Fibroblast Growth Factor 2 Restrains Ras-Driven Proliferation of Malignant Cells by Triggering RhoA-Mediated Senescence


Departmento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil

Abstract
Fibroblast growth factor 2 (FGF2) is considered to be a bona fide oncogenic factor, although results from our group and others call this into question. Here, we report that exogenous recombinant FGF2 irreversibly inhibits proliferation by inducing senescence in Ras-dependent malignant mouse cells, but not in immortalized nontumorigenic cell lines. We report the following findings in K-Ras–dependent malignant Y1 adenocortical cells and H-Ras V12–transformed BALB-3T3 fibroblasts: (a) FGF2 inhibits clonal growth and tumor onset in nude and immunocompetent BALB/c mice, (b) FGF2 irreversibly blocks the cell cycle, and (c) FGF2 induces the senescence-associated β-galactosidase with no accompanying signs of apoptosis or necrosis. The tyrosine kinase inhibitor PD173074 completely protected malignant cells from FGF2. In Y1 adrenal adenocarcinoma cells, reducing the constitutively high levels of K-Ras-GTP using the dominant-negative RasN17 mutant made cells resistant to FGF2 cytotoxicity. In addition, transfection of the dominant-negative RhoA-N19 into either Y1 or 3T3-B61 malignant cell lines yielded stable clonal transfectants that were unable to activate RhoA and were resistant to the FGF2 stress response. We conclude that in Ras-dependent malignant cells, FGF2 interacts with its cognate receptors to trigger a senescence-like process involving RhoA-GTP. Surprisingly, attempts to select FGF2-resistant cells from the Y1 and 3T3-B61 cell lines yielded only rare clones that (a) had lost the overexpressed ras oncogene, (b) were dependent on FGF2 for proliferation, and (c) were poorly tumorigenic. Thus, FGF2 exerted a strong negative selection that Ras-dependent malignant cells could rarely overcome. [Cancer Res 2008;68(15):6215–23]

Introduction
Fibroblast growth factor 2 (FGF2) is well known for playing crucial roles in embryogenesis, morphogenesis, tissue turnover, and wound-healing (1–5). However, the role of FGF2 in the development and maintenance of tumors remains an open question, and constitutes the central focus of this article. Early and more recent reports have implicated FGF2 in oncogenesis. For example, ectopic FGF2 overexpression in transformed mouse 3T3 fibroblasts was found to be oncogenic (4), and more recently, FGF2 expression has been found to be up-regulated in several types of cancer and to protect cancer cells from chemotherapy (5–8). On the other hand, other laboratories have reported that exogenous FGF2 inhibits proliferation, causes death, and chemosensitizes human and rodent tumor cell lines (9–13), implying that FGF2 can restore tumor defense mechanisms in malignant cells.

To analyze whether and how exogenous FGF2 can trigger tumor-defense mechanisms in malignant cells, we chose a pair of Ras-dependent mouse malignant cell lines that are resistant to apoptosis or senescence. The first was the Y1 adenocortical carcinoma cell line, which was isolated and cloned (14), and later discovered to carry the amplified and overexpressed K-ras oncogene (15). The second is a set of highly tumorigenic clonal sublines of BALB-3T3 fibroblasts that were previously transformed with both the H-ras V12 oncogene and a neomycin marker gene (16).

Mutated ras oncogenes are frequently found in human cancers (17, 18) and are sufficient to induce malignant transformation of almost any kind of immortalized cell line (17, 19, 20). On the other hand, ectopic expression of the activated H-Ras-V12 oncoprotein triggers premature senescence or apoptosis in primary cultures of mouse embryo fibroblasts or human cells, instead of malignant transformation (21, 22). This phenomenon, designated the oncogene stress response, constitutes one of several checkpoints characterizing the complex tumor-defense system of mammalian cells. This system activates well-established apoptotic pathways or still poorly defined senescence pathways (22). When an immortalized cell is transformed with one of the ras oncogenes, a malignant cell line is produced that has passed several tumor-defense checkpoints and is now resistant to programmed cell death (19, 20). A timely question in cancer biology and cancer therapy is whether and how one can elicit tumor-defense mechanisms that can lead to the demise of Ras-dependent malignant cells.

