Inhibition of Tumor Growth by a Dominant Negative Mutant of the Insulin-like Growth Factor I Receptor with a Bystander Effect

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ABSTRACT

The insulin-like growth factor I receptor is known to play a major role in transformation and apoptosis. The dominant negative mutant of the insulin-like growth factor I receptor, designated 486/STOP, causes massive apoptosis of tumor cells and inhibition of tumor growth and metastases. We now show that: (a) the stable expression of 486/STOP inhibits transformation (colony formation in soft agar) and/or tumor growth in nude mice of five different types of human tumor cell lines; and (b) more importantly, it has a bystander effect, inhibiting the growth of wild-type tumor cells when cells expressing 486/STOP are cojected with wild-type tumor cells. These findings suggest that it is not necessary to infect all tumor cells with 486/STOP to inhibit tumor growth, and they also open the possibility of using the product of 486/STOP directly against tumor cells.

INTRODUCTION

The IGF-IR, activated by its ligands, is emerging as a powerful inhibitor of apoptosis induced by a variety of agents. Thus, addition of IGF-I (or insulin-like growth factor II) inhibits apoptosis induced by interleukin 3 withdrawal (1, 2), c-myc overexpression (3), interleukin 1β-converting enzyme protease expression (4), serum withdrawal (5), anticancer drugs (6), and transforming growth factor-β (7). An overexpressed and activated IGF-IR protects cells from apoptosis induced by a variety of agents, including etoposide (8), interleukin 3 withdrawal (1, 9, 10), osmotic shock (11), tumor necrosis factor-α (12), p53 (10, 13), ionizing and non-ionizing radiation (14–16), and okadaic acid (17). Conversely, impairment of the IGF-IR function by antisense strategies (8, 18–26), dominant negative mutants (27, 28), or triple-helix formation (29) causes large-scale apoptosis of tumor cells in vivo and abrogation of tumor growth and metastases (30, 31). The variety of the procedures used to induce apoptosis suggests that the overexpressed wild-type IGF-IR may have a widespread antiapoptotic effect. In fact, Evan and collaborators (32) have suggested that only Bcl-2 and the IGF-IR truly decrease the probability of apoptosis, whereas other procedures may inhibit the apoptotic pathway(s), without actually preventing cell death.

Targeting of the IGF-IR may, therefore, be of practical interest in the treatment of cancer, especially in view of the fact that, when the IGF-IR function is impaired or abrogated, normal cells seem to be less affected than tumor cells (33). Among the various strategies used to down-regulate the IGF-IR function (see above), the use of dominant negative mutants has recently received some attention. A number of mutants of the IGF-IR have been described that act as dominant negative mutants, but some of these, although they may inhibit colony formation in soft agar, are not effective against tumor cells in vivo (34). Two dominant negative mutants of the IGF-IR have been shown to be effective against rodent and/or human tumors in vivo: (a) 952/STOP (27), which inhibits tumorigenesis in nude mice and includes the α subunit and part of the β subunit up to the juxtamembrane region and is thus anchored to the membrane; and (b) 486/STOP (28), which has a frameshift mutation resulting in a stop codon at residue 486 (not including the signal peptide). The RNA is, therefore, of normal length, but it codes only for the first 481 amino acids of the α subunit plus 4 amino acids that are not present in the original sequence. 486/STOP, not having a transmembrane domain can be secreted in the medium (28), where it causes apoptosis of C6 rat glioblastoma cells, inhibits tumor growth in nude mice (28) and prevents human breast cancer metastases, also in a nude mouse model.

We have extended our studies on 486/STOP to several human tumor cell lines, and here, we show that it has a bystander effect, when cells expressing it are cojected with wild-type human tumor cells in nude mice.

MATERIALS AND METHODS

Strategy for the Construction of the IGF-IR Truncated at Residue 486. The CVN-IGF-IR plasmid (35) containing the full-length coding sequence cDNA of the human IGF-IR under the control of the SV40 promoter was digested with PinAI, which cuts at nucleotide 1574 [numbering according to Ullrich et al. (35)]. The overlaps were filled with Klenow, and the plasmid was religated. This procedure generated a frameshift

Received 4/20/98; revised 8/17/98; accepted 8/20/98.

