[Frontiers in Bioscience 14, 159-166, January 1, 2009]

Epidermal growth factor receptor modulates the tumorigenic potential of melanoma

Arlhee Diaz[1], Eduardo Suarez[1], Rances Blanco[1], Armando Lopez[1], Enrique Montero[1]

[1]Department of Experimental Immunotherapy, Center of Molecular Immunology, P.O. Box 16040, Havana 11600, Cuba

TABLE OF CONTENTS

1. ABSTRACT

Potential contribution of the Epidermal Growth Factor Receptor (EGFR) in melanoma immunobiology remains unclear, in part due to a lack of experimental models. We demonstrated previously that B16F10 melanoma transfected with the full length cDNA of the human EGFR increases the tumor cell proliferation *in vitro*. To further study its contribution *in vivo*, EGFR-transfected B16F10 cells were inoculated in syngenic C57BL/6 mice and its tumorigenic capacity was compared with the parental melanoma. Contrary to the observed *in vitro* effect, EGFR-transfected B16F10 cells displayed a delayed tumor growth rate *in vivo*, correlating inversely to the transgene expression. Interestingly, resulting tumors showed a downregulation of the EGFR transgene expression. Contrastingly, parental and EGFR-transfected B16F10 cells exhibited a similar tumorigenic potential in immunocompromised subjects, persisting the EGFR transgene expression. These results document the adaptability of melanoma to growth in immunocompetent individuals. Moreover, the potential EGFR expression during the melanoma outgrowth that would be downregulated by interacting with the host immune system during the tumor evolution is not excluded and which may be dissected in this model.

2. INTRODUCTION

Over the past few decades, there has been considerable interest in developing new agents to improve the outcome for patients with solid tumors (1). One cell membrane receptor, which has been shown to play an important role in the growth and survival of many solid tumors, is the epidermal growth factor receptor (EGFR), a transmembrane glycoprotein with protein kinase activity (2,3). Activation of the EGFR has shown to enhance processes responsible for tumor growth and progression, including proliferation, angiogenesis, invasion/metastasis, and inhibition of apoptosis (4,5). Overexpression of the EGFR has been tightly associated with malignant progression and worse clinical behavior of different tumors types, such as the metastatic NSCLC (6), invasion and metastasis of bladder cancer (7,8), and poor survival in patients with breast cancer (9). These findings prompted the scientific community to consider EGFR as an important target for rational drug design (4). However, the role of the EGFR in melanoma remains unclear, appearing as an area of recent interest and controversy (10). Contradictory reports have emerged from evaluating the role of EGFR either in normal human melanocytes as well as in malignant melanoma (10-12). Indeed, the importance of EGFR in melanoma immunobiology is still limited and

should be established by the ongoing investigation. A better understanding of the role of the EGFR in the biology of this tumor will permit to establish its contribution on the progression of the disease as well as the development of new successful molecular-targeted anti-EGFR therapeutic strategies. Therefore, we questioned whether EGFR expression might be associated with changes in the melanoma biology by transfecting B16F10 melanoma cells with the full length of the human EGFR cDNA sharing a substantial homology with its mouse counterpart. C57BL/6 mice were inoculated with the EGFR-transfected melanoma to evaluate their capacity to form tumors at the site of injection compared with parental B16F10 cells. We found an impaired capacity to form tumors when injected into normal but not immunocompromised mice, for cells with the highest level of EGFR expression as compared to non-transfected parental cells. Additionally, changes on the tumorigenic potential correlated directly with transgene expression level suggesting an inverse relation between the tumorigenic potential of B16F10 tumor cells and the EGFR expression.

3. MATERIALS AND METHODS

3.1. Antibodies

For the study were used: Mouse monoclonal antibody IgG2a (ior egf/r3) (13) and humanized monoclonal antibody IgG1 (TheraCIM h-R3, Nimotuzumab) (14) directed against human EGFR, obtained at Center of Molecular Immunology (CIM), Havana, Cuba; RPMI 1640 and Dulbecco´s Modified Eagle's Medium (DMEM) from Gibco-BRL (Paisley, UK).

3.2. Animals

Female C57BL/6 mice, 6 to 8 weeks old, were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba), and maintained at the CIM animal house. The study was conducted under CIM's Institutional Animal Care and Use committee's regulations.

3.3. Cell culture

Human vulvar epithelial carcinoma A431 (ATCC CRL 1555) is a highly EGFR expressing human cell line. Cells were grown in DMEM supplemented with 10% v/v FCS (foetal calf serum; Hyclone) containing 100 IU/ml of penicillin, 100 µg/ml streptomycin and 0.025 µg/ml amphotericin B. B16F10 is a metastasizing subline of the B16 melanoma that arose spontaneously and it is a syngeneic tumor in C57BL/6 mice. Transplantation into C57BL/6 mice at 10^5 cells/animal establishes tumors in 100% of mice. Cells were grown in RPMI 1640 medium supplemented with 5% FCS containing 100 IU/ml of penicillin, 100 µg/ml streptomycin and 0.025 µg/ml amphotericin B. All cell lines were maintained at 37ºC in a humidified incubator with 5% CO_2. Freshly medium was added every two days.

