Inhibition of Tumorigenesis and Induction of Apoptosis in Human Tumor Cells by the Stable Expression of a Myristylated COOH Terminus of the Insulin-like Growth Factor I Receptor

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ABSTRACT

The insulin-like growth factor I receptor (IGF-IR) plays an important role in cell transformation and in protection from apoptosis. Although the wild-type IGF-IR generally has an antiapoptotic effect, there are reports that its COOH terminus may actually generate a proapoptotic signal. Three different expression plasmids, all coding for the COOH-terminal sequences of the human IGF-IR, MyCF, CF, and MyKCF, were stably transfected into human ovarian carcinoma CaOV-3 cells. All three plasmids had no effect on monolayer growth but strongly inhibited colony formation in soft agar. Only one of the plasmids, MyCF, expressing the last 112 amino acids of the IGF-IR and carrying a myristylation signal, caused large-scale apoptosis of CaOV-3 cells in vitro and abrogation of tumorigenesis in nude mice. The plasmid expressing the MyCF sequence was also introduced into human glioblastoma T98G cells, where it decreased the clonogenicity of cells, caused a marked inhibition of colony formation in soft agar, and induced apoptosis in vivo. A double mutation at residues 1293 and 1294 of MyCF completely abrogated its inhibitory and proapoptotic activities. Neither the autophosphorylation of the IGF-IR nor the tyrosyl phosphorylation of IRS-1 was affected by the mutations. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

INTRODUCTION

The IGF-IR activated by its ligands plays an important role in cell proliferation in at least three ways: (a) it is required, in cooperation with other growth factors, for the optimal growth of cells in vitro (1) and in vivo (2, 3); (b) it is quasi-obligatory for the establishment and maintenance of the transformed phenotype, at least in a variety of cell types (4); and (c) it protects cells from apoptosis. The evidence for an antiapoptotic function of the IGF-IR is substantial. Thus, an overexpressed IGF-IR is substantially inhibited by antisense strategies, dominant negative mutants, or triple-helix formation, tumor cells undergo massive apoptosis, which is impaired by antisense strategies, dominant negative mutants, or triple-helix formation (5). These cells can also be protected from apoptosis induced by IL-3 withdrawal by simple addition of IGF-I (6). IGF-I also protects cells overexpressing c-myc, which undergo apoptosis in the absence of other growth factors (7), as well as human cancer cells from cell death induced by diverse anticancer drugs (8). When the function of the IGF-IR is decreased or otherwise impaired by antisense strategies, dominant negative mutants, or triple-helix formation, tumor cells undergo massive apoptosis, which is more prominent in vivo than in vitro (9–11). This results in a dramatic inhibition of tumorigenesis (11–19) and metastases (20). Conversely, an overexpressed and functional IGF-IR protects cells from apoptosis induced by etoposide (21), tumor necrosis factor α (22), transforming growth factor β (23), p53 (24), ionizing and nonionizing radiation (25, 26), okadaic acid (27), and IL-3 withdrawal (28). Mutational analyses of the IGF-IR have indicated that the three functions mentioned above (mitogenesis, transformation, and protection from apoptosis) map on separate domains of the receptor itself (28–30).

In a previous report (28), we had found that IGF-IRs truncated at residues 1229 or 1245 (108 and 92 amino acids from the COOH terminus, respectively) were even more efficient than the wt receptor in protecting FL5.12 cells from apoptosis induced by IL-3 withdrawal. We postulated that the COOH terminus of the IGF-IR may transmit a proapoptotic signal, which is usually neutralized by the antiapoptotic function of the whole receptor. To test this hypothesis, several plasmids were generated expressing COOH terminus sequences of the IGF-IR (31, and see below). These plasmids were shown to be cytotoxic in transient expression assays in human breast cancer cell lines (31). We report here that the stable expression of these COOH terminus sequences of the IGF-IR inhibits colony formation in soft agar, and that one of these sequences (carrying a myristylation signal) induces large-scale apoptosis of human ovarian carcinoma cells and human glioblastoma cells in vitro and in vivo and can abrogate their ability to form tumors in nude mice.