As a step in this direction, we report here that FGF2 blocks the cell cycle of Ras-dependent Y1 and 3T3-B61 malignant cells, irreversibly inhibiting proliferation and inducing senescence-associated β-galactosidase (SA-β-Gal) without signs of apoptosis or necrosis. In addition, whereas inoculating Y1 and 3T3-B61 cells under the skin of BALB/c mice initiates rapid tumor growth, this growth is inhibited by injections of FGF2 at the site of inoculation. RhoA-GTP mediates this FGF2-induced senescence response in both malignant cell types. In fact, prolonged exposure of Y1 and 3T3-B61 malignant cells to FGF2 exerts strong selective pressure against the cells and favors the emergence of poorly tumorigenic variants devoid of overexpressed Ras oncoproteins that are dependent on FGF2 for proliferation and survival. Taken together, these results lead us to propose that, similar to the oncogene stress response of normal cells, some malignant cells resistant to
apoptosis and senescence exhibit a novel FGF2 stress response. This FGF2 stress response is unlikely to be unique to this growth factor and is unquestionably of great interest for cancer biology and therapy.

Materials and Methods

Cell lines. The homogeneously stained chromosome region (HSR) subline (23) of the mouse Y1 adenocarcinoma carcinoma cell line (14) was obtained from the American Type Culture Collection in 1973. The early passage BALB/c mouse embryo fibroblast cell line, BALB-3T3 clone A31, was obtained in 1984 from the laboratory of Dr. Charles D. Stiles (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Parental Y1 and BALB-3T3 cells and multiple sublines derived from them in this laboratory were cultured in DMEM containing 10% FCS (10% FCS-DME).

Clonogenic assays and growth curves. For clonogenic assays on a solid substrate, cells were plated at 80 to 120 cells/cm² in 10% FCS-DME. For suspension cultures, suspensions of single cells in 0.3% agarose in 10% FCS-DME were overlaid on the solid phase of 0.6% agarose-10% FCS-DME suspension cultures, suspensions of single cells in 0.3% agarose in 10% FCS-DME were inoculated in either immunodeficient nude or immunocompetent BALB/c mice. Samples of 5×10⁵ cells were cultured to 50% confluence in 100 mm dishes in 10% FCS-DME. For flow cytometry, cells fixed with 75% ethanol were suspended in 150 μL of PBS plus 0.1% sodium citrate, 0.1% Triton X-100, 50 μL of 1 mg/mL RNase A (Sigma-Aldrich), and 100 μL of 100 μg/mL propidium iodide (Sigma-Aldrich) and incubated overnight at 4°C. The suspensions were then analyzed in a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Inc.) with an argon laser (250 mW, 488 nm; Coherent, Inc.). Red fluorescence due to the propidium iodide was detected at 560/580 nm. Data from samples of 10,000 cells were collected and analyzed using Cyclone (Cytogem, University of Wales College of Medicine, United Kingdom). Light scattering was used to monitor cell size and granularity or internal complexity.

SA-β-Gal assay. Monolayers of cells were fixed for 5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde. The fixed cells were rinsed and incubated for 8 h at 37°C with a staining solution of 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (100 μmol/L of NaCl, 2 mmol/L of MgCl₂, 40 mmol/L of citric acid, and sodium phosphate (pH 6.0). The staining solution was diluted from a 20 mg/mL stock solution in dimethylformamide shortly before use. The percentage of SA-β-Gal–stained cells was estimated in photomicrographs by counting a total of 500 cells per duplicate plates. Results from independent experiments were pooled and statistically analyzed using the χ² test of homogeneity.

Results

FGF2 triggers irreversible senescence and inhibits the proliferation of two Ras-dependent malignant cell lines. FGF2 severely inhibited colony development in clonogenic assays with Y1 cells in both solid substrate cultures (Fig. 1A) and agarose suspension cultures (Fig. 1B). FGF2 was a much stronger inhibitor than FGF1, FGF4, or FGF5 (Fig. 1A), even though all of them activated the ERK1/2 mitogenic pathway to similar extents (Supplementary Data, Fig. S1A). Growth curve analysis shows that FGF2 blocked Y1 cell proliferation (Fig. 1C) with an ED₅₀ of 65 pmol/L (data not shown), whereas FGF1 caused only minor inhibition (Fig. 1C). In addition, injections of FGF2 strongly inhibited the rapid tumor growth of Y1 cells previously inserted under the skin of immunodeficient nude mice (Fig. 1D). The FGF receptor (FGFR) tyrosine kinase inhibitor PD173074 completely protected Y1 cells from FGF2 inhibition (Supplementary Data, Fig. S1B), implying that the inhibitory effect of FGF2 is mediated by FGFRs. These inhibitory effects of FGF2 were not unique to our Y1 strain and were also observed in another ras-dependent malignant cell line, which carried amplified K-ras genes in the DM instead of HSR chromosomes (Supplementary Data, Fig. S1C; ref. 23).