3.4. Immunoprecipitation

Cultured cells were harvested with trypsine, scraped in ice-cold PBS and adjusted at $5x10^5$ cell/mL. Alternatively, tumors were rinsed twice with ice-cold PBS. Then, RIPA lysis buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 10 µg/mL PMSF and Na_3VO_4, sodium orthovanadate) was added and incubated for 1 hour at 4ºC while rocking. Clarified protein lysates (500 µg/mL) were precipitated with 10 µg of specific monoclonal anti-EGFR antibody ior egf/r3 and 50 µL protein A-agarose overnight at 4ºC while rocking. The next day, beads were collected by centrifugation, denatured by boiling and subjected to western blot analysis.

3.5. Western blots

Lysates were cleared by centrifugation (10 min at 10000 rpm, 4ºC) and pellets were denatured by boiling for 5 min in 40 µL of buffer sample (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 50mM 2-mercaptoethanol). 20 µg protein were resolved by SDS-polyacrylamide (7.5%) gel electrophoresis and transferred onto nitrocellulose membranes (Hybond-N^+, Amersham) by electroblotting. The membrane was blocked in wash solution (Tris-buffered saline pH 7.6, 0.1% Tween) containing 5% non-fat dry milk and subsequently incubated overnight with EGFR 1005, a rabbit polyclonal IgG recognizing the human EGFR at residues 1005-1016 (Santa Cruz Biotechnology, Inc) at 1:1000 dilution. After washing, the membrane was incubated for 1 hour at 4ºC with horseradish peroxidase-conjugated goat anti human IgG (Santa Cruz Biotechnology, Inc). Bound antibody was visualized using a western blotting chemiluminescence luminol reagent system (Santa Cruz Biotechnology, Inc).

3.6. Total RNA isolation

For total RNA isolation, cultured cells or tumors were prepared similarly as described above. Approximately 10^6 cells from transfectant A5 or A431 control tumor cells were pelleted, washed twice with PBS and total cellular RNA was extracted with Trizol reagent (Gibco-BRL, Paisley, UK).

3.7. cDNA synthesis and polymerase chain reaction (PCR) amplification of human EGFR

Reverse transcription polymerase chain reaction (RT-PCR) was carried out with the Access RT-PCR System (Promega, Madison) according to the instructions of the manufacturer, using approximately 0.2 µg total RNA per reaction, as described previously by Suarez *et al*. (15). Specific 5'-ACC AGA GTG ATG TCT GGA GC-3' human sense primer (hEGFRs) and 5'-GAT GAG GTA CTC GTC GGC AT-3' human antisense primer (hEGFRas) were used for human EGFR detection. For reference, control G3PDH was amplified from identical amounts of the same RNA samples using the primers 5'-TGA GGA CGG GAA GCT TGT CAT-3' G3PDH human sense primer (hG3PDs); 5'-AAC ATC ATC CCT GCC TCT ACT-3' human antisense primer (hG3PDas); 5'- ACC ACA GTC CAT GCC ATC AC -3' G3PDH murine sense primer (mG3PDs); 5'- TCC ACC ACC CTG TTG CTG TA -3' murine antisense primer (mG3PDas). The amplified products were visualized in 1.5% agarose gel by ethidium bromide staining.

3.8. Immunohistochemistry

Cultured cells or tumors were treated as described above. Extensively rinsed and washed with ice-cold PBS,

adjusted at $5x10^5$ cells/mL, deposed in slides and dried. Cells were fixed in ice-cold acetone for 90 s. The slides were incubated for 1 hour with human monoclonal antibody (Nimotuzumab), diluted 1:100 at room temperature. Primary antibody was developed with biotin-conjugated secondary rabbit antihuman antisera and avidin-biotin peroxidase complex (ABC) using a DAKO ABC kit. Staining was visualized with 3-amino-9-ethylcarbazole (AEC). The nuclei were lightly counterstained with Mayer's hematoxylin.

3.9. Animal irradiation

Mice were irradiated with a single exposure of 600cGy by a Cobalt therapy tube (Alcyon II) of whole-body exposure delivered with an output of 84cGy/min. The distance to target was 100 cm. Tumor inoculation was carried out 72 hours after irradiation.

3.10. Tumor growth in mice

$5x10^5$ viable tumor cells were inoculated subcutaneously into the right flank of female C57BL/6 mice (five mice per group) using a 26-gauge needle. Animals were monitored for 30-60 days after tumor challenge. Time of latency was determined, and the largest perpendicular diameters of the resulting tumors were measured with a caliper every three days. Animals were sacrificed when tumor sizes exceeded $1x10^3$ mm^2. A second set of experiments were designed to evaluate the tumor growth in immunosuppressed mice. These experiments were conducted identically to the one described above, except that mice were inoculated with $2x10^5$ viable tumor cells.

3.11. Statistical analysis

Means were calculated using GraphPAD version 4.0 software (GraphPAD, San Diego, C.A.). Statistical analysis was performed using the GraphPAD InStat software. The significance of differences in tumor size was compared using a Mann-Whitney test. Difference in time of tumor latency and survival was analyzed using the Kaplan-Meier method and groups were compared using the log-rank test. Only p values < 0.05 were considered as significant.