MATERIALS AND METHODS

Plasmids. Three different constructs containing cDNA sequences coding for the COOH-terminal region of the human IGF-IR (CF, MyCF, and MyKCF; Fig. 1) were subcloned into expression vector pcDNA3 (Invitrogen). The CF sequence begins from the methionine residue at 1225 and ends at the COOH terminus, residue 1337 [here we follow the numbering of Ulrich et al. (32), which excludes the signal peptide]. The MyCF sequence begins at proline residue 1223 fused at its NH2 terminus with the first 16-amino acid consensus sequence myristylation signal from Src (31), which allows the translated polypeptide to localize on the inner surface of the plasma membrane. MyKCF is also fused with the myristylation signal but also has the kinase region sequence myristylation signal from Src (31), which also contains the translated polypeptide to localize on the inner surface of the plasma membrane. The MyKCF plasmid was then introduced into human glioblastoma T98G cells by a mechanism that avoids the protective effect of the IGF-IR.

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3 The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; IL-3, interleukin 3; IGF-I, insulin-like growth factor I; SFM, serum-free medium; PBS, fetal bovine serum; wt, wild-type; poly(EHMA), poly(2-hydroxyethyl methacrylate); PDEK, phosphatidylino-
generates the CaOV-3/CF, CaOV-3/MyCF, and CaOV-3/MyKCF cell lines. Clones were isolated in 600 µg/ml G418. Clones were also obtained from CaOV-3 cells transfected with the empty pcDNA3 vector. CaOV-3/MyCF clone 2 stably expressing the MyCF protein was transfected again with wt pcDNA3 to generate the CaOV-3/MyCF 2/IGF-IR cell lines. The wt pcDNA3 plasmid was cotransfected with the pLHL4 hygromycin resistance gene (35), and clones were selected in DMEM with 300 µg/ml G418 and 100 µg/ml hygromycin B.

The human glioblastoma cell line T98G was obtained from the American Type Culture Collection and kept in Earle's MEM with 10% FBS, 1% β-mercaptoethanol, 1% NEAA, and 1% sodium pyruvate. T98G cells were transfected by using Transfectam (Promega) and selected in media with 1400 µg/ml G418.

Colony Formation in Soft Agar. Cells were plated at 3 × 10⁴ cells/35-mm plate in DMEM with 10% FBS and 0.2% agar. Colonies more than 100 µm in diameter were counted after 1–3 weeks of incubation.

Tumorigenesis in Nude Mice. Cells were washed three times with HBSS and incubated in SFM for 24 h, trypsinized and washed twice with PBS, and resuspended in PBS. Cells (5 × 10⁶) resuspended in 100 µl of sterile PBS were injected s.c. above the hind leg of male BALB/c nude mice (Charles River Breeders).

Diffusion Chamber Assay. This assay was carried out as described previously (9).

Poly(HEMA) Cultures. Petri dishes (Falcon) were coated with a film of poly(HEMA) (Sigma Chemical Co.), following the protocol reported by Folkman and Moscona (36). Cells were seeded at concentrations varying from 5 to 10 × 10⁴ cells/ml with or without IGF-I (Life Technologies, Inc.) at 20 ng/ml unless otherwise noted.

Western Blots and Immunoprecipitation. For expression levels of the plasmids transfected into CaOV-3 cells, 1.0 mg of whole cell lysates from various clones was immunoprecipitated with anti-FLAG M2 antibody (Eastman Kodak) and separated on a gel, stained with anti-FLAG M2 antibody for the detection of CF and MyCF, or stained with an anti-IGF-IRβ subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for the detection of MyKCF. For the phosphorylation of the IGF-IR and IRS-1 in CaOV-3 cells, the cells were kept in SFM (DMEM with 0.1% BSA and 50 µg/ml transferrin) for 48 h and stimulated with 20 ng/ml human recombinant IGF-I (Life Technologies, Inc.) for 5 min. Cell lysates (600 µg) were immunoprecipitated with an anti-IGF-IR antibody (Ab1; Oncogene Science), or 300 µg of lysates were immunoprecipitated with an anti-IRS-1 antibody and then blotted with an antiphosphotyrosine antibody (PY20H; Transduction Laboratory). The Bcl-2 antibody was a kind gift from Dr. Carlo Croce (Kimmel Cancer Center).

Cloning Efficiency. After the transfection of each plasmid, T98G cells were cultured in media with 1400 mg/ml G418 for 2 weeks and then washed with PBS and stained with crystal violet.