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The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; IGF-I, insulin-like growth factor I; EMEM, Eagle’s MEM; FBS, fetal bovine serum; SFM, serum-free medium; CM, conditioned medium.

mutation that resulted in the creation of an early stop codon 12 nucleotides downstream from the PinAI site (28). The receptor was designated 486/STOP, following the nomenclature of Prager et al. (27), whereas the expression plasmid was designated pIGFIRsol. This plasmid, like the original plasmid, contains a neo resistance gene.

**Generation of Cell Lines Expressing 486/STOP IGF-IR.**

The parental cell lines used here include: Calu-6, anaplastic human carcinoma from lung (ATCC HTB-56); DU145, human prostate carcinoma, metastasis to brain (ATCC HTB-81); LNCaP, human metastatic prostate adenocarcinoma (ATCC CRL-1740); HCT116, human colon carcinoma; Caov-3, human ovarian adenocarcinoma (ATCC HTB-75); and COS-7, green monkey SV40-transformed kidney cells (ATCC CRL-1651). All cell lines, with the exception of LNCaP, were cultured in EMEM (Life Technologies, Inc., Grand Island, NY), supplemented with 1% Basal Medium Eagle vitamins, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS (Sigma Chemical Co., St. Louis, MO). LNCaP cells were cultured in RPMI supplemented with 10% FBS. Semiconfluent cultures were transfected with pIGFIRsol or with pCVN empty vector in the presence of Transfectam Reagent (Promega), as recommended by the manufacturer’s protocol. Selection of neomycin-resistant clones was performed in the presence of 1 mg/ml G418 (Life Technologies, Inc.).

**PCR.** PCR was performed directly on cells treated with proteinase K (1 mg/ml) at 50°C for 30 min. Subsequently, samples were boiled for 10 min (to inactivate proteinase K) and spun down to pellet debris. Two μl of the cell lysates were used in a 100-μl PCR, performed according to the protocol given by Boehringer Mannheim. Primers to detect 486/STOP were chosen from the sequence of human IGF-IR cDNA (National Center for Biotechnology Information accession no. M24599). Primer sequences were: 5’ sense primer, positions 1152–1171 bp, 5’-CATTGCTTCCAGCTGGAAG-3’; and 3’ antisense primer, positions 1763–1782 bp, 5’-AACCGCGTACTGAGTCAG-3’. These primers span several introns and do not amplify genomic DNA. The amplification product (630 bp) contains a PinAI site that is lost in the 486/STOP cDNA. PinAI digestion of the PCR product amplified from the wild-type IGF-IR cDNA generates 423- and 208-bp fragments. PCR product amplified from 486/STOP cDNA cannot be cut by PinAI (Fig. 1B).

**Northern Blot.** Total RNA was extracted following the methodology described by Chomczynski and Sacchi (36), and RNA blots were carried out by standard procedures (37). A radioactive probe was prepared by the Random Primed DNA Labeling Kit (Boehringer Mannheim) and [α-32P]dCTP (~3000 Ci/mmol; Amersham, Arlington Heights, IL). The probe used was a Spht fragment of the wild-type IGF-IR cDNA (2.2 kb).

**Western Blot.** Protein levels for IGF-IR α and β subunits and 486/STOP were examined in all cell lines used in this study. Cells were lysed on ice with a 400 μl of lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 1% aprotinin]. Protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and samples of 20 μg or 100 μg (where indicated) of total proteins were separated on a 4–15% gradient SDS-PAGE (Bio-Rad) and transferred into nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 and probed with rabbit anti-IGF-IRα (N-20) or rabbit anti-IGF-IRβ (C-20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was an antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz), which was subsequently visualized by enhanced chemiluminescence detection reagents (Amersham). N-20 antibody was also used to detect 486/STOP protein because it recognizes amino acids 31–50 mapping at the NH2 terminus of human IGF-IRα. Proteins isolated from three cell lines of mouse fibroblasts that express known numbers of IGF-IR, R508 (15 × 103 IGF-IRs/cell), R503 (22 × 103 IGF-IRs/cell), and R600 (30 × 103 IGF-IRs/cell), were used to estimate the levels of IGF-IR in tumor cell lines.