The FGF2-induced inhibition of malignant cell proliferation seems to be due to irreversible cell cycle arrest (senescence). Bromodeoxyuridine (BrdUrd) labeling of Y1 cells arrested in G₀/G₁ shows that FCS, FGF2, or FCS + FGF2 stimulate the completion of G₁ and entry into S phase (Table 1), in agreement with our previous results (25). However, Y1 cells treated with FGF2 or FCS + FGF2 showed significantly less uptake of ³H-thymidine into DNA compared with serum-treated cells (Table 1; Supplementary Data, Fig. S2). In addition, Y1 cells treated with FGF2 or FCS + FGF2 had extremely low mitotic indices by 18 hours (Table 1), indicating that
they fail to reach mitosis because they are trapped in S phase. Furthermore, DNA fragmentation characteristic of the later stages of apoptosis remained at negligible levels even after 48 hours of FGF2 treatment, as shown by flow cytometry analysis (Fig. 2A) and terminal nucleotidyl transferase–mediated nick end labeling assays (data not shown). To detect early signs of apoptosis and necrosis, we used standard assays of FITC–Annexin V labeling and propidium iodide exclusion, respectively. Our results showed that the vast majority of FGF2–treated Y1 cells were negative for both green (FITC–Annexin V) and red (propidium iodide) fluorescent labeling (Fig. 2B). In addition, Y1 cells treated with FGF2 for 24-48 hours did not show any activation of caspases 3 or 7, and their ATP levels were normal and stable, confirming that the mitochondrial respiratory chain remained functional (data not shown).

Consistent with these results, the strong and specific caspase inhibitor Ac–DEVD–CMK did not protect Y1 cells from FGF2 toxicity in clonogenic assays (data not shown). In fact, FGF2 increased Y1 cell granularity or internal complexity (see side-scattered light in Fig. 2C) and induced SA-β-Gal expression in 50% of the cells within

### Table 1. BrdUrd labeling, 3H-thymidine incorporation, and mitotic index in Y1 adrenocortical cells arrested in G<sub>0</sub>-G<sub>1</sub> and subsequently stimulated with FCS and/or FGF2

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<th>3H-thymidine incorporation (cpm x10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>3H-TdR / BrdUrd</th>
<th>Mitotic index (%)</th>
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<td>FGF2</td>
<td>60</td>
<td>17.5 ± 5.2</td>
<td>0.29</td>
<td>0.9</td>
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</table>

NOTE: G<sub>0</sub>-G<sub>1</sub>-arrested Y1 cells were stimulated with 10% FCS and/or 1 nmol/L of FGF2 for 18 h; cells were allowed to incorporate BrdUrd (50 μmol/L) or 3H-thymidine (3H-TdR, 1 μCi/mL, 10<sup>-7</sup> mol/L) for 8 h (between 10 and 18 h). 3H-TdR incorporation results are presented as the means of two independent experiments ± SD. BrdUrd-labeled nuclei (n = 1,500 cells) and mitotic indices (n = 6,000 cells) were obtained from two independent experiments.
6 hours and in >80% of the cells by 24 hours (Fig. 2D). All of these observations indicate that FGF2, although acting as a canonical mitogen in promoting the G0-G1 → S transition, also triggers a senescence-like process and irreversibly blocks the cell cycle at the S phase in Y1 cells.