4. RESULTS

4.1. Expression of the EGFR in B16F10 cells modulates their tumorigenic potential *in vivo*

The EGFR overexpression has been associated with a more aggressive phenotype in solid tumors (1), whereas its activation has usually correlated with the disease progression, poor survival and bad outcome of patients (4). In addition, the introduction and overexpression of human EGFR cDNA in NIH 3T3 cells has lead to a marked increase in DNA synthesis in response to EGF, conferring a transformed phenotype to these cells (16). To evaluate the contribution of EGFR in melanoma biology, we have established previously two B16F10 melanoma cell clones transfected with the human EGFR, namely D3 and A5 (17). A previous immunocytochemical analysis has revealed difference in the EGFR protein expression between control B16F10 melanoma cells and the transfected clones. Parental B16F10 cells were negative for the endogenous EGFR expression, whereas transgenic clones exhibited difference in the percent of EGFR-positive population, being 6 to 8-fold higher in the A5 clone (17). Moreover, the EGFR transgene expression increases cell proliferation enhancing its tumorigenic potential *in vitro*. To examine further the role of the human EGFR expression in tumor proliferation *in vivo*, groups of syngenic C57BL/6 mice were inoculated subcutaneously into the right flank with $5x10^5$ cells from transfected clones D3 and A5, and tumor latency was evaluated and compared to wild type B16F10 cell line. As shown in Figure 1A, transfected clone A5 formed palpable tumors considerably later compared to B16F10 control group; whereas D3 clone expressing intermediate amounts of EGFR showed an intermediate growth level correspondingly. Indeed, the latency of tumors was significantly delayed in mice injected with A5 ($p=0.0128$), but not for D3 cells ($p=0.1762$), compared to non-transfected B16F10 group. Thus, the median for tumor latency among the different groups was increased from 11 days in mice inoculated with B16F10 cells, to 14 and 17 days for D3 and A5 respectively. Furthermore, 14 days post-inoculation all mice from the control group developed palpable tumors at the site of inoculation, contrasting with 80% of mice in group D3 and 20% of mice in group A5. Additionally, tumors in the control group grew progressively, and all mice had to be sacrificed within 21 days after tumor inoculation, similarly to those mice which received D3 cells (Figure 1B). At this time, the tumors formed at the site of injection in both groups had reached a tumor size >9.0 cm^2. In contrast, injection of A5 transfected clone into the mice resulted in tumors that grew considerably more slowly than control, exhibiting a ~3.1 cm^2 average tumor size by week 3. Thus, EGFR expression was associated to a reduced tumor frequency (Figure 1A) and growth (Figure 1B) in a dose-dependent manner, and the inhibition was statistically significant. Consequently, a significant increase in the median survival time was observed in animals receiving A5 cells (45 days), compared to D3 (34 days) and B16F10 (31 days) (data not shown).

4.2. Transgenic cells downregulate the human EGFR expression after growing as a solid tumor *in vivo*

To investigate whether the transgene expression was associated to the altered tumorigenesis observed in mice, tumors were resected and subjected to western blotting and immunohistochemistry for EGFR protein detection. As shown in Figure 2A, tumors arose in mice inoculated with transfected clones D3 or A5 exhibited a significant downregulation of EGFR levels, with no distinguishable protein bands at 170 kD corresponding to EGFR transgene expression. In contrast, they were readily detected in transfectant cells maintained in culture and subjected to immunoblotting under the same conditions. Those results were confirmed by immunohistochemistry analysis, as shown in Figure 2B; however, no changes on cell morphology were observed among these cell lines grown *in vivo*. Previous reports have revealed the occurrence of post-transcriptional events leading to the lack of recombinant protein expression (18). To determine whether the downregulation of EGFR protein expression found here depend upon post-transcriptional events, the