![Diagram of the three IGF-IR COOH-terminal sequences used in these experiments. The IGF-IR numbers are those used by Ullrich et al. (32). The myristylation signal is a 16-amino acid sequence from Src (31). These sequences were cloned into expression plasmids (see "Materials and Methods").](image)
RESULTS

Expression of Transfected Plasmids. The clones of CaOV-3 cells obtained were monitored for the expression of the transfected plasmids and their relationship to IGF-IR levels. Lysates were immunoprecipitated with an anti-FLAG antibody and blotted with either the same antibody (Fig. 2A) or an antibody to the β subunit of the IGF-IR (Fig. 2B). Three clones were found to express significant amounts of the CF construct (COOH terminus without the myristylation signal; Fig. 2A, Lanes 1–3), and four clones were positive for the expression of MyCF (COOH terminus with the myristylation signal; Fig. 2B). In addition, three clones expressed the larger myristylated sequence (Fig. 2B), which includes the tyrosine kinase domain (see the diagram in Fig. 1).

We then compared the expression of the MyCF plasmid with that of the β subunit of the IGF-IR, using the same clones as in Fig. 2A. The results show that both the COOH terminus polypeptides and the IGF-IR can be detected in the same blot, but that the expression levels of the COOH terminus plasmids are lower than the receptor levels (Fig. 2C, Lanes 1–4).

Effect on Monolayer Growth. The cell lines described in the previous sections were tested for their ability to grow in monolayers. We tested them under three different conditions: (a) growth in SFM; (b) growth in SFM supplemented with IGF-1; and (c) growth in SFM in 10% FBS. The results are shown in Fig. 3. CaOV-3 cells grow in SFM, and their growth is augmented by IGF-1 and further increased by 10% serum. Expression of the COOH terminus plasmids does not seem to greatly affect the growth of these cells. Clones MyCF6 and MyKCF15 have significantly reduced growth rates, but this seems to be due to clonal variation, because some other clones (for instance, MyCF2) grow almost as well as the parental cell line.

Colonies of Cells in Soft Agar of CaOV-3 and Transfectant Cell Lines. The same cell lines were then tested for their ability to form colonies in soft agar (see “Materials and Methods”). The results are summarized in Table 1. Parental CaOV-3 cells grow in soft agar, but they do not make large colonies, as some other transformed cells do. In all transfectants, there was a marked reduction in the ability to form colonies in soft agar. Although we noticed some variability among the clones, we could not relate this variability to the level of expression of the COOH terminus polypeptides. The tendency is for cells expressing MyCF or MyKCF to be more affected than cells expressing the nonmyristoylated COOH terminus sequence, but there is no question that all three plasmids have a substantial inhibitory effect on the ability of CaOV-3 cells to form colonies in soft agar.

Tumorigenesis in Nude Mice. The effect of these stably expressed plasmids on the ability of CaOV-3 cells to form tumors in nude mice was then tested as described in “Materials and Methods.” Table 2 shows that parental CaOV-3 cells and CaOV-3 cells expressing either the CF plasmid or the MyKCF plasmid formed tumors in nude mice that became palpable within 3–4 days after injection. The CaOV-3/MyCF 2 clone was the only one that did not form tumors in nude mice, although the animals were kept under observation for a period of 3 months.

The Myristylated COOH Terminus of the IGF-IR Induces Apoptosis in Vivo. The abrogation of tumorigenesis suggests that in the case of CaOV-3/MyCF cells, the cells that were injected s.c. are probably undergoing programmed cell death. This was confirmed in diffusion chambers implanted into the s.c. tissue of rats (37). The results with CaOV-3 and CaOV-3-derived cell lines showed that the wt cells doubled in number in 24 h, whereas the CaOV-3 cells expressing the MyCF terminus died on a large scale, with the recovery of viable cells being less than 1% of the initial number. None of the other stably transfected plasmids had a similar effect, with recovery varying between 150 and 238% of the initial number (data not shown).