H-RasV12–transformed BALB-3T3 fibroblasts express constitutively high levels of H-RasV12-GTP (Supplementary Data, Fig. S3). Similar to its effect on Y1 cells, FGF2 induces a senescence-like process in these fibroblasts. Thus, both FGF2 and FGF1 strongly inhibited colony growth of the transformed 3T3-B61 subline in clonogenic assays (Fig. 3A), and this inhibition depended on the activation of FGFRs (Fig. 3B). In addition, FGF2 inhibited the proliferation of 3T3-B61, regardless of whether the cells existed as monolayers (Fig. 3C), as suspension cultures (data not shown), or as tumors in immunocompetent BALB/c mice (Fig. 3D). Furthermore, FGF2 inhibited the proliferation of the Y1-RasN17-3.1 subline in the absence of dexamethasone (Fig. 4A), but not in its presence (Fig. 4B). Y1-RasN17-3.1 cells behaved identically to parental Y1 cells in clonogenic assays, showing reduced colony growth after 24 hours of FGF2 treatment (Fig. 4C). However, prior treatment with dexamethasone rendered Y1-RasN17-3.1 cells resistant to FGF2, whereas colony development of parental Y1 cells was still strongly inhibited by FGF2 (Fig. 4C). On the other hand, the expression of cyclins D1 and E, the activation of CDK2, and the phosphorylation of Rb protein in G0-G1–arrested parental Y1 cells and Y1-RasN17-3.1 cells stimulated with either FCS or FGF2 were essentially the same, irrespective of dexamethasone treatment (Supplementary Data, Fig. S5). These results imply that serum or FGF2 promotes progression through G1 in Y1 cells, and that this promoting effect is independent of the levels of K-Ras-GTP. Therefore, high levels of K-Ras-GTP in Y1 cells cause FGF2 to induce a senescence-like response consisting of a block in S phase.

Constitutively high levels of Ras-GTP in malignant cells make their proliferation vulnerable to inhibition by FGF2. As seen most clearly in the Y1 adrenocortical cell line, the inhibitory effect of FGF2 on cell proliferation depends on constitutively high levels of K-Ras-GTP. In stable clonal transfectant Y1 sublines (26) carrying the human dominant-negative mutant H-rasN17 (27) under the control of the mouse mammary tumor virus promoter, dexamethasone induced the expression of the H-rasN17 transgene and caused a severe reduction in endogenous levels of KRas-GTP levels (Supplementary Data, Fig. S4; ref. 26). This reduction, in turn, led to changes in cell phenotype. FGF2 inhibited the proliferation of the Y1-RasN17-3.1 subline in the absence of dexamethasone (Fig. 4A), but not in its presence (Fig. 4B). Furthermore, Y1-RasN17-3.1 cells behaved identically to parental Y1 cells in clonogenic assays, showing reduced colony growth after 24 hours of FGF2 treatment (Fig. 4C). However, prior treatment with dexamethasone rendered Y1-RasN17-3.1 cells resistant to FGF2, whereas colony development of parental Y1 cells was still strongly inhibited by FGF2 (Fig. 4C). On the other hand, the expression of cyclins D1 and E, the activation of CDK2, and the phosphorylation of Rb protein in G0-G1–arrested parental Y1 cells and Y1-RasN17-3.1 cells stimulated with either FCS or FGF2 were essentially the same, irrespective of dexamethasone treatment (Supplementary Data, Fig. S5). These results imply that serum or FGF2 promotes progression through G1 in Y1 cells, and that this promoting effect is independent of the levels of K-Ras-GTP. Therefore, high levels of K-Ras-GTP in Y1 cells cause FGF2 to induce a senescence-like response consisting of a block in S phase.

Cells rescued from tumors growing in FGF2-injected animals are not resistant to FGF2. Y1 cells taken from tumors growing in FGF2-injected nude mice were sensitive to FGF2 growth.
inhibition and did not yield clonal sublines resistant to FGF2 (data not shown). These observations show that the tumor growth observed in FGF2-injected animals was not due to the emergence of FGF2-resistant cells; rather, it was more likely because the FGF2 injection procedure did not provide sufficient access to all tumor cells. Thus, cell culture conditions are more appropriate for analyzing the frequency of spontaneous emergence of FGF2 resistance among Y1 cells.

Y1 colonies grown from single cells that had been treated with FGF2 for 24 hours produced Y1 clonal sublines that maintained the amplified K-ras gene and exhibited high basal levels of K-Ras-GTP (Fig. 4D; Supplementary Data, Fig. S6). These sublines were not FGF2-resistant and gave rise to rapidly growing tumors when inoculated into nude mice; this tumor growth was inhibited by FGF2 injections, as in the parental Y1 line (Supplementary Data, Fig. S6).