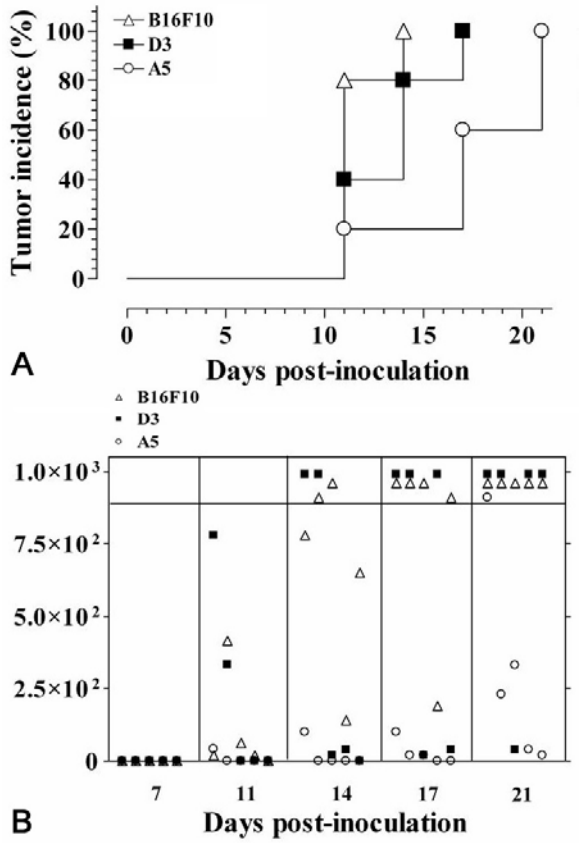

Figure 1. Tumor growth delay of B16F10 melanoma cells transfected with the Epidermal Growth Factor Receptor. (A) Animal experiments were performed with the parental B16F10 cell line (Δ) and transfected B16F10 with intermediate (■) levels (D3 clone) or high (○) levels of EGFR expression (A5 clone); $5x10^5$ viable cells were injected subcutaneously into the right flank of syngenic C57BL/6 mice. Tumor establishment was determined by palpation every other day. Shown are representative results from one of three separate experiments expressed as the percentage of tumor-bearing mice (n=5) at the indicate time points. The tumor outgrowth in mice injected with the A5 clone occurred significantly later ($p<0.05$) compared to D3 and B16F10 groups; Mann-Whitney test. (B) Tumor sizes were measured every three days and indicated at the time thereafter.

human EGFR transgene expression was analyzed by RT-PCR. Total RNA was isolated from tumors or cultured cells and the human EGFR mRNA expression was analyzed. The specificity of the RT-PCR reaction for the transgene was confirmed by using total RNA from wild type B16F10 cells, which yielded no amplification product (data not shown). Whereas EGFR mRNA was not detected in tumors arisen from mice inoculated with transfected clone A5, it was readily detected in these cells cultured *in vitro*, expressing high amounts of EGFR mRNA (Figure 3). Highest EGFR mRNA expression was found in tumor tissues from A431 cells, which were stained and used as positive control. These findings strongly correlate with the downregulation of EGFR protein expression observed by immunoblotting (Figure 2A) and immunohistochemistry (Figure 2B), suggesting that the occurrence of post-transcriptional events does not seem to be the cause leading to the downregulation of the transgene expression.

4.3. Persistent expression of EGFR in transfected B16F10 melanoma developed in immunocompromised C57BL/6 mice

Because the results obtained above suggest that EGFR transgene expression may affect the normal growth of B16F10 melanoma *in vivo*, a second set of experiments were designed to evaluate the growth of transgenic cells A5 in immunocompromised mice. Female C57BL/6 mice were sublethally irradiated by a single exposure of 600cGy Cobalt therapy (whole-body irradiation) and inoculated with the half of the tumor burden used in previous experiments ($2.5x10^5$) of B16F10 cells or transfectants and monitored for tumor outgrowth. Mice were sacrificed when tumors exceeded 9.0 cm^2. Additional mice were sacrificed 72 hours post-irradiation to corroborate the efficacy of ionizing therapy on depleting T and B lymphocytes (data not shown). Tumors developed in mice implanted with the B16F10 cell clone receiving no additional irradiation therapy grew as described above and 15 days post-inoculation all mice in this group exhibited palpable tumors at the site of injection (Figure 4A). However, transfected cells A5 failed to growth as a solid tumor at this dose and none of the mice left untreated developed palpable tumors by week 3. Contrastingly, transfected cells formed tumors with a roughly equal growth rates compared to the non-transfected B16F10 cell line when injected in mice previously irradiated. In this group, 60% of irradiated mice injected with cells expressing high amounts of EGFR developed tumors that reached a size >9.0 cm^2 after 20 days (Figure 4B). Additionally, tumors developed earliest in the group of mice carrying the B16F10 non-transfected cells in addition to radiation therapy showing the highest tumor growth rates, with a median tumor latency of 10 days, and 100% of the animals reached the threshold size of 9.0 cm^2 at day 20. More interesting, tumors developed in irradiated mice implanted with the A5 clone were found positive for the transgene expression as assessed by immunohistochemistry (Figure 5). These results confirmed that tumor growth of B16F10 melanoma cells correlates to the transgene expression in immunocompetent, but not immunocompromised mice, suggesting a relationship between the expression of EGFR and the host status.

4.4 EGFR permanent transgene downregulation in B16F10 melanoma after *in vivo* passage in immunocompetent C57BL/6 mice

It is possible that promoter silencing or a loss of the transgene could have led to the drop in transgene expression in the B16F10 tumors. Despite the results derived from the above experiments in mice underwent irradiation pointed out a loss in transgene expression as the cause leading to the downregulation in EGFR protein expression, we decided to further clarify this issue. Hence, syngenic immunocompetent C57BL/6 mice were inoculated with $5x10^5$ of the high-expressing EGFR-transfected B16F10 cell line (A5 clone) and left tumors growth until reaching a size >9.0 cm^2. Then, mice were sacrificed and tumors were excised and re-transplanted

