Intracellular Expression of an Antibody Fragment-neutralizing p21 Ras Promotes Tumor Regression

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ABSTRACT

Mutated ras genes are found in a large number of human tumors and, therefore, constitute one of the primary targets for cancer treatment. Microinjection of the neutralizing anti-Ras monoclonal antibody Y13-259 was previously reported to induce transient phenotype reversion of ras-transformed rodent fibroblasts in vitro. We have prepared a single-chain Fv fragment (scFv) derived from Y13-259, and here, we show that intracellular expression of the scFv led to the specific inhibition of the Kas signaling pathway in Xenopus laevis oocytes and NIH3T3 fibroblasts. Moreover, neutralizing Ras with the scFv specifically promoted apoptosis in vitro in human cancer cells but not in untransformed cells. As a step toward cancer gene therapy, we finally demonstrated that intratumor transduction of HCT116 colon carcinoma cells with the anti-Ras scFv using an adenoviral vector elicited sustained tumor regression in nude mice.

INTRODUCTION

Mutated ras genes have been detected in a large number of carcinomas, as well as in many other cancers (1, 2). Most of these cancers have a poor prognosis (3) with currently available therapies, probably partly because cells harboring ras oncogenes become more resistant to radiotherapy and chemotherapy (4, 5). However, it was recently shown that inhibition of the expression of the Ki-ras oncogene in human tumor cell lines caused complete regression in animal models (6, 7). These results clearly demonstrate that ras oncogenes offer an excellent target for a therapeutic intervention.

ras genes encode GTP-binding proteins (p21 Ras) located at the inner face of the plasma membrane as a result of posttranslational modification by a farnesyl moiety (8). In their active, GTP-bound form, p21 Ras stimulate a signaling cascade that can lead to cancerous cell growth when it is deregulated, for instance, following a point mutation that locks the proteins under their active conformation. This cascade is also activated in cells transformed by other oncogene products with activities that depend on Ras, such as human epidermal growth factor receptor type 2 (9) and Src family kinases (10). In past years, intense efforts have been undertaken to control the biological activity of Ras. Strategies currently being developed mainly target the specific inhibition of Ras farnesylation (11, 12) or are based upon the expression of Ras-neutralizing molecules in cancer cells by gene transfer techniques (6). Among the latter approaches, intracellular expression of the Vh and Vk regions from a MAb as a scFv fragment has made it possible to neutralize a variety of harmful molecules, including viral proteins (13, 14). In the case of Ras, the cloning of a scFv derived from the neutralizing MAb Y13-259 (Ref. 15; hereafter referred to as Y259-scFv) has already been described (16) and been shown to interfere with Ras-mediated transactivation of a reporter gene in Jurkat cells (17). Likewise, expression of Y259-scFv in Xenopus oocytes caused inhibition of insulin-induced, Ras-dependent meiotic maturation (18, 19). A similar approach has also been developed to prevent the expression of oncogenic human epidermal growth factor receptor type 2 and to inhibit the development of breast and ovarian carcinoma cell lines (20, 21).

We took advantage of two anti-Ras MAbs (15), one neutralizing antibody (Y13-259) and one control, nonneutralizing antibody (Y13-238), to study their effects in human cancer cells. Microinjection of Y13-259 MAb into ras-transformed cells has been shown to abolish their transformed phenotype, as well as that caused by other oncogenes, such as Src and Src-related kinases (10, 22). In addition, Y13-259 inhibits all biological responses that require Ras proteins (23–25). Y13-238, on the other hand, has no influence on these events. We show here that microinjection of Y13-259 MAb but not of Y13-238 specifically kills tumor cells and provides evidence, for the first time, that neutralization of Ras with Y259-scFv promotes apoptosis. Finally, expression of the scFv in tumors derived from HCT116 colon carcinoma cells established in nude mice is shown to dramatically delay tumor growth, thus demonstrating that intracellular immunization against Ras represents a potentially attractive approach to cancer gene therapy.