With sustained FGF2 treatment, it was possible to select rare, stable Y1 sublines lacking K-Ras overexpression (Fig. 4D). These lines require FGF2 for growth in both monolayer and suspension cultures and are poorly tumorigenic in nude mice. A complete characterization of these FGF2-resistant Y1 sublines will be described in a separate publication. These results show that FGF2 strongly selects against K-Ras overexpression in Y1 cells, instead favoring the emergence of sublines whose growth requires FGF2.

Likewise, the 3T3-B61 malignant line yielded FGF2-resistant sublines, which require FGF2 for growth, are non-tumorigenic in BALB/c mice but have lost both the neomycin marker gene and the H-ras V12 trans-oncogene (Supplementary Data, Fig. S7). The results therefore show that, surprisingly, it is very difficult for malignant cells from both Y1 and 3T3-B61 lines to overcome the negative selection exerted by FGF2 in culture.

**Tumor cells deficient in RhoA activity are resistant to FGF2-induced senescence.** FGF2 causes migration and morphologic changes in Y1 adrenal cells and BALB-3T3 fibroblasts, indicating that the cell adhesion-cytoskeleton system has been activated. This activation is coordinated by the Rho family of GTPases (reviewed in ref. 28). In serum-deprived Y1 or 3T3-B61 cells, the level of RhoA-GTP was below the detection limit (Fig. 5A), but exogenous FGF2 was sufficient to elevate the level of RhoA-GTP and maintain it at a high level (Fig. 5B). Thus, cell cycle arrest by serum deprivation correlated with RhoA deactivation, whereas cell cycle re-stimulation by FCS or FGF2 led to elevated levels of RhoA-GTP. However, blocking RhoA activation by constitutively expressing the dominant-negative mutant RhoAN19 (Fig. 5C and D) severely inhibited cell migration (data not shown) without reducing proliferation, demonstrating that RhoA activity is not essential for cell cycle completion or cell division in either the Y1 or 3T3-B61 cell lines.

### Figure 3.

FGF2 inhibits both in vitro and in vivo proliferation of mouse 3T3-B61 fibroblasts transformed with H-RasV12. A, clonogenic assays in solid substrate cultures; the number of colonies in FCS + (FGF2—24 h) is significantly lower than in FCS, FCS + (FGF4—24 h), and FCS + (FGF5—24 h; P < 0.0001; \( \chi^2 \) test) but not FCS + (FGF1—24 h). B, clonogenic assays. The FGFR kinase inhibitor PD173074 protects cells from FGF2 cytotoxicity (P < 0.0001, \( \chi^2 \) test). C, growth curves (P < 0.01; Mann-Whitney test). D, onset of 3T3-B61 tumors in immunocompetent BALB/c mice injected with FGF2; controls did not receive FGF2 (P < 0.01, Mann-Whitney test).
In addition, clonal sublines stably transfected with the gene for RhoAN19, typified by Y1-RhoAN19-2.1 (Fig. 5C) and B61-RhoAN19-C.1 (Fig. 5D), were resistant to FGF2-induced senescence, as shown by clonogenic assays, growth curves (Fig. 5C and D), and SA-β-Gal activity (data not shown). On the other hand, the clonal sublines Y1-RhoAV14-1.1 (Fig. 5C) and B61-RhoAV14-A.1 (Fig. 5D) maintained the phenotype of their respective parental cell lines: FGF2 inhibited their proliferation (Fig. 5C and D) and induced the expression of SA-β-Gal (data not shown). Furthermore, inhibition of RhoA activity with C3 exoenzyme protected Y1 and 3T3-B61 cells from the noxious effect of FGF2 in clonogenic assays and abolished FGF2 induction of SA-β-Gal activity (Supplementary Data, Fig. S8).

Thus, the results from the ectopic expression of the negative-mutant RhoAN19 and the inhibitory effects of the C3 exoenzyme suggest that RhoA mediates the senescence-like process triggered by FGF2 in Y1 and 3T3-B61 cells.