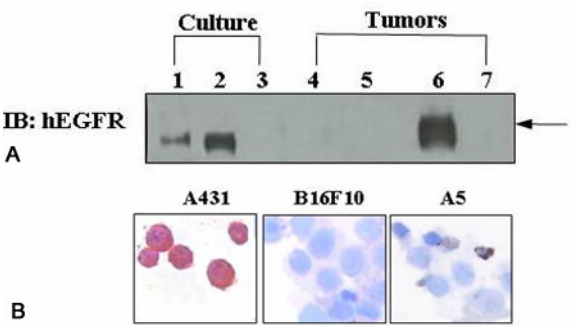

Figure 2. Downregulation of the EGFR protein expression in transfected B16F10 melanoma inoculated in immunocompetent mice. $5x10^5$ viable cells from parental B16F10 cells or EGFR transgenic clones D3 and A5 were injected subcutaneously into the right flank of syngenic C57BL/6 mice. (A) EGFR protein expression determined in a cell extract prepared from cells grown either in culture cells (lanes 1-3) or tumors (lanes 4-7) by immunoprecipitation with a specific anti-human EGFR monoclonal antibody (ior egf/r3). Immunoblotting analysis was performed as described under "Materials and Methods". A431 human tumor cells were used as positive control. D3 (lanes 1, 4); A5 (lanes 2, 5); B16F10 (lanes 3, 7); A431 (lane 6). (B) Immunohistochemical assessment was performed using a specific anti-EGFR monoclonal antibody (TheraCIM h-R3, Nimotuzumab). Staining was visualized with AEC. The nuclei were counterstained with Mayer's hematoxylin. Original magnification 40x. Positive (red) staining indicates expression of human EGFR.

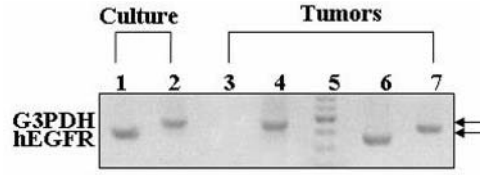

Figure 3. Downregulation of the EGFR transgene in transfected B16F10 melanoma inoculated in immunocompetent mice. Total cellular RNA from cultured cells (lanes 1, 2) or tumors (lanes 3-7) was extracted and RT-PCR was carried out as described under "Materials and Methods". The amplified products of the human EGFR region (lanes 1, 3 and 6) and GAPDH ('G3PDH') (lanes 2, 4 and 7) were visualized in 1.5% gel by ethidium bromide staining. Arrows point to the human EGFR and G3PDH gene products. Lanes 1 and 2, A5 cells; lanes 3 and 4, A5 tumor; lane 5, 100 bp ladder molecular size marker; lanes 6 and 7, A431 tumor. Detailed information on the primers used is given in the "Materials and Methods" section.

either into immunocompetent mice or mice underwent previous irradiation (Figure 5A). After the *in vivo* passage, tumor incidence and tumor latency were similar in both groups of mice and comparable to the parental B16F10 cell line. Moreover, the tumor growth rate of re-transplanted cells was also similar in both groups, contrasting with the original EGFR-transfected B16F10 cell line, which displays a reduced tumor growth rate in immunocompetent but not in immunocompromised mice (Figure 4). Additionally, re-transplanted tumors were excised and subjected to immunohistochemistry for EGFR detection as described previously. As expected, no protein staining for EGFR was visualized in tumors growing in normal mice, corroborating the above results. Interestingly, EGFR expression was not recovered in re-transplanted tumors re-growing in immunocompromised mice (Figure 5B). Thus, it supports a permanent downregulation of the EGFR transgene, unlike a promoter silencing process, remaining unaffected the tumorigenic potential of the melanoma.

5. DISCUSSION

Increasing development in the field of molecular biology, genetics, and immunology converge to a better understanding of the molecular characteristics of tumors. Moreover, this has opened new possibilities to improve survival and overall prognosis of cancer patients. Understanding the role of the EGFR in cellular signalling processes underlying malignancy has enabled the development of rationally designed EGFR-targeted therapeutics. Melanoma is a solid tumor particularly resistant to conventional cancer therapies: this is responsable of the lack of the significant increase patient survival (19). Therefore, the importance of EGFR signalling in melanoma development has become an area of growing interest. Contradictory reports have emerged from the evaluation of the role of EGFR either in normal human melanocytes as well as in malignant melanoma (10). In contrast to the study published by Grahn and Isseroff (2004) (11) indicating the absence of EGF-mediated signalling through EGFR, others have reported tyrosine kinase–mediate signalling in normal human melanocytes in response to recombinant EGF, accounting for transforming potential *in vivo* (10,12). However, many of these results have been obtained in cultured melanoma cells; therefore the knowledge of the contribution of EGFR in melanoma immunobiology is still limited. Based on these considerations we have developed a system to express a functional EGFR in a murine melanoma cell line to further study its contribution in melanoma biology. In our model we have provided evidences that transfection of EGFR in B16F10 melanoma cells increased cell proliferation *in vitro* in the presence of EGF in a dose-dependent manner, while increases the sensitivity to an anti-EGFR monoclonal antibody (17). These results obtained *in vitro* encouraged us to evaluate the potential tumorigenesis *in vivo* for such transfected cells. Unexpectedly, the EGFR expression correlated to a reduced melanoma tumorigenic potential *in vivo*. Moreover, there was an inverse association between the EGFR expression levels ad the tumor growth development. However, in this study the expression of EGFR in the B16F10 melanoma cells did not completely blocks subcutaneous primary tumor growth in mice, but prolonged survival by significantly delaying tumor outgrowth. The suppression of primary B16F10 melanoma correlated directly with transgene expression levels. A relationship between EGFR transgene expression level and tumor growth inhibition has not been described previously. Instead, we have found that high expression of a functional human EGFR transgene had direct effect on promoting