**Growth Curves.** To compare growth in monolayer of human cancer cell lines transfected with pIGFIRsol or with pCVN empty vector, we plated selected clones at 1 × 105 cells/35-mm dish and cultured for 24 h. in the presence of FBS. Subsequently, the medium was changed to serum-free medium (SFM) [EMEM, 0.5 mg/ml BSA, and 50 μg/ml transferrin

Fig. 1. Detection of 486/STOP in stably transfected human tumor cells. A. RNA expression. Lane 1, Northern blot of p6 cells (controls); Lane 2, LNCaP cells transfected with an empty vector; Lane 3, LNCaP cells transfected with 486/STOP. All three cell lines express the endogenous LNCaP cells transfected with an empty vector; 2, transfected with 486/STOP. A clones transfected with 486/STOP: Lane 6–13, clones transfected with 486/STOP. Lane 14–17, clones transfected with empty vector.

B. mRNA. Lane 1, PCR product of wild-type IGF-IR; Lane 3, PCR product of 486/STOP, digested (resistant to digestion); Lane 4, undigested PCR product of wild-type receptor. Lane 5, H2O; Lanes 6–13, clones transfected with 486/STOP; Lanes 14–17, clones transfected with empty vector.
wild-type Calu-6 cells injected remaining constant at $1 \times 10^6$ cells. Wild-type and 486/STOP-expressing cells were co-injected into NIH Swiss nude mice. The size of the tumors was evaluated 3 weeks after co-injection.

**Tumorigenesis and CM.** To prepare CM from COS-7 or COS/486/STOP, cells were plated at $1.5 \times 10^6$ cells/100-mm plate in 10% FBS for 24 h. The cells were then washed three times with HBSS, and the semiconfluent cultures were incubated in SFM for an additional 48 h. Both media were concentrated 20× by Centriprep-50 centrifugal concentration (Amicon, Inc., Beverly, MA) and frozen at −80°C. Aliquots of the CM (100 μl) were injected into the lower neck of nude mice 7 days after the initial injection of Caov-3 cells ($3 \times 10^6$ cells). The size of the tumors was evaluated 4 weeks later.

**RESULTS**

A number of human tumor cell lines were transfected either with the plasmid expressing 486/STOP or with the empty vector. Clones were selected and screened for the presence of 486/STOP by PCR, taking advantage of the fact that the engineering of 486/STOP eliminated a restriction site. Digestion of the PRC products with PinAI cut the endogenous receptor into two bands, leaving 486/STOP undigested (630 bp). Fig. 1B shows one of these screenings, with controls: clones 7–13 are positive for 486/STOP (although some clones are only weakly positive), and only clone 6 is negative. Fig. 1A shows a representative Northern blot of one clone of LNCaP cells stably transfected with 486/STOP: the probe (see "Materials and Methods") recognizes the endogenous message (~11 kb) in all cell lines. Only p6 cells, carrying a wild-type human IGF-IR cDNA (Fig. 1A, Lane 1) and LNCaP/486/STOP (Fig. 1A, Lane 3) express the mRNA originating from the transfected cDNA (4 kb). The p6 lane is purposely overexposed, to show the endogenous signal for LNCaP. Other cell lines gave similar results (data not shown).

**Receptor Levels.** Fig. 1 shows that the level of endogenous IGF-IR mRNA in LNCaP cells is low (when compared to p6 cells, for example). Because it was the same with the other cell lines, we wanted to determine the levels of IGF-IR in all tumor cell lines used, the protein level being a better indicator of IGF-IR function than the mRNA levels. To obtain a semiquantitative measurement of receptor levels, we included in our Western blots (Fig. 2) the lysates of three mouse fibroblast cell lines that express known numbers of human IGF-IR (38): R600 (30 × 10^3 receptors/cell), R503 (22 × 10^3 receptors/cell), and R508 cells (15 × 10^3 receptors/cell). With the exception of Calu-6 cells, the other human tumor cell lines express very low levels of IGF-IR, <15,000 for LNCaP and DU145 and probably ~2000–3000 for Caov-3 and HCT116 (notice the difference in the amount of protein used for the Western blots Fig. 2, A and B). The α subunit of the IGF-IR in LNCaP cells reproducibly migrates slightly faster than the α subunits of the other cell lines. This is perhaps due to a decreased level of glycosylation (39).