MATERIALS AND METHODS

Cells and Antibodies. Rat anti-Ras Y13-259 and Y13-238 and mouse anti-c-Myc 9E10 hybridoma cells were grown in DMEM supplemented with 10% FCS (HyClone), 1 mM sodium pyruvate, 2 mM glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. MAbs were purified from culture supernatants using protein G-Sepharose (Pharmacia). Cell lines listed in Table 1 were obtained from American Type Culture Collection, except for NHDF (normal human dermal fibroblasts) and NHBE (normal human bronchial epithelial cells) lines, which were purchased from Clonetics and grown in the medium supplied by the manufacturer.

Plasmids. The prokaryotic pSWI expression vector (26) was a gift from Dr. G. Winter (Medical Research Council, Cambridge, United Kingdom). It contains the pelB signal peptide for periplasmic expression, Ncol and NotI cloning sites, and a c-myc-derived tag. pcDNA3 was from Invitrogen. The pMT3 vector was described previously (27). 

Cloning of Y238-scFv and Y259-scFv. Cloning and assembly of rearranged Vh (Y13-259) or Vh (Y13-238) and Vl genes were carried out by a three-step reverse transcription-PCR method using Taq polymerase (Applied Biosystems). Mouse Vh1Back (28) and Vh1For-2 (26) amplimers and mouse Vl2Back and Vl4For amplimers (29), as well as scMRL2-Back and scMRL-For, were used for the first Vh, Vl, and Vl reverse transcription-PCR amplifications, respectively. PCR conditions were 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. Purified PCR products (Wizard PCR Prep; Promega) were then submitted to a second PCR with nested primers (adding restriction sites to the 5' Vh or 3' Vl or Vl ends) and overlapping complementary sequences encoding the (GGGGS) linker to allow for cloning.
Table 1 Effect of microinjection of Y13-238 and Y13-259 MAbs into human normal
and tumor cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ras status</th>
<th>p53 status</th>
<th>Antibody</th>
<th>Cell death</th>
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<tbody>
<tr>
<td>Normal cells</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MRC5</td>
<td>WT</td>
<td>WT</td>
<td>Y13-259</td>
<td>–</td>
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<tr>
<td>NHBE</td>
<td>WT</td>
<td>WT</td>
<td>Y13-259</td>
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<tr>
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<td>WT</td>
<td>WT</td>
<td>Y13-259</td>
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<tr>
<td>NHD6</td>
<td>WT</td>
<td>WT</td>
<td>Y13-259</td>
<td>–</td>
</tr>
<tr>
<td>Osteosarcoma Saos</td>
<td>WT</td>
<td>–/-</td>
<td>Y13-259</td>
<td>++</td>
</tr>
<tr>
<td>Cervix carcinoma HeLa</td>
<td>WT</td>
<td>WT, inactivated</td>
<td>Y13-259</td>
<td>++</td>
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<tr>
<td>Breast carcinoma SK-BR-3</td>
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<td>Mutant</td>
<td>Y13-259</td>
<td>++</td>
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<tr>
<td>Lung carcinoma H322</td>
<td>WT</td>
<td>Mutant</td>
<td>Y13-259</td>
<td>+</td>
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<tr>
<td>H460</td>
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<td>WT</td>
<td>Y13-259</td>
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<td>H460</td>
<td>Ki, mutant</td>
<td>WT</td>
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<td>Colon carcinoma HCT116</td>
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<td>WT</td>
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<td>HT29</td>
<td>WT</td>
<td>Mutant</td>
<td>Y13-259</td>
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WT, wild type.

Experiments were done at least four times.

the 3' Vβ4 and 5' Vα1, or Vα2 ends. PCR conditions were 2 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. PCR products (about 415 bp for Vαn and 375 bp for Vαn and Vαj) were purified from agarose gels with the GeneClean kit (BIO 101). The third PCR (assembly PCR) was conducted in two steps: purified VH and VK or VA were mixed in equimolar gels with the Geneclean kit (BIO 101). The third PCR (assembly PCR) was conducted in two steps: purified VH and VK or VA were mixed in equimolar quantities and first assembled by 10 PCR cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. Amplification was then followed by 15 PCR cycles of 1 min at 94°C and 2 min at 74°C after addition of Back and For corresponding amplifiers. PCR products were purified as before, digested with NcoI and Nol, and ligated into pSW1 (26). Clones were tested by PCR, and the selected pY259-scFv and pY238-scFv clones were sequenced using the ABI Prism DNA sequencing kit (Perkin-Elmer Corp.). The sequence of Y238-scFv will be published elsewhere.