There is therefore a discrepancy between the inhibition of colony formation in soft agar, on one hand, and tumorigenesis and the induction of apoptosis in vivo, on the other. This discrepancy between colony formation in soft agar and in vivo results has already been noticed previously with certain dominant negative mutants of the IGF-IR (38). In contrast, tumorigenesis in nude mice correlates with the extent of apoptosis in the diffusion chamber.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Palpable tumors (days)</th>
</tr>
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<tbody>
<tr>
<td>CaOV-3 wt</td>
<td>3–4</td>
</tr>
<tr>
<td>Empty vector clone 2</td>
<td>3–4</td>
</tr>
<tr>
<td>CaOV-3/CF 3</td>
<td>3–4</td>
</tr>
<tr>
<td>CaOV-3/MyCF 2</td>
<td>No tumors detected after 3 months</td>
</tr>
<tr>
<td>CaOV-3/MyKCF 3</td>
<td>3–4</td>
</tr>
</tbody>
</table>
TUMORICENESIS AND APOPTOSIS IN HUMAN TUMOR CELLS

Fig. 4. CaOV-3/MycF cells undergo apoptosis in vivo. The cells were analyzed as described in "Materials and Methods" before loading into the diffusion chamber (left panel) and 16 h after loading into the diffusion chamber (right panel). An apoptotic peak is clearly visible in the latter panel.

Fig. 5. Phosphorylation of the IGF-IR and IRS-1 in CaOV-3 cells. The cells were either unstimulated (−) or stimulated with IGF-1 (+) for 10 min. A, lysates (600 μg) were immunoprecipitated with an antibody to the IGF-IR and blotted with antiphosphotyrosine antibody (see "Materials and Methods"). B, lysates (300 μg) were immunoprecipitated with an antibody to IRS-1 and blotted again with antiphosphotyrosine antibody. Lanes 1 and 2, CaOV-3 cells; Lanes 3 and 4, CF 3; Lanes 5 and 6, MyCF 2; Lanes 7 and 8, MyKCF #3; Lane 9, no lysate.

Evidence for Apoptosis. Apoptosis could be demonstrated by several methods. In Fig. 4, we show the results of fluorescence-activated cell-sorting analysis, which was paralleled by DNA laddering (21). The CaOV-3/MycF cells are shown before being loaded into the diffusion chamber (left panel) and 16 h after incubation in the s.c. tissue of a rat (right panel). The cell population shows a typical apoptotic peak (pre-G₁) after 16 h in the diffusion chamber. As expected, there is no apoptotic peak in cells in monolayer because, as mentioned above, none of the COOH terminus plasmids has any effect on monolayer growth. The apoptotic effect of MyCF in transient expression assays has been previously documented in breast carcinoma cells (31).

Phosphorylation of the IGF-IR and IRS-1. Because the activated IGF-IR protects cells from apoptosis (5, 7, 9, 10, 21, 24, 28), and because apoptosis is induced in CaOV-3 cells by the expression of a myristylated COOH terminus of the receptor, it is reasonable to inquire whether the expression of this construct affects IGF-IR signaling. Selected clones were tested, and the results are shown in Fig. 5. In all cases, there is good autophosphorylation of the IGF-IR after stimulation by IGF-1, and there does not seem to be any inhibition by any of the three plasmids tested (Fig. 5A). The same results were obtained when the cells were tested for tyrosyl phosphorylation of IRS-1 (Fig. 5B), one of the major substrates of the IGF-IR, which is required for mitogenic signaling (39–43).

Effect of an Overexpressed IGF-IR on Apoptosis of CaOV-3 Cells. Because protection from apoptosis in several cell lines and by different modalities often involves an overexpression of the receptor (44), we transfected CaOV-3/MycF 2 cells with a plasmid expressing human IGF-IR cDNA (32, 45). Two clones that overexpressed the IGF-IR (Fig. 6, top panel) were isolated. By densitometry, the level of IGF-IR expression in these two clones was 2.9-fold above that of the parental CaOV-3/MycF 2 cells. Fig. 6 also shows that there is essentially no difference in survival between the clones transfected with the IGF-IR cDNA and parental cell line CaOV-3/MycF 2 when tested in the diffusion chamber.

Cloning Efficiency of T98G Cell Transfectants. CaOV-3 cells are difficult to transfect, and we have noticed that the transfected plasmids are often poorly expressed. We therefore decided to extend