Fig. 2 shows detection of 486/STOP in DU145, Caov-3, and LNCaP, respectively. Lysates of the same cell lines transfected with the empty vector are shown in Lanes 4–6, which are completely negative. Although the prod-
Growth in Monolayer Cultures. Although the down-regulation of the IGF-IR, either by antisense strategies or dominant negative mutants, inhibits colony formation in soft agar and abrogates tumorigenesis (see “Introduction”), its effect on monolayer cultures is usually much less dramatic (18, 28, 33). This is confirmed in Fig. 3, where the cells expressing 486/STOP were tested in monolayer. The control cells were stably transfected with an empty vector. To avoid confusion, we are giving the results with representative clones for each cell line, but the results were reproducible with other clones. Expression of 486/STOP causes a modest inhibition of monolayer growth in Calu-6 and DU145 cell lines (~25–40%), but in the other cell lines, it is essentially inactive.

Colony Formation in Soft Agar. Table 1 summarizes several experiments in which the ability to form colonies in soft agar was tested in the five different human tumor cell lines. As control, we are presenting here the tumor cell lines transfected with an empty vector (E clones), but no differences were noted between the parental cell lines and the clones of cells transfected...
with the empty vector. The clones expressing 486/STOP are designated Sol (followed by the clone number), and the cells transfected with the empty vector are designated E clones. Although there is variability among clones, in every tumor cell line, the expression of 486/STOP causes marked inhibition of colony formation in soft agar. When cells are in anchorage-independent conditions than when they are in monolayers.

**Tumor Growth in Nude Mice.** The animals were injected as described in “Materials and Methods.” Table 2 summarizes the results obtained. With all human tumor cell lines, it is safe to say that expression of 486/STOP causes a marked inhibition of tumor growth in nude mice. LNCaP cells could not be tested in this assay because the parental cell line did not form tumors in nude mice. The best inhibition was obtained with Calu-6, 75%; for Caov-3, 83%; for DU145, 79%; for HCT116, 79%; and for LNCaP, 81%. The extent of inhibition is remarkably constant, considering the differences among the various tumor cell lines, and the various clones. We, therefore, confirm again that interference with the function of the IGF-IR causes more dramatic effects when cells are in anchorage-independent conditions than when they are in monolayers.

**Table 1 Colony formation in soft agar**

<table>
<thead>
<tr>
<th>Clone name</th>
<th>No. of colonies</th>
<th>Clone name</th>
<th>No. of colonies</th>
<th>Clone name</th>
<th>No. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-6</td>
<td></td>
<td>Caov-3</td>
<td></td>
<td>DU145</td>
<td></td>
</tr>
<tr>
<td>Sol1</td>
<td>29 ± 9</td>
<td>Sol3</td>
<td>5 ± 1</td>
<td>Sol1</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Sol9</td>
<td>32 ± 4</td>
<td>Sol6</td>
<td>11 ± 1</td>
<td>Sol3</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>Sol10</td>
<td>44 ± 13</td>
<td>Sol9</td>
<td>8 ± 3</td>
<td>Sol7</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>E1</td>
<td>155 ± 19</td>
<td>E3</td>
<td>52 ± 13</td>
<td>E1</td>
<td>117 ± 34</td>
</tr>
<tr>
<td>E2</td>
<td>138 ± 16</td>
<td>E6</td>
<td>37 ± 11</td>
<td>E2</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>E3</td>
<td>123 ± 24</td>
<td>E7</td>
<td>45 ± 9</td>
<td>E3</td>
<td>101 ± 10</td>
</tr>
</tbody>
</table>

**Table 2 Tumor growth in nude mice**

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>No. of mice with tumors/total no. of mice</th>
<th>Tumor weight (mg), mean (SD)</th>
<th>Days after injection</th>
<th>Time of appearance (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-6/E1</td>
<td>4/4</td>
<td>388 (84)</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>Calu-6/Sol10</td>
<td>1/4</td>
<td>70</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>HCT116/E1</td>
<td>3/3</td>
<td>347 (73)</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>HCT116/Sol</td>
<td>3/3</td>
<td>62 (38)</td>
<td>28</td>
<td>14–21</td>
</tr>
<tr>
<td>DU145/E1</td>
<td>4/5</td>
<td>8.2 (5)</td>
<td>49</td>
<td>25–35</td>
</tr>
<tr>
<td>DU145/Sol</td>
<td>1/5</td>
<td>4.0</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>Caov-3/E1</td>
<td>3/3</td>
<td>15.3</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td>Caov-3/Sol</td>
<td>0/3</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

growth in some of the mice injected with 486/STOP cells or control cells (empty vectors).