4 Subcloning of Y259-scFv and Y238-scFv. pSW1-Y259-scFv and pSW1-Y238-scFv plasmids were first digested with NcoI and EcoRI. The blunt inserted clones were cloned into EcoRV-digested pcDNA3 or Smal-digested pMT3. Correct orientation was assessed by restriction digestion.

Adeno-Y259-scFv Construct. The HnisP11-blunted fragment digested from pcDNA3-Y259-scFv and containing the sequences cytomegalovirus promoter-Y259-scFv cDNA-bovine growth hormone poly(A) was inserted into pCOS shuttle plasmid at the EcoRV site. pCOS contains the left inverted terminal repeat, the Y sequences, multiple cloning sites, pIX, and part of IVa2 regions as recombination sequences. The recombinant Ad was obtained following in vivo homologous recombination in human 293 cells cotransfected with pCOS-Y259-scFv linearized with BstBl and Ad-Rous sarcoma virus ß-galactosidase (30) genomic DNA digestion with Cial. Selection of the recombinant Ad was performed by two successive rounds of plaque assay purification. The purity of the recombinant virus was checked by restriction digestion and Southern blot hybridization of restricted viral DNA. The recombinant Ad was propagated in 293 cells and purified by two cesium chloride density centrifugations (31) and dialyzed against PBS. Titters of the viral stocks were determined by plaque assay in 293 cells.

Oocyte Assays. Groups of 30 Xenopus laevis oocytes (27) were microinjected in the nucleus with 1 ng of empty pMT3, pMT3-Y259-scFv, or pMT3-Y238-scFv. Thirty h later, oocytes were left in MBS buffer, stimulated with 1 μM progesterone, or received a cytosolic injection of 10 ng of p21 Ha-Ras Lyn12. Eighteen h after stimulation, oocytes were scored for GVBD and then homogenized in 200 μl of lysis buffer (80 mM sodium β-glycerophosphate (pH 7.4), 20 mM EGTA, 15 mM MgCl2, 1 mM sodium orthovanadate, 1 mM 4-phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After centrifugation, lipid-free cytosolic supernatants were collected. To determine the level of expression of the scFvs, 50 μg of protein from each lysate were separated by 14% SDS-PAGE and transferred onto a PVDF membrane that was incubated with anti-Myc 9E10 Mab, followed by peroxidase-labeled goat antimouse IgG (Nordic Immunology). The Amersham ECL detection system was used to reveal the scFvs.

Coimmunoprecipitation of Ras with Y259 and Y238-scFvs in Xenopus Oocytes. Oocytes received a first nuclear injection of 1 ng of pMT3, pMT3-Y59-scFv, or pMT3-Y238-scFv, and the next day, a cytoplasmic injection of 10 ng p21 Ha-Ras Lyn12. Four h later, oocytes were lysed as above in the presence of 1% Triton X-100, and the scFvs were immunoprecipitated with 10 μg of 9E10 Mab using protein G-Sepharose beads. scFv-bound p21 Ha-Ras was detected after SDS-PAGE of the samples and transfer onto PVDF using the pan-Ras Ab3 Mab (Oncogene Science), followed by antimouse IgG coupled to peroxidase.

MAPK Gel Shift Assay. Fifty μg of proteins from oocyte lysate were run on a 10% SDS-PAGE and transferred onto a PVDF membrane, which was probed with an anti-MAPK (ERK1 and ERK2) mouse Mab (Zymed). Detection was performed with goat antimouse alkaline phosphatase-conjugated IgG (Promega) using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Transfection and CAT Assay. Transfection of NIH3T3 cells and measurement of CAT activity were carried out as described (32). Cells were transfected with 0.5 μg of Py-TK-CAT and 0.5 μg of pSV2-Ha-Ras Val12 or 0.5 μg of pSV2-v-Raf in the presence of 0.2 μg of pcDNA3-Y259-scFv or pcDNA3-Y238-scFv. The total amount of DNA per dish was 5 μg (buffered with empty pSV2), and 30 μl of Lipofectamine (Life Technologies, Inc.) were added. After transfection, cells were maintained in the presence of serum, and CAT activity was assayed 48 h later by thin-layer chromatography. The radioactivity of the spots corresponding to the acetylated forms of chloramphenicol was quantitated using a PhosphorImager (Packard).