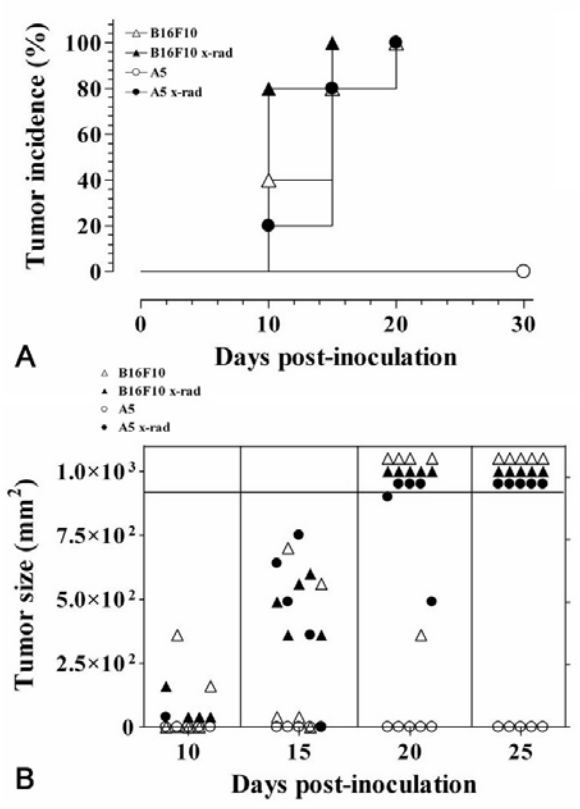

Figure 4. EGFR-transfected B16F10 melanoma growth is influenced by host immunocompetence. (A) Animal experiments were performed with parental B16F10 cells (triangles), or the clone A5 with high levels of EGFR expression (circles); $2.5x10^5$ viable cells were injected subcutaneously into the right flank of either normal (empty symbols) or sublethally irradiated (filled symbols) syngenic C57BL/6 mice. Irradiation was performed exposing mice to a single dose of 6Gy of whole-body irradiation, 72 hours before tumor implantation. Tumor establishment was determined by palpation every other day. Shown are representative results from one of three separate experiments expressed as the percentage of tumor-bearing mice (n=5) at the indicate time points. Tumor outgrowth in mice injected with the A5 clone receiving no irradiation therapy occurred significantly later ($p<0.05$) compared to the one receiving the A5 clone and radiation therapy; Mann-Whitney test. (B) Tumor sizes were measured every three days and indicated at the time thereafter.

B16F10 cell growth *in vitro* (17). This paradoxical observation seems to be related with a downregulation of the EGFR either at the protein and mRNA expression level. Our data suggest that the loss of the transgene could have led to the drop in transgene expression in the B16F10 tumors. Indeed, B16F10 cells have been classified as "genomically unstable" (20), which could explain a loss of the transgene. More interesting, whereas tumors arisen in normal mice showed a significant downregulation of the transgene expression, those from immunocompromised mice did not. Similarly, the tumor growth in those immunocompromised mice was not affected by the transgene expression, suggesting a role of the host condition for the normal growth of B16F10 melanoma. The tumor growth *in vivo* requires additional events such as angiogenesis, invasion of host tissues, and interactions with normal surrounding cells of the host (21,22). The control of this complex network of communication between the tumor and surrounding cells relays on many different types of regulatory ligands and proteins, resulting particularly relevant the host immunity (23,24). In this context, the impaired tumorigenesis of transfected cells in immunocompetent mice indicates that human EGFR may modulate normal growth of B16F10 tumors *in vivo*. Thus, in our model EGFR may function in opposing fashions to determine the net outcome and development of B16F10 tumors under *in vivo* condition. An explanation to these results may be that EGFR expressed in transfected cells might increase the immunogenicity of the tumor cells, thereby facilitating tumor recognition by the host immune system when injected into immunocompetent mice. Indeed, B16F10 melanoma is considered among the most aggressive and poorly immunogenic murine tumors (25). However, based on our previous observations we have found that the transduction of the B16F10 cells with the human EGFR cDNA leads to an increased expression of the EGFR transcript when compared with non-transfected cells (17). Therefore, the antigen expression in these cells could expose or generate new epitopes that are presented on the context of MHC class I/II molecules and probably recognized by the host immune system. Despite the murine and the human EGFR share a high homology, estimated on 88 % identity at amino acid level for the extracellular domain, recent reports have demonstrated that it is possible to break the immune tolerance against EGFR in a cross-reaction between the xenogenic homologous and self EGFR (26). Despite we were not able to find significant antibody responses in sera from mice injected with transfected cells (data not shown), other immune mechanisms (*e.g.* cellular response) would be involved. Downregulation of EGFR transgene expression was detected not only at protein, but also at the transcript level (Figure 3). In this respect, we may hypothesize that those cells expressing high levels of the human EGFR are removed during the process of tumor development. Therefore, only a minor fraction of the tumor potentially negative for EGFR and thus non-sensitive to the immune recognition may proliferate, leading to a significant delay in tumor outgrowth. However, it is also possible that EGFR positive tumor cells may edit that antigen during their interaction with the immune system, resulting in a subsequent tumor cell with a reduced capacity to be recognized and escaping to the immune effector mechanisms (27), which may operate in melanoma patients expressing the EGFR constitutively. These observations are in agreement with those from Zou *et al*. (2004) (28) which demonstrated that the inoculation of renal carcinoma cells expressing influenza HA antigen, which induces tumor recognition and early activation of tumor-specific $CD4^+$ T cells, alters the antigenic profile of progressing tumor. In that study, the authors reported the initial activation of the immune system of mice resulting in the selection of tumor escape variants that lost the expression of the target antigen measured by antibody staining. However, downregulation