Fig. 6. Overexpression of the IGF-IR does not protect CaOV-3/MycF cells from apoptosis in vivo. CaOV-3/MycF cells were stably transfected with a plasmid expressing wt human IGF-IR cDNA. Top panel, the levels of expression of the receptor in the different cell lines. Bottom panel, the percentage of cells recovered after a 24-h incubation in vivo (see "Materials and Methods").
our observations to another human tumor cell line, T98G glioblastoma (46). In the first experiment, we tested the effect of MyCF or its mutants on the clonogenicity of T98G cells. The T98G cells were transfected as described in “Materials and Methods” with various plasmids expressing MyCF (IGF-IR wt COOH terminus) or its mutants. After transfection, the cells were selected with G418 for 2 weeks, and the plates were then stained with crystal violet. Fig. 7 shows that there is a substantial difference in the number of clones obtained with the different constructs. T98G cells transfected with MyCF gave very few clones, of small size (Fig. 7B), whereas the number of clones obtained by transfection with the 93/94 mutant (Fig. 7E) was very close to that obtained by transfection with the vector only (Fig. 7F). The number of clones obtained by transfection with mutants 50/51 (Fig. 7C) or 4A (Fig. 7D) was intermediate between the number obtained by transfection with wt MyCF and that obtained by transfection with the 93/94 mutant. Fig. 7A shows mock-transfected T98G cells. Thus, MyCF is inhibitory on another type of human tumor cell line, and its inhibitory activity is abrogated by a mutation at residues 1293 and 1294.

Levels of Expression of the MyCF Constructs in T98G Cells. Clones selected in parallel to the clones shown in Fig. 7 were then studied in more detail. Fig. 8 shows the level of expression of the various wt and mutant MyCFs determined as described in “Materials and Methods.” Lanes 1–3 are clones stably transfected with wt MyCF; Lanes 4–6 are clones transfected with the 50/51 mutant; Lanes 7–9 are clones transfected with the 4A mutant; and Lanes 10–12 are clones transfected with the 93/94 mutant. There are variations in the level of expression among the various clones, as one would expect, but each construct has at least one clone that expresses it quite well.

For a proper evaluation of the subsequent experiments, it should be noted that two clones of the 93/94 mutant express high levels of the MyCF plasmid. The same clones shown in Fig. 8 were tested for their ability to grow in monolayer cultures under three different conditions: (a) SFM; (b) SFM supplemented with IGF-I (20 ng/ml); or (c) SFM supplemented with 10% FBS. Again, as in the case of the CaOV-3 cells, T98G transfectants grew in monolayers as well as the parental cell line did (data not shown).

Colony Formation in Soft Agar. When the same T98G clones were tested for their ability to form colonies in soft agar, there were dramatic differences, as shown in Table 3. Clones expressing wt MyCF were markedly inhibited, whereas the number of colonies formed by cells expressing the 93/94 mutant was only slightly fewer than that formed by T98G cells transfected with the empty vector. In fact, colony formation in soft agar is roughly proportional to the cloning efficiency shown in Fig. 8. It is quite clear that a mutation at residues 1293 and 1294 of the IGF-IR COOH terminus inactivates the inhibitory effect of the expressed COOH terminus.

Apoptosis in Vitro. Cell survival was first determined in cells seeded on poly(HEMA) plates (36), in which cells were denied substrate attachment (see “Discussion”). In this assay, cells in SFM usually die, but they are protected to a certain extent if they are incubated with IGF-I only.4 This is also true of T98G cells (Fig. 9A), which survive better on poly(HEMA) plates when the SFM is supplemented with IGF-I. However, T98G/MyCF cells die under these conditions, even when IGF-I or 10% serum is added, confirming that these cells have a decreased survival in anchorage-independent situations. T98G cells stably transfected with the 1293/1294 mutant of MyCF are resistant to apoptosis (at least as resistant as the parental T98G cells), especially when IGF-I is added (Fig. 9A). An overexpressed Bcl-2 (Fig. 9B) protects MyCF cells from death in poly(HEMA) plates, indirectly confirming the apoptotic nature of cell death, caused by MyCF.

The wt IGF-IR was transfected into T98G/MyCF cells. This time, the expression was quite good (Fig. 10B), calculated at approximately 300,000 receptors/cell. However, there was no protection of T98G/MyCF from death, caused by MyCF.
MyCF cells in the poly(HEMA) assay (Fig. 10A), even after the addition of IGF-I.

The effect of COOH terminus expression on resistance to apoptosis was confirmed in diffusion chambers (data not shown). The T98G/MyCF clone stably transfected with the plasmid expressing human IGF-IR cDNA (see above) was also tested in vivo. Despite the very high level of expression of the IGF-IR, the clone was not protected from apoptosis when tested in the diffusion chamber (data not shown), confirming the poly(HEMA) data.