 Apoptosis Assay. H460 cells were seeded onto Cellocate coverslips (Eppendorf). The plasmids pcDNA3-Y238 or pcDNA3-Y259-scFv were microinjected in the nucleus (0.25 mg/ml) along with 2 mg/ml mouse IgG as a marker. Twenty h later, cells were fixed in 4% formaldehyde and permeabilized in 0.2% Triton X-100. Coverslips were incubated 1 h with P(ab’)2 antimouse labeled with Texas Red (Jackson ImmunoResearch) in Power Block (BioGenex). Apoptotic cells were stained by incorporation of FITC-conjugated DUTP into DNA breaks using the In Situ Cell Death Detection Kit (Boehringer Mannheim).

Intratumoral Injection of Ad-Y259-scFv in Nude Mice. All in vivo experiments were reviewed and approved by an internal ethical committee. HCT116 cells (105) were injected s.c. to nude mice. Eight days later, when tumors have reached a volume of 100 mm3, mice were anesthetized, and a single (on day 8) or four intratumoral injections of Ad-Y259-scFv, Ad-ß-gal (on the same days), or vehicle (PBS containing 3% glycerol) on days 8, 12, 14, and 16, was performed. Each injection consisted of a dose of 2 × 1010 pfu of recombinant Ad, which was delivered in two sites within the tumor mass. Tumors were measured three times a week using a caliper. Tumor volumes were calculated as follows: small diameter × large diameter × height/2.

RESULTS

Microinjection of Y13-259 MAb into Cancer Cells Induces Cell Death. Y13-259 MAb was injected into human lung carcinoma H460 cells that express a mutant Ki-ras gene. Within the first 15 h, some cells carried on dividing, whereas a number of others died (Fig. 1A). This can be explained by the fact that Ras activity is necessary for the G1-S transition (33) and that cells that had already passed this point when they received Y13-259 MAb completed their cycle in a Ras-independent manner. Alternatively, the antibody may kill cells only when they are in a certain phase of the cycle. Later on, the antibody was clearly lethal and killed about 50% of the cells initially injected within 40 h. Accordingly, the rate of cell division considerably slowed down (Fig. 1A). The number of deaths decreased thereafter, possibly due to intracellular degradation of the antibody. It has also been
reported that Swiss 3T3 fibroblasts scrape-loaded with Y13-259 can up-regulate their Ras levels within hours, eventually causing the cells to escape the neutralizing effect of the antibody (34). The way cells died is consistent with apoptosis and accompanied by shrinkage of the cytoplasm and nuclear condensation followed by cell fragmentation and was actually verified by TUNEL staining (data not shown). This toxic effect was specific for Y13-259 MAb because injection of Y13-238 MAb at the same concentration into H460 cells did not cause any cell death (Fig. 1B).

To determine whether this observation applied to other cell lines, we injected Y13-259 MAb into a panel of transformed cells (Table 1). We found that Y13-259 was able to kill most of the cells tested, although to a varying extent. Interestingly, the lethal effect of the antibody was independent of the ras status because H460 cells, which express mutant ras genes, were killed as efficiently as HeLa cells, which have wild-type ras. Moreover, p53 was not required for death induced by Y13-259 because Saos and HeLa cells, which are defective for p53 function, were very sensitive to the antibody. In contrast, Y13-259 MAb was devoid of toxicity to normal cells (Table 1), in agreement with previous observations (33). On the total duration of the experiments (2-3 days), we did not see any significant alteration of the growth rate in normal cells injected with the antibody (see “Discussion”). Altogether, these results indicate that most transformed cell lines require a functional Ras pathway to survive, at least in vitro. Injection of the control antibody Y13-238 into normal or tumor cells had no effect (Table 1).