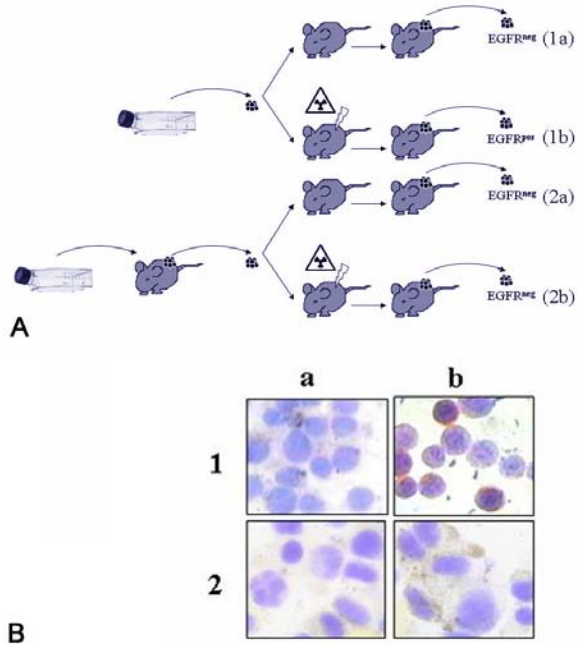

Figure 5. Permanent downregulation of the EGFR transgene in B16F10 melanoma after *in vivo* passage. (A) Cartoon depicting the experimental approach to determine whether downregulated EGFR would be recovered re-transplanting the tumor cells coming from normal mice. (B) Immunohistochemical assessment was performed using a specific anti-EGFR monoclonal antibody (TheraCIM h-R3, Nimotuzumab). Staining was visualized with AEC. The nuclei were counterstained with Mayer's hematoxylin. Original magnification 40x. Positive (red) staining indicates expression of human EGFR.

of the antigen was not complete and a HA mRNA threshold still remain detectable. Here we showed that the EGFR editing is promoting not only the downregulation of the target antigen expression, but also the transcript. In summary, we have developed a transformed B16F10 melanoma transfected with a vector containing the human EGFR cDNA. Since the EGFR inhibition is effective in the tumor growth suppression, this transgenic cell line would permit to study further the direct role of the EGFR in melanoma biology as well as evaluating different anti-EGFR candidates for a potential use in the clinical setting. Additionally, the precise mechanisms responsible for the EGFR downregulation might provide new insights on the role of the immune system in the detection and eradication of solid tumor in cancer patients.

6. ACKNOWLEDGMENT

We thank Mr. Michael Rodriguez for his skilful technical assistance with the experimental animal irradiation studies.

7. REFERENCES

1. Baselga J.: Why the epidermal growth factor receptor? The rationale for cancer therapy. *Oncologist* 7 Suppl 4, 2-8 (2002)

2. Raymond E., S. Faivre & J. P. Armand: Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* 60 Suppl 1, 15-23; discussion 41-12 (2000)

3. Carpenter G.: Receptors for epidermal growth factor and other polypeptide mitogens. *Annu Rev Biochem* 56, 881-914 (1987). 10.1146/annurev.bi.56.070187.004313

4. Woodburn J.R.: The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* 82, 241-250 (1999)

5. Hanahan D. & R. A. Weinberg: The hallmarks of cancer. *Cell* 100, 57-70 (2000).

6. Veale D., T. Ashcroft, C. Marsh, G. J. Gibson & A. L. Harris: Epidermal growth factor receptors in non-small cell lung cancer. *Br J Cancer* 55, 513-516 (1987)

7. Neal D.E., C. Marsh, M. K. Bennett, P. D. Abel, R. R. Hall, J. R. Sainsbury & A. L. Harris: Epidermal-growth-factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *Lancet* 1, 366-368 (1985)

8. Neal D.E., L. Sharples, K. Smith, J. Fennelly, R. R. Hall & A. L. Harris: The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer* 65, 1619-1625 (1990)

9. Sainsbury J.R., J. R. Farndon, G. V. Sherbet & A. L. Harris: Epidermal-growth-factor receptors and oestrogen receptors in human breast cancer. *Lancet* 1, 364-366 (1985)