**Discussion**

In this study, we show that FGF2 stimulates the growth and proliferation of immortalized mouse cell lines, but it triggers senescence in Ras-dependent malignant cells that are resistant to programmed cell death. This FGF2 stress response is observed at physiologic FGF2 concentrations (ED_{50} \sim 60 pmol/L), it depends on FGFR kinase activity (Fig. 3B; Supplementary Data, Fig. S1B), and it occurs through the activity of RhoA-GTP (Fig. 5). This stress response does not involve the MEK/ERK or phosphoinositide-3-kinase/Akt mitogenic pathways because inhibitors of these pathways (PD98059 and LY294002, respectively) failed to protect cells from FGF2 toxicity in clonogenic assays (data not shown).

Intriguingly, FGF2 causes severe toxicity, specifically in malignant cells, in sharp contrast with serum, which promotes the robust growth of malignant cells. Selection with FGF2 caused the appearance of rare revertants that could be amplified into sublines characterized by (a) a lack of Ras overexpression, (b) low tumorigenicity, and (c) a requirement of FGF2 for growth. Surprisingly, in spite of the known genetic instability of malignant cells, FGF2-resistant sublines exhibiting high tumorigenicity and Ras overexpression rarely arose spontaneously in Y1 and 3T3-B61 stock cultures.1 These results highlight the robustness with which extracellular FGF2 blocks the proliferation of Ras-driven malignant cells.

Y1 adrenocortical cells and 3T3-B61 fibroblasts suffer different types of cell cycle deregulation. In Y1 cells, the cell cycle is partially deregulated by two independent oncogenic lesions. First, constitutively high levels of K-Ras-GTP lead to the elevated activity of the

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1 T.G.F. Matos and H. Armelin, manuscript in preparation.
phosphoinositide-3-kinase/Akt pathway (26, 29, 30); second, cyclins D1 and E are simultaneously induced instead of being induced sequentially (Supplementary Data, Fig. S5), meaning that progression through G1 lies under the exclusive control of the signaling pathway: ERK1/2 \rightarrow \text{cyclin E/CDK2} \rightarrow \text{pRB-phosphorylation} \rightarrow \text{initiation of DNA synthesis. Thus, both serum and FGF2 stimulated the ERK} \rightarrow \text{E/CDK2} \rightarrow \text{pRB pathway, leading to the induction of fos and jun expression (25) and promoting progression through G1, irrespective of the levels of K-Ras-GTP (Supplementary Data, Figs. S1A, S1B, and S5). At the same time, FGF2 activated a novel}

A

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C

Y1-RhoAN19-2.1

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Figure 5. Inhibition of RhoA activation with the dominant-negative RhoA-N19 mutant renders both Y1 and 3T3-B61 cells resistant to FGF2 cytotoxicity. A, FGF2 activated RhoA in G0-G1–arrested Y1 parental cells but not in the Y1-RhoAN19-2.1 subline; Y1-RhoAV14-1.1 cells had constitutively activated RhoA. B, likewise, FGF2 activated RhoA in 3T3-B61 parental cells but not in the B61-RhoAN19-C.1 subline; B61-RhoAV14-A.1 cells exhibited constitutively activated RhoA. C, Y1-RhoAN19-2.1 cells were resistant to FGF2 cytotoxicity, as shown by growth curves and clonogenic assays; on the other hand, Y1-RhoAV14-1.1 cells were sensitive to FGF2 cytotoxicity, as shown by growth curves (P < 0.05, Mann-Whitney test) and clonogenic assays (P < 0.01, χ² test). D, B61-RhoAN19-C.1 cells were resistant, whereas B61-RhoAV14-A.1 cells were sensitive to FGF2 cytotoxicity.
signaling route (FGFR → RhoA-GTP) to trigger a stress response independent of constitutively high levels of K-Ras-GTP (Fig. 4C) but dependent on the ERK pathway. Therefore, the cell cycle arrest caused by the FGF stress response in Y1 cells is unlikely to involve p16/pRb. However, FGF2 induced the expression of p21 in Y1 cells (data not shown), but further study is needed to determine whether the p53/p21 pathway plays a relevant role in the cell cycle arrest.