**Fate of Expressed 486/STOP Plasmid in Nude Mice.** We had noticed that those nude mice that eventually developed tumors with cells expressing 486/STOP not only had small tumors, but they had tumors with a delayed appearance, i.e., they became detectable 2–3 weeks later than they did with the parental cell lines (see Table 2). We suspected that these tumors developed from surviving cells that divided and eventually lost the 486/STOP plasmid. We, therefore, tested one of these tumors (from Calu-6/Sol10 cells), and, as Fig. 5 shows, the 486/STOP plasmid was no longer detectable by PCR. The results are essentially the same as those obtained with a plasmid expressing an antisense RNA to the IGF-IR in human melanoma cells in nude mice (20). In those experiments, the appearance of late tumors was also characterized by plasmid loss.

**486/STOP Product Has a Bystander Effect.** For these experiments, we selected a sixth cell line, COS-7, because in these cells, 486/STOP is expressed at higher levels than in any other cell line. Parental COS-7 cells do not form tumors in nude mice (see below). To test for an inhibitory effect of the expressed 486/STOP, we carried out experiments in nude mice, in which the number of wild-type Calu-6 cells injected remained constant, whereas the number of coinjected cells expressing 486/STOP was progressively increased. Two different clones of 486/STOP-expressing cells were used: Sol10, derived from Calu-6, and COS/486/STOP, derived from COS-7 cells. The target always remained wild-type Calu-6 cells. The results are summarized in Fig. 6A, in which the stippled bars represent experiments with wild-type Calu-6 cells and COS/486/STOP cells. The same numbers of parental Calu-6 cells and COS/486/STOP cells (also designated as COS-sol cells) were injected, the number and the size of tumors was significantly reduced. When the number of COS-sol cells coinjected was twice the number of parental Calu-6 cells, there was a dramatic inhibition of tumor growth and, in fact, some mice did not develop tumors at all. At a ratio of 1:4 (parental Calu-6:COS-sol), three of six mice developed no tumors at all, whereas the other three developed tumors that were 15% the size of tumors generated by the parental cells alone. Several controls (not shown) were also used in these experiments: as already mentioned, parental COS-7 cells do not form tumors in nude mice, and increasing the number of parental cells used proportionately increased the weight of the tumors. The last bar on the right in Fig. 6A shows the tumor weight in nude mice when 5 × 10⁵ Calu-6 cells are coinjected with parental COS-7 cells. Please
Fig. 5 Fate of expressed 486/STOP plasmid in nude mice. PCR detection of 486/STOP. Lane 1, markers; Lane 2, Calu-6/Sol10 cells; Lane 3, 1 μg of 486/STOP plasmid; Lanes 4–7, tumors grown from Calu-6/Sol10 cells.

Fig. 6 A, tumor growth in nude mice. The number of wild-type Calu-6 cells injected was always 10⁶ cells per mouse. Abscissa, the ratio of coinfected wild-type Calu-6 cells and cells expressing the 486/STOP. ■, wild-type Calu-6 plus Calu-6/Sol10; ◊, wild-type Calu-6 plus COS-sol (486/STOP) cells; ■, Calu-6 only; ◊, 5 × 10⁶ wild-type Calu-6 cells plus 2 × 10⁶ of parental COS-7 cells. B, expression of 486/STOP in COS-7 cells. COS-7 cells were stably transfected with an empty vector (Lane 2) or with a vector expressing 486/STOP (Lanes 3–7). Western blots using an antibody against the α subunit of the human IGF-IR. M₇, 77,000 marker indicates the product of 486/STOP. The band at M₉ ~ 121,000 kDa is the endogenous receptor α subunit. Lane 1, lysate from R503 cells that have 22,000 receptors per cell.