Y259-scFv and Y238-scFv Interact with p21 Ras in Vivo. The cDNAs encoding the Vh and Vl chains of both MAbs were amplified and assembled by PCR, as described in “Materials and Methods.” To verify that the Myc-tagged scFvs retained a good affinity for Ras, they were expressed in Xenopus oocytes in the presence of human recombinant Ha-Ras p21, which is the only Ras protein recognized by Y13-238, whereas Y13-259 recognizes all three Ras p21s (15). Anti-Myc immunoprecipitates were analyzed by Western blot for the presence of Ras. Fig. 2A shows that, compared to control oocytes (Lane a), Ras coprecipitated with Y238-scFv (Lane b) and Y259-scFv (Lane c), indicating that they were correctly folded and able to bind their target efficiently in cells.

Y259-scFv Blocks Ras Signaling in Xenopus Oocytes. The left data set in Fig. 2B shows the level of maturation (GVBD) achieved in oocytes exposed to p21 Ras or progesterone in the absence of scFvs. Y259-scFv inhibited GVBD induced by oncogenic Ras by 70% on average (compare columns 3 in the left and right data sets), whereas it had virtually no effect (~10% inhibition) on progesterone-induced maturation, which uses a Ras-independent pathway (Ref. 24; columns 2, left and right data sets). Y238-scFv, on the other hand, did not prevent GVBD by either stimulus (Fig. 2B, middle data set). These results closely reflected the state of activation of p42 MAPK, which was estimated by gel shift (Fig. 2D). The slow-migrating band corresponding to the activated, phosphorylated form of MAPK that was detected in matured oocytes exposed to Ras or progesterone (Lanes 2 and 3, left data set) was absent in Ras-stimulated, Y259-scFv-expressing oocytes (Lane 3, right data set), whereas the level of MAPK activation was never affected in progesterone-treated oocytes (Lane 2, right data set). Y238-scFv did not inhibit activation of MAPK (Fig. 2D, middle data set). Fig. 2C shows that similar amounts of Y238-scFv and Y259-scFv were expressed in oocytes at similar levels.

Y259-scFv Inhibits Ras-dependent Transactivation in NIH3T3 Cells. The effect of Y259-scFv was tested on Ras-stimulated transcription of a reporter gene driven by the polyoma virus enhancer in NIH3T3 fibroblasts. Cells were cotransfected with a plasmid containing the CAT gene along with expression vectors encoding oncogenic Ha-Ras, in the presence or absence of Y259-scFv or Y238-scFv. Under our conditions, more than 40% of the cells were transfected (data not shown). As shown in Fig. 3, the anti-Ras scFvs did not inhibit basal transcription of the reporter gene in the absence of oncogenic stimulation, indicating that intracellular expression of scFv molecules is not toxic per se. Expression of Ha-Ras oncprotein enhanced the level of CAT activity up to 12-fold over the basal level, and this effect was inhibited by 70% when cells coexpressed Y259-scFv. To further demonstrate the specificity of Y259-scFv, we determined whether it would affect Raf transcripcional activity, which has been shown to be independent of Ras (35). Like Ras, v-Raf stimulated CAT expression from the construct, but it was not inhibited by Y259-scFv (Fig. 3). Y238-scFv had no effect on either Ras or Raf-mediated signaling in NIH3T3 cells.
Fig. 2. Y259-scFv but not Y238-scFv interferes with Ras signaling in Xenopus oocytes. A, Western blot of 9E10 (anti-Myc) immunoprecipitates of oocytes injected with the control vector (Lane a), Y238-scFv (Lane b), or Y259-scFv (Lane c) expression plasmids was probed with the pan-Ras Ab3 antibody to detect scFv-bound Ras. B, effect of Y259 and Y238-scFvs on Ras-induced GVBD. Oocytes injected with the control (left data set) or expression plasmids for Y238-scFv (middle data set) or Y259-scFv (right data set) were unstimulated (column 1), stimulated by progesterone (column 2), or microinjected with p21 Ha-ras (column 3). GVBD was scored 18 h later. Columns, means of six experiments; bars, SE. Expression of the scFvs was verified by Western blot with the anti-c-myc MAb (C), and the state of activation of p42 MAPK was assessed with an anti-ERK antibody (D). Molecular mass markers are indicated in kilodaltons.

