,[a] Federico Centeno,[b] Jorge Reyes-Esparza,[c] Alejandro García-Carrancá[d,e] and Efraín Garrido[b]

[a]*Departamento de Biomedicina Molecular,* [b]*Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México, D.F., México*
[c]*Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México*
[d]*Unidad de Investigación Biomédica en Cáncer, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D.F., México*
[e]*Instituto Nacional de Cancerología, Secretaría de Salud, México, D.F., México*

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Background. Human papillomavirus (HPV) infection is the most prevalent factor in anogenital cancers. However, epidemiological surveys and molecular data indicate that viral presence is not enough to induce cervical cancer, suggesting that cellular factors could play a key role. One of the most important genes involved in cancer development is the *RAS* oncogene, and activating mutations in this gene have been associated with HPV infection and cervical neoplasia. Thus, we determined the effect of *HRAS* oncogene expression on cell proliferation in a cell line immortalized by E6 and E7 oncogenes.

Methods. HPV positive human cervical carcinoma-derived cell lines (HeLa), previously transfected with the *HRAS* oncogene or the empty vector, were used. We first determined the proliferation rate and cell cycle profile of these cells by using flow cytometry and BrdU incorporation assays. In order to determine the signaling pathway regulated by *HRAS* and implicated in the alteration of proliferation of these cells, we used specific chemical inhibitors to inactivate the Raf and PI3K pathways.

Results. We observed that HeLa cells stably transfected with oncogenic *HRAS* progressed faster than control cells on the cell cycle by reducing their G1 phase. Additionally, *HRAS* overexpression accelerated the G1/S transition. Specific chemical inhibitors for PI3K and MEK activities indicated that both PI3K/AKT and RAF/MEK/ERK pathways are involved in the *HRAS* oncogene-induced reduction of the G1 phase.

Conclusions. Our results suggest that the *HRAS* oncogene could play an important role in the development of cervical cancer, in addition to the presence of HPV, by reducing the G1 phase and accelerating the G1/S transition of infected cells.

Key Words: Cervical carcinoma, HPV, *RAS*, Flow cytometry.

Introduction

Cervical carcinoma accounts for about 10% of all newly diagnosed cancers in women worldwide (1) and is one of the most frequent tumors in Mexico (2). Human papillomavirus infection (HPV) is the main factor associated with cervical carcinoma (3). The HPV gene products E6 and E7 play a critical role in cervical carcinogenesis by interfering with

Address reprint requests to: Dr. Efraín Garrido, Departamento de Genética y Biología Molecular, CINVESTAV-IPN, Av. IPN No. 2508, Col. San Pedro Zacatenco, 07360 México D.F., México. Phone: (+52) (55) 5061-3800 ext. 5373; FAX: (+52) (55) 5061-3800 ext. 5372; E-mail: egarrido@mail.cinvestav.mx

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p53 and Rb functions, respectively, and deregulating the cell cycle (4). It has been realized that HPV infection is not sufficient for cervical carcinoma development, and that the presence of additional factors is needed (3).

RAS proto-oncogenes are among the most frequently mutated genes in different human tumors (5,6). Mutations in *RAS* genes have been shown to coexist with oncogenic HPV in cervical cancer (7). In addition, *RAS* mutations are associated with a faster progression of HPV-induced lesions of uterine cervix (8). In mammals there are at least three *RAS* genes: *NRAS*, *KRAS*, and *HRAS* and the corresponding proteins function as regulators of different proliferation and survival signals (9). Although several putative *RAS* effectors have been reported, the RAF/MAPK-ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI-3K)/AKT pathways have been described as the most important (10,11).

Although it has been described that in the absence of a functional Rb protein all the effects of *RAS* in cell cycle regulation are lost (12,13), recently several reports suggest that *RAS* have additional functions on cell cycle progression (14–16). In fact, neutralizing antibodies against Ras protein still inhibit DNA replication in Rb knockout cells (14,15). Additionally, it has been shown that MYC and *RAS* cooperate to induce cell cycle progression into S phase in an Rb-independent manner (16). These observations suggest that *RAS* oncogene could regulate G1/S progression independently of Rb protein. Therefore, despite the absence of a functional Rb protein in cervical cancer, *RAS* could participate in the development of this kind of tumor.