Fig. 4 Growth of tumors in nude mice. Nude mice injected with 1 × 10⁶ Calu-6 (A and B) or Calu-6/Sol10 (C and D) cells. The animals were photographed at 4 weeks after injection.

Note that, in this particular experiment, the number of tumor Calu-6 cells injected was half the number injected in the other experiments of Fig. 6A, which accounts for the lower tumor weight. Similar results were obtained when wild-type Calu-6 cells were coinfected with increasing numbers of Calu-6/Sol10 cells (left-hatched bars in Fig. 6A). Fig. 6B shows that COS-sol cells express high levels of 486/STOP. These experiments establish unequivocally that cells expressing 486/STOP can inhibit the growth of wild-type tumor cells in vivo.

Effect of Injection in Tumor-bearing Mice of CM. We also tested the effect of injecting medium conditioned by COS-sol cells into tumor-bearing mice. The mice were first injected

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with Caov-3 cells \((3 \times 10^6 \text{ cells in } 100 \mu l)\), and a week later CM (prepared as described in "Materials and Methods") was injected nearby. The results are summarized in Table 3. In all cases, the mice injected with CM from COS-sol cells had smaller tumors than the controls, and in one mouse, no tumor could be detected at the termination of the experiment, another 3 weeks later. An ANOVA indicated that the difference in tumor weights was significant at a value of <0.05 confidence level.

### DISCUSSION

Our results can be summarized as follows. (a) 486/STOP, a dominant negative mutant of the IGF-IR, when stably expressed, causes growth inhibition in five human tumor cell lines. In all of the five cell lines tested, colony formation in soft agar was markedly inhibited (75–80%). (b) Tumor growth in nude mice was also dramatically inhibited in four cell lines (the fifth could not be tested, because the parental cell line, LNCaP, did not form tumors in our nude mice). The number of mice with tumors was markedly reduced, and when the tumors appeared, they were smaller. In Caov-3 cells, there was complete inhibition of tumor development. (c) 486/STOP had little effect on the growth of the same cell lines in monolayer cultures, confirming that the effect of targeting the IGF-IR is much more pronounced when the cells are challenged with anchorage-independent condition, as in soft agar and in xenotransplants (33, 40). (d) 486/STOP has a bystander effect, causes inhibition or even abrogation of tumor growth, when cells expressing it are co-injected with a tumor-forming cell line. Even CM from cells expressing 486/STOP has an inhibitory effect on already established tumors in nude mice. In addition, we are showing here, for the first time, that the product of 486/STOP can be detected in lysates of cells stably transfected with the plasmid.

In a previous paper (28), 486/STOP was shown to inhibit tumor growth in nude mice injected with C6 rat glioblastoma cells. Dunn et al.\(^4\) found that the expression of 486/STOP inhibits metastases from human breast cancer cells in nude mice but that the reduction in size of primary tumors was not statistically significant. We now show that the expression of 486/STOP markedly inhibits colony formation in soft agar and/or inhibits tumor growth in nude mice with five other human tumor cell lines. The cell lines are of varied origin: two prostate cancer, one colon cancer, one ovarian carcinoma, and one lung cancer cell line. HCT116 cells are the most resistant to inhibition: this is a very aggressive cell line, with tumors reaching an average weight of 346 mg in 4 weeks (range, 290–430 mg). HCT116 cells expressing 486/STOP did form tumors in xenotransplants, but the tumors appeared later and were of smaller size, with an average of 62 mg at 4 weeks (range, 23–100 mg). It may or may not be coincidental that HCT116 cells express very low levels of IGF-IR (see Fig. 2).

Unfortunately, the LNCaP cell line does not form tumors in nude mice, at least under the conditions we used. LNCaP cells are not sensitive to IGF-I, even in monolayers, a property probably due to their hypoglycosylated receptor, the hypoglycosylation often indicating that the receptor is poorly expressed on the surface of the cell (39). Still, colony formation in soft agar of LNCaP cells is markedly inhibited when the cells express 486/STOP, again indicating that cells in anchorage-independent conditions are much more sensitive to down-regulation of the IGF-IR than cells in monolayer.