H460 Cells Undergo Apoptosis upon Expression of Y259-scFv. H460 cells were microinjected with Y259-scFv- or Y238-scFv-encoding plasmids, along with a marker IgG to facilitate the detection of injected cells. When cells were analyzed 20 h later, the marker IgG was detected predominantly in the cytosol (Fig. 4, A and C), either because it had been cleaved and the resulting fragments translocated to the cytosol or because the injected cells had gone through a round of division and the antibody distributed between the cytoplasms of the daughter cells. Fig. 4, C and D, shows that cells that had received the control Y238-scFv were perfectly viable and incorporated the fluorescent nucleotide dUTP into their DNA at a very low background level (around 4%), identical to that produced by injection of the empty vector pcDNA3. In contrast, ~21% of the cells injected with pcDNA3-Y259-scFv clearly exhibited condensed and fragmented DNA, which was labeled with dUTP, demonstrating that they were undergoing apoptosis (Fig. 4, A and B). Fig. 4, A and C, illustrates the dramatic morphological changes that accompany death by apoptosis in cells expressing Y259-scFv. Interestingly, Y259-scFv retained the properties of the parental MAb, in that it did not cause lethality in normal Swiss 3T3 fibroblasts. However, the scFv was perfectly functional in these cells because it was able to prevent the synthesis of the c-fos protein stimulated by serum (data not shown).

Y259-scFv Promotes Tumor Regression in Nude Mice. To enable expression of the scFv in tumor cells in vivo, Y259-scFv cDNA was inserted into an adenoviral vector derived from human Ad type 5, downstream of a cytomegalovirus promoter. Preliminary experiments with Ad-β-gal showed that H460 were not easily infected in vitro. Thus, the colon carcinoma cell line HCT116, which also has a mutant Ki-ras gene, was used instead. Following in vitro infection of HCT116 cells at different multiplicities of infection, Y259-scFv could be easily detected by Western blot using the anti-Myc antibody (data not shown) for several days. Fig. 5A illustrates that injection of a single dose of $2 \times 10^9$ pfu of Ad-Y259-scFv into HCT116 tumors preestablished in nude mice dramatically affected tumor growth,
which was efficiently stopped for 24 days. However, tumor growth resumed after that period. As a control, injection of $2 \times 10^9$ pfu of Ad-β-gal had no effect. We estimated that ~5% of the tumor cells were transduced following a single injection of $3 \times 10^9$ pfu of Ad-β-gal (data not shown). When four injections of $2 \times 10^9$ pfu of Y259-scFv-expressing Ad were performed, tumor regression was much more pronounced, and the animals remained tumor free for an additional 20 days (Fig. 5B). It is noteworthy that in this experiment, one mouse of seven was completely cured and showed no sign of relapse for over 4 months (data not shown). As previously reported (36), some delay in the tumor growth rate was also noticed upon multiple injections of Ad-β-gal (Fig. 5B). Interestingly, under these conditions (four intratumor injections of $2 \times 10^9$ pfu of Ad-Y259-scFv), both the colon carcinoma cell line HT29 (wild-type ras) in nude mice and a ras-transformed murine fibroblastic line established in DBA/2 mice were also susceptible to the growth-inhibitory effect of Y259-scFv, although to a lesser extent (data not shown).

**DISCUSSION**

This study demonstrates that Y259-scFv, derived from the Ras-neutralizing Y13-259 MAb, is a potent effector molecule that blocks oncogenic Ras-mediated cell activation in different model systems and triggers apoptosis in tumor cells. It is worth noting that most of the cell lines described in Table 1 have either a mutant Ki-ras or a
The most striking feature of Y13-259 MAb is that it specifically caused the death of tumor cell lines. To our knowledge, this essential property has never been reported before, most likely because either injected cells were not recorded individually throughout the duration of the experiments or the experiments were done in normal cells (25). Y13-259 MAb also induced apoptosis in tumor cells with no ras mutations. It is well known that a number of oncogenes require Ras proteins to be fully transforming. Furthermore, p21 Ras are necessary for transformation by proteins that are effectors of very different signaling pathways, such as the eIF-4E initiation/translation factor, which regulates the translation of mRNAs into proteins (37, 38). Blocking Ras in such genetic backgrounds will, therefore, have the same beneficial outcome as in ras-transformed cells. On the other hand, we found that Ras inhibition in normal cells does not trigger cell death, probably because of the redundancy of survival pathways that are defective in cancer cells or, alternatively, because wild-type and mutant Ras may fulfill different biological functions.