10. Mirmohammadsadegh A., M. Hassan, A. Gustrau, R. Doroudi, N. Schmittner, S. Nambiar, A. Tannapfel, T. Ruzicka & U. R. Hengge: Constitutive expression of epidermal growth factor receptors on normal human melanocytes. *J Invest Dermatol* 125, 392-394 (2005)

11. Grahn J.C. & R. R. Isseroff: Human melanocytes do not express EGF receptors. *J Invest Dermatol* 123, 244-246 (2004)

12. Gordon-Thomson C., R. S. Mason & G. P. Moore: Regulation of epidermal growth factor receptor expression in human melanocytes. *Exp Dermatol* 10, 321-328 (2001)

13. Fernandez A., E. Spitzer, R. Perez, F. D. Boehmer, K. Eckert, W. Zschiesche & R. Grosse: A new monoclonal antibody for detection of EGF-receptors in western blots and paraffin-embedded tissue sections. *J Cell Biochem* 49, 157-165 (1992)

14. Mateo C., E. Moreno, K. Amour, J. Lombardero, W. Harris & R. Perez: Humanization of a mouse monoclonal antibody that blocks the epidermal growth factor receptor: recovery of antagonistic activity. *Immunotechnology* 3, 71-81 (1997)

15. Suarez Pestana E., T. Tenev, S. Gross, B. Stoyanov, M. Ogata & F. D. Bohmer: The transmembrane protein

tyrosine phosphatase RPTPsigma modulates signaling of the epidermal growth factor receptor in A431 cells. *Oncogene* 18, 4069-4079 (1999)

16. Di Fiore P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger & S. A. Aaronson: Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell* 51, 1063-1070 (1987)

17. Diaz A., E. Suarez, R. Blanco, T. Badia, D. Rivero, A. Lopez-Requena, A. Lopez & E. Montero: Functional expression of the human epidermal growth factor receptor in a melanoma cell line. *Biotechnol Appl Biochem* 48, 21-27 (2007)

18. Barnes L. M., C. M. Bentley & A. J. Dickson: Stability of protein production from recombinant mammalian cells. *Biotechnol Bioeng* 81, 631-639 (2003)

19. Pilla L., R. Valenti, A. Marrari, R. Patuzzo, M. Santinami, G. Parmiani & L. Rivoltini: Vaccination: role in metastatic melanoma. *Expert Rev Anticancer Ther* 6, 1305-1318 (2006)

20. Cillo C., J. E. Dick, V. Ling & R. P. Hill: Generation of drug-resistant variants in metastatic B16 mouse melanoma cell lines. *Cancer Res* 47, 2604-2608 (1987)

21. Gupta N., H. Wang, T. L. McLeod, C. C. Naus, S. Kyurkchiev, S. Advani, J. Yu, B. Perbal & R. R. Weichselbaum: Inhibition of glioma cell growth and tumorigenic potential by CCN3 (NOV). *Mol Pathol* 54, 293-299 (2001)

22 Hofmann. U. B., R. Houben, E. B. Brocker & J. C. Becker: Role of matrix metalloproteinases in melanoma cell invasion.
Biochimie 87, 307-314 (2005)

23. MacKie R. M., R. Reid & B. Junor: Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. *N Engl J Med* 348, 567-568 (2003)

24. Koebel C. M., W. Vermi, J. B. Swann, N. Zerafa, S. J. Rodig, L. J. Old, M. J. Smyth & R. D. Schreiber: Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450, 903-907 (2007).

25. Fidler I. J.: Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. *Cancer Res* 35, 218-224 (1975)

26. Lu Y., Y. Q. Wei, L. Tian, X. Zhao, L. Yang, B. Hu, B. Kan, Y. J. Wen, F. Liu, H. X. Deng, J. Li, Y. Q. Mao, S. Lei, M. J. Huang, F. Peng, Y. Jiang, H. Zhou, L. Q. Zhou & F. Luo: Immunogene therapy of tumors with vaccine based on xenogeneic epidermal growth factor receptor. *J Immunol* 170, 3162-3170 (2003)

27. Dunn G. P., L. J. Old, R. & D. Schreiber: The three Es of cancer immunoediting. *Annu Rev Immunol* 22, 329-360 (2004)

28. Zhou G., Z. Lu, J. D. McCadden, H. I. Levitsky & A. L. Marson: Reciprocal changes in tumor antigenicity and antigen-specific T cell function during tumor progression. *J Exp Med* 200, 1581-1592 (2004)

Abbreviations: DMEM: Dulbecco´s modified Eagle's medium, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, FCS: Foetal calf serum, G3PDH: glyceraldehyde-3-phosphate dehydrogenase, HA: haemaglutinin, MHC I/II: major histocompatibility complex class I/II, RT: Reverse transcriptase

Key Words: B16F10, Epidermal Growth Factor Receptor, Melanoma, Tumor Editing, Tumor Growth

Send correspondence to: Arlhee Diaz, Center of Molecular Immunology, 216 St. Havana, Cuba, Tel: 53-7-2717933, Fax: 53-7-2720644, E-mail: arlhee@cim.sld.cu

http://www.bioscience.org/current/vol14.htm