The product of 486/STOP is known to be secreted into the medium (Ref. 28; see below), but it is also detectable in lysates of cells. It is not clear why nor do we know whether its presence in lysates may be part of its mechanism of action. In a purely speculative way, we may point out that molecules traveling through the endoplasmic reticulum easily form disulfide bonds, due the abundance of disulfide isomerase (reviewed in Ref. 41). Because the product of 486/STOP has an abundance of cysteines, it is possible that it may interact, in the endoplasmic reticulum, with the nascent subunits of the endogenous receptor. This is compatible with the observation that, in some but not all cases, the levels of endogenous IGF-IR are reduced in tumor cells expressing 486/STOP.

The delayed appearance of tumors in some cases with cells expressing 486/STOP is apparently due to the fact that surviving cells lose the plasmid and, therefore, the inhibition. This had already been observed with another plasmid expressing an antisense RNA to the IGF-IR RNA (20). This is not surprising because cells no longer under the pressure of selective agents (neomycin, puromycin, and so on) tend to lose plasmids rather rapidly. An important consideration in this respect is that the inhibitory effect of IGF-IR targeting is based on the induction of apoptosis in tumor cells (17-21), not on a slowing down of their growth; if the tumor cells survive, they resume cell division, and in so doing, rapidly eliminate the plasmid. Indeed, a careful observation of growth rates in the experiments described above (data not shown) indicated that the delay in the time of appearance of palpable tumors was the determinant factor. Once the 486/STOP tumors made their appearance, they grew at the same rate as parental cell tumors. This behavior is best explained by assuming that a large fraction of tumor cells expressing 486/STOP die in the first 24 h after s.c. injection, with the survivors becoming indistinguishable from parental cells.

By far, the most important aspect of these experiments is the finding that cells overexpressing 486/STOP can inhibit or even abrogate tumor growth of wild-type Calu-6 cells. The most reasonable explanation is that sufficient 486/STOP products are released into the environment to cause death of wild-type Calu-6 cells. There is a rough dose response, with the effect on tumor growth increasing with the number of 486/STOP cells co-injected (remembering that the number of Calu-6 cells injected was constant). Thus, when Calu-6 cells were co-injected with clone Calu-6/Sol10 (Calu-6 cells stably transfected with 486/STOP), the average weights of the Calu-6 tumors at 3 weeks were: 131 mg (all Calu-6 cells), 100 mg (ratio of Calu-6 to Calu-6/Sol10 cells, 2:1), 55 mg (ratio, 1:1), and 38 mg (ratio,
Similar results were obtained when Calu-6 cells were coinjected with COS-7 cells expressing 486/STOP. At higher ratios, indeed, there was even complete inhibition of tumor growth. Thus, at a 1:4 ratio of Calu-6 to COS-sol cells, three of six mice did not develop tumors at all, whereas the other three developed late smaller tumors. The CM was somewhat less effective, but one should consider that it was a single injection of CM 7 days after inoculation of tumor cells. Still, one of the animals did not develop any tumor at all. It is reasonable to assume that repeated injections may have more dramatic effects.

These findings open the way to the use of the product of 486/STOP to induce death of tumor cells in vivo. First, it is suggested that not all tumor cells have to be transfected or infected with 486/STOP to completely inhibit tumor growth: one can calculate that 80% efficiency is sufficient to inhibit the growth of wild-type tumor cells. This is probably an overestimate because these experiments were done, by necessity, in nude mice. In syngeneic models (19, 42), the down-regulation of the IGF-IR causes also an abscopal effect that leads to the killing of surviving tumor cells. Thus, in syngeneic tumors, it is possible that the percentage of tumor cells that have to be directly infected with 486/STOP could be <80%.

The bystander effect, however, also opens another, more important, possibility, and that is that inhibition of tumor growth could also be achieved by the injection of sufficient amounts of the 48-amino acid polypeptide. One advantage of targeting the IGF-IR is that the targeting (whether by antisense strategies, antibodies, or dominant negative mutants) seems to affect tumor cells much more than normal cells, at least in animals (reviewed in Ref. 33). Injection of the 486/STOP product could, therefore, discriminate between normal and abnormal growth. This approach is now being pursued.

REFERENCES


Inhibition of tumor growth by a dominant negative mutant of the insulin-like growth factor I receptor with a bystander effect.

K Reiss, C D'Ambrosio, X Tu, et al.