A lot of attention was recently raised by the implication of Ras in cell survival. It has been reported that oncogenic Ras maintains the levels of bcl-2 mRNA and protein following interleukin 3 withdrawal (39). Although these data were generated in hematopoietic cells, Ras might also be able to stimulate bcl-2 expression in epithelial tumor cells. More recently, it has been shown that, in fibroblasts undergoing c-myc-driven apoptosis, a mutant Ras p21 could rescue cells through the specific activation of the phosphatidylinositol-3-kinase/protein kinase B pathway (40). In addition, there are indications that activation of the Ras/Raf/MAPK cascade has a role in blocking apoptosis in other cell systems (41, 42), yet the underlying mechanism is unknown. Likewise, p21 Ras can activate transcription from the mdr promoter and, thus, be partially responsible for the multidrug-resistant phenotype by keeping down the intracellular concentration of anticancer drugs (43), possibly in combination with other mechanisms (44, 45).

It is, therefore, possible that Ras activate multiple pathways that cooperate and result in enhanced tumor cell survival. Recent articles demonstrating that inhibition of Ras processing in rat fibroblasts restores their susceptibility to killing by ionizing radiations (46) or serum removal (47) fully support this hypothesis. Therefore, one can reasonably foresee that neutralizing Ras will restore a functional cell death pathway in tumor cells. We are currently investigating whether the scFv could exert its apoptotic effect through up-regulation of Ras ligand (48).

The specific and important apoptosis caused in vitro by Y13-259 MAb and the related scFv prompted us to evaluate the efficacy of the latter as an antitumor agent in animal models. Significant tumor regression was, indeed, observed following infection with an Ad expressing Y259-scFv of HCT116 colon carcinoma cells that have a mutant K-ras gene. We were able to detect expression of the scFv in vivo through its myc tag; furthermore, apoptotic cells were clearly identified by TUNEL staining in the scFv-treated tumors but not in control tumors (data not shown). Control experiments realized with Ad-β-gal showed that, at most, 10% of the total number of tumor cells were transduced in vivo in the four-administration protocol. Thus, the efficacy of the scFv must be accounted for by some "bystander" mechanism that may involve subapoptotic bodies containing toxic molecules that are taken up by neighboring, noninfected cells (49), the release of cytotoxic factors by immune cells, or the disruption of cell-to-cell contacts and subsequent collapse of the cytoskeleton (50). It can also be imagined that the scFv has some antiangiogenic activity because the process is partially controlled by Ras (51). However, we did not notice a major impact of the anti-Ras antibody on the growth rate of normal cells, which was roughly estimated by video recording.

Another strong advantage of this approach is that it provides a growth inhibition very specific for cancer cells but has no deleterious effect in normal human fibroblasts and epithelial cells. Mulcahy et al. (33) have reported that injection of Y13-259 MAb into growing, asynchronous NIH3T3 fibroblasts is not toxic (33). They found that the antibody transiently (between 11 and 22 h after injection) inhibited cell cycle progression and DNA replication only in those cells that had been injected before they had reached the S phase (33). More recent work suggested, however, that the effect of Y13-259 may be more subtle and that the antibody actually causes a delay in S-phase entry rather than a complete block (34). These data are consistent with the fact that we did not notice a major impact of the anti-Ras antibody on the growth rate of normal cells, which was roughly estimated by video recording.

Another strong argument in favor of the efficacy of the anti-ras scFv is that it specifically caused the death of tumor cell lines. To our knowledge, this essential property has never been reported before, most likely because either injected cells were not recorded individually throughout the duration of the experiments or the experiments were done in normal cells (25). The most striking feature of Y13-259 MAb is that it specifically caused the death of tumor cell lines. To our knowledge, this essential property has never been reported before, most likely because either injected cells were not recorded individually throughout the duration of the experiments or the experiments were done in normal cells (25). Y13-259 MAb also induced apoptosis in tumor cells with no ras mutations. It is well known that a number of oncogenes require Ras proteins to be fully transforming. Furthermore, p21 Ras are necessary for transformation by proteins that are effectors of very different signaling pathways, such as the eIF-4E initiation/translation factor, which regulates the translation of mRNAs into proteins (37, 38). Blocking Ras in such genetic backgrounds will, therefore, have the same beneficial outcome as in ras-transformed cells. On the other hand, we found that Ras inhibition in normal cells does not trigger cell death, probably because of the redundancy of survival pathways that are defective in cancer cells or, alternatively, because wild-type and mutant Ras may fulfill different biological functions.