In this work, we investigated whether the overexpression of oncogenic *HRAS* could alter cell cycle progression in the HeLa cell line containing the HPV-18 genome. We found that *HRAS* overexpression reduced G1 phase duration. Moreover, the G1/S transition was faster in overexpressing *HRAS* HeLa cells. Finally, using *RAS*-signaling chemical inhibitors, we showed that RAF/MEK/ERK and PI3K/AKT pathways participate in the G1 phase reduction mediated by *HRAS*.

Materials and Methods

Cell culture. HeLa cells were stably transfected with plasmid pSV2neo *HRAS* Val^{12} (HeLa-EJ) or pSV2neo empty vector (HeLa-neo) and have already been described (17,18). Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM) and 10% newborn calf serum at 37°C in 5% CO_2 atmosphere. Experiments were performed on subconfluent cultures not less than 24 h after plating.

Drug treatments. The different treatments were performed at a cell density of 3.5×10^5 cells per 60-mm plate. Thymidine [50-89-5] (Amersham, Piscataway, NJ) was used at 2 mM (final concentration) for 24 h, and the cells were trypsinized at different times post-treatment. For mitotic arrest, cells were incubated for 24 h with 2 mM thymidine, washed and nocodazole [31430–18–9] (Sigma, St. Louis, MO) was added at 0.1 µg/mL for 15 h. Floating cells from the supernatant (mitotic cells) were recovered, washed out extensively, re-seeded and harvested at different time intervals.

HeLa-EJ and neo cells were synchronized at mitosis as described above. After releasing them from mitotic arrest, different concentrations of the chemical inhibitors PD 098059 [167869–21–8] (Sigma) or wortmannin [19545–26–7] (Sigma) were added and cells harvested and analyzed by flow cytometry.

Flow cytometry analysis. One million cells treated or not with the different drugs were pelleted by low-speed centrifugation and the pellet resuspended with PBS. One mL of ice-cold 80% ethanol was added and the cell suspension was incubated overnight at 4°C. Fixed cells were harvested by centrifugation, resuspended in PBS containing RNase A (100 µg/mL) and propidium iodide (50 µg/mL), then incubated in the dark at 4°C for 1 h. The ethanol-fixed and propidium iodide-stained cells were analyzed for their DNA content in a FACS sort flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) (BDIS). Data analysis was performed using CellQuest (BDIS) and Modfit software (Verity Software House, Topsham, ME).

Cell lysates and Western blot. Cells were harvested by trypsinization, washed with PBS and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS, pH 7.4) supplemented with a mixture of phosphatase and protease inhibitors (1 mM sodium orthovanadate and 2 mM sodium fluoride, 1 mM PMSF, aprotinin 100 µg/mL, leupeptin 100 µg/mL, pepstatin 10 µg/mL, benzamidin 500 µg/mL, antipain 50 µg/mL and chymostatin 50 µg/mL). After incubation for 30 min on ice, lysates were cleared by centrifugation at 10,000 rpm for 10 min and protein concentration quantified with BioRad DC protein assay (BioRad, Hercules, CA). Equal amounts of proteins from the different samples were electrophoretically separated on a denaturating polyacrylamide gel (SDS-PAGE). Proteins were transferred to Hybond membrane (Amersham) and blocked with 5% non-fat milk in PBS. Membranes were incubated with anti-phosphospecific ERK antibodies (Cell Signaling, Beverly, MA), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (Zymed, South San Francisco, CA). Proteins were observed using the ECL detection system (Amersham) followed by exposure to XO-MAT films (Kodak, Rochester, NY). Membranes were stripped and re-blotted with anti-ERK antibodies (Cell Signaling).

Statistical analysis. The statistical significance of the difference between intergroup comparisons was obtained using