It has been known for years that ectopic expression of the H-RasV12 oncogene in mouse 3T3 fibroblasts leads to silencing of c-fos and fosB genes and constitutive expression of cyclin D1, leading to cell cycle deregulation (31). In fact, when serum-starved 3T3-B61 fibroblasts transfected with RasV12 were stimulated to grow with FGF2 in the presence or absence of serum, none of the canonical mitogenic pathways were activated. Indeed, ERK1/2 were poorly phosphorylated, essential immediate-early genes such as c-fos and fosB were not induced, and cyclin D1 remained constitutively expressed (data not shown). Despite this severe deregulation of G1 phase progression, the cell cycle in 3T3-B61 cells could be arrested irreversibly by FGF2 in a RhoA-dependent manner (Fig. 5B and D). Thus, as mentioned above for Y1 cells, it is unlikely that the p16/pRb pathway plays a role in FGF2-induced cell cycle arrest in 3T3-B61 cells. In contrast, FGF2 induced p21 expression in 3T3-B61 cells (data not shown), implying that the p53/p21 pathway may be involved in the FGF2 stress response. However, BALB-3T3 sublines classically transformed with SV40 were also susceptible to the noxious effect of FGF2 in clonogenic assays (data not shown), despite the inactivation of the p53/p21 pathway by SV40 T-antigen in these transformed cells. Thus, a functional p53/p21 pathway may be unnecessary for triggering the FGF2 stress response in malignant transformed 3T3 cells.

A recent study in human ovarian carcinoma cell lines clearly showed that p53 and caspase 2 are required for apoptosis induced by the DNA-damaging drug cisplatin (32). In the absence of functional p53, cisplatin leads ovarian carcinoma cells into mitotic catastrophe followed by necrosis-like lysis (32). In fact, several stressful stimuli—including DNA damage, certain active oncogenes such as ras and myc, and telomere shortening—trigger senescence in normal cells by a process involving the p16/pRb and p53 pathways, which irreversibly block the cell cycle at the G1 phase (reviewed in ref. 33). Malignant transformation requires overcoming these barriers that protect cells and organisms from unchecked malignant proliferation. We propose that the RhoA-dependent, FGF2-induced senescence reported in this article is an example of exogenously induced defenses that restrain tumor growth by a mechanism independent of the p16/pRb and p53 pathways. To our knowledge, this is the first report of a senescence-like state in malignant cells induced by mechanisms involving RhoA pathways. Our findings indicate that FGF2, both a paracrine and autocrine tissue homeostatic factor, can initiate senescence in cells that have already passed through most or all of the intracellular barriers to malignant transformation. This novel proposal contradicts the long-standing suspicion that FGF2 is an oncogenic factor.

Numerous publications over the last 15 to 20 years have dealt with the role of FGF signaling in cancer development. The importance of understanding FGF signaling for advancing cancer biology and cancer therapy seems indisputable. However, the literature contains numerous and apparently contradictory results describing a diversity of epiphenomena, which makes it difficult to develop a coherent picture of FGF signaling in cancer. Indeed, the paracrine and autocrine roles of FGF2 in cancer development and maintenance remain ambiguous. Reports of high levels of FGF2 in several types of cancer (9–11) do not prove that FGF2 promotes tumor growth. It is also possible that during oncogenesis, FGF2 paracrine and autocrine circuits function as antitumor defenses that are eventually surpassed as the cancer progresses. In this way, the high levels of FGF2 in aggressive advanced tumors may simply be vestiges of the overproduction of antitumor factors. It is understandable that the data from FGF signaling is so complex, given that tumor growth involves somatic evolution within a genetically unstable and highly diverse cell population made up of both malignant and stromal cells. The hypothesis that FGF2 has an antitumor function may help to simplify this complex situation. Further elucidation of the mechanisms underlying the FGF2 stress response described here may provide insight into how malignant cells resist programmed cell death and suggest practical methods to reduce this resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

12. Coleman AB, Metz MZ, Donohue CA, Schwarz RE.
FGF2 Triggers Senescence in Malignant Cells via RhoA

Kane SE. Chemosensitization by fibroblast growth factor-2 is not dependent upon proliferation, S-phase accumulation, or p53 status. Biochem Pharmacol 2002; 64:1111–23.


Forti FL, Schwindt TT, Moraes MS, Eichler CB, Armelin HA. ACTH promotion of p27(Kip1) induction in mouse Y1 adrenocortical tumor cells is dependent on both PKA activation and Akt/PKB inactivation. Biochemistry 2002;41:10133–40.

Feig LA, Cooper GM. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 1988;8:3235–43.


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