A lot of attention was recently raised by the implication of Ras in cell survival. It has been reported that oncogenic Ras maintains the levels of bcl-2 mRNA and protein following interleukin 3 withdrawal (39). Although these data were generated in hematopoietic cells, Ras might also be able to stimulate bcl-2 expression in epithelial tumor cells. More recently, it has been shown that, in fibroblasts undergoing c-myc-driven apoptosis, a mutant Ras p21 could rescue cells through the specific activation of the phosphatidylinositol-3-kinase/protein kinase B pathway (40). In addition, there are indications that activation of the Ras/Raf/MAPK cascade has a role in blocking apoptosis in other cell systems (41, 42), yet the underlying mechanism is unknown. Likewise, p21 Ras can activate transcription from the mdr promoter and, thus, be partially responsible for the multidrug-resistant phenotype by keeping down the intracellular concentration of anticancer drugs (43), possibly in combination with other mechanisms (44, 45).

It is, therefore, possible that Ras activate multiple pathways that cooperate and result in enhanced tumor cell survival. Recent articles demonstrating that inhibition of Ras processing in rat fibroblasts restores their susceptibility to killing by ionizing radiations (46) or serum removal (47) fully support this hypothesis. Therefore, one can reasonably foresee that neutralizing Ras will restore a functional cell death pathway in tumor cells. We are currently investigating whether the scFv could exert its apoptotic effect through up-regulation of Ras ligand (48).

The specific and important apoptosis caused in vitro by Y13-259 MAb and the related scFv prompted us to evaluate the efficacy of the latter as an antitumor agent in animal models. Significant tumor regression was, indeed, observed following infection with an Ad expressing Y259-scFv of HCT116 colon carcinoma cells that have a mutant K-ras gene. We were able to detect expression of the scFv in vivo through its myc tag; furthermore, apoptotic cells were clearly identified by TUNEL staining in the scFv-treated tumors but not in control tumors (data not shown). Control experiments realized with Ad-β-gal showed that, at most, 10% of the total number of tumor cells were transduced in vivo in the four-administration protocol. Thus, the efficacy of the scFv must be accounted for by some "bystander" mechanism that may involve subapoptotic bodies containing toxic molecules that are taken up by neighboring, noninfected cells (49), the release of cytotoxic factors by immune cells, or the disruption of cell-to-cell contacts and subsequent collapse of the cytoskeleton (50). It can also be imagined that the scFv has some antiangiogenic activity because the process is partially controlled by Ras (51).

Our study demonstrates that the anti-ras scFv may be an effective anticancer agent able to induce tumor regression even at low tumor growth in cancer patients at an early stage of the disease. Intracellular Ras targeting by the means of Y259-scFv could also be combined
with farnesyltransferase inhibitory drugs (12) or associated with radiotherapy or chemotherapy, which might reverse the loss of sensitivity of many cancer cells to these treatments (52).

ACKNOWLEDGMENTS

We are grateful to H. Paterson (Institute of Cancer Research, London, United Kingdom) and J. Schrader (University of British Columbia, Vancouver, Canada) for the gift of ras-transformed hamster fibroblasts (RAS1 and RAS2 MABs, respectively) as well as to T. Sasazuki (Kyuushu University, Japan) for HCT116 cells. Y13-259 MAB preparation was given by J. Grassi (Centre d’Etudes de Saclay, Gif-sur-Yvette, France). We thank C. Orsini, C. Deleforterie, and I. Barlat for help with the Ad construct; J.M. Guillaume, S. Benoist, and N. Coutaeul for Ad stock production; M. Janicot and E. Adeline for help and support; and F. Risbec, N. Gruel, M.H. Dubois, and F. Lacroix for technical expertise.

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