**DISCUSSION**

The rationale behind these experiments was based on previous observations that: (a) the IGF-IR protects tumor cells from apoptosis (see above); and (b) mutant IGF-IRs truncated at residues 1229 or 1245 provided better protection from apoptosis than did wt receptors (28). We postulated that the COOH terminus may send a proapoptotic signal that is usually overwhelmed by the antiapoptotic signal of the remainder of the receptor. We therefore designed three constructs expressing the COOH terminus of the receptor to test whether the COOH terminus itself actually did send a proapoptotic signal. The construction of these plasmids is given in more detail in the study by Liu et al. (31) in which they were shown to be cytotoxic in transient expression assays.

The novel findings in the present experiments are: (a) COOH terminus sequences of the IGF-IR can be expressed stably in human tumor cell lines; (b) none of the three plasmids used inhibits the growth of human tumor cells (CaOV-3 and T98G) in monolayer cultures, but all three greatly diminish the ability of these cells to form colonies in soft agar; (c) only the MyCF plasmid (the last 112 amino acids of the IGF-IR with a myristylation signal) inhibits tumorigenesis in nude mice by causing large-scale apoptosis of tumor cells; (d) a double mutation at residues 1293 and 1294 of the COOH terminus sequences completely abrogates the inhibitory and proapoptotic effects of the MyCF plasmid; (e) Bcl-2 overexpression protects MyCF-transfected cells from apoptosis in anchorage-independent conditions; and (f) the IGF-IR, which has a widespread antiapoptotic effect against a variety of apoptotic injuries, does not protect cells from the apoptotic signals originating from MyCF. These findings are discussed separately, because they establish a basis for a detailed investigation of the mechanism(s) by which the myristylated COOH terminus of the IGF-IR, when expressed separately, can induce the apoptosis of tumor cells.

None of the three expression plasmids (stably transfected) inhibited the monolayer growth of CaOV-3 or T98G cells, whereas all three were quite effective in inhibiting colony formation in soft agar, again confirming that there are substantial differences when cells are tested in monolayer culture or under anchorage-independent conditions. We have now noticed several times that the targeting of the IGF-IR has a very modest effect on monolayer growth but has a very strong inhibitory effect on colony formation in soft agar (9–11). This discrepancy between the effect on monolayers and that on cells under anchorage-independent conditions is not limited to a functionally impaired IGF-IR. Rak et al. (47) found that cells transformed by an activated ras underwent apoptosis when cultured as multicellular spheroids on a nonadhesive surface and were thus forced into anchorage-independent growth, although they grew normally in monolayers. A similar result was obtained by Lebowitz et al. (48) with a farnesyl transferase inhibitor that lacked significance in cell toxicity in monolayer cultures but became a potent activator of apoptosis when cell attachment to the substrate was prevented. The higher susceptibility of some cells to apoptosis when under anchorage-independent conditions and the protective effect of substratum attachment have also been reported before (49–53). In some of these experiments, the poly(HEMA) system was used to show the induction of apoptosis caused by anchorage-independent conditions (48, 52, 53).

There are also striking differences between the inhibition of colony formation in soft agar and tumorigenesis in nude mice (Tables 1 and 2). As previously shown, the inhibition of tumorigenesis in nude mice correlates with the extent of apoptosis (9, 11). The extent of apoptosis was determined by the use of a diffusion chamber that allows quantitative recovery of cells implanted into the s.c. tissue of animals (9). In the case of the three plasmids, only MyCF can induce apoptosis in vivo and inhibit tumorigenesis in nude mice.

In a previous study (31), these three plasmids were tested for cytotoxicity on MCF-7 breast cancer cells in a transient expression assay. The MyCF construct was found to be cytotoxic, whereas the CF construct (no myristylation signal) was poorly cytotoxic. The MyKCF, which encoded the kinase domain as well as the COOH terminus (and the myristylation signal), had an intermediate effect. We have now extended these studies to stably transfected clones.

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**Table 3** Colony formation in soft agar of T98G cells and derivatives

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<thead>
<tr>
<th>Cell line</th>
<th>No. of colonies in soft agar after 1 week</th>
</tr>
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<tbody>
<tr>
<td>Empty vector clones 1, 2, and 3</td>
<td>130/130; 109/107; 74/74</td>
</tr>
<tr>
<td>wt MyCF clones 2, 4, and 8</td>
<td>7/11; 9/5; 3/2</td>
</tr>
<tr>
<td>50/51 clones 3, 9, and 10</td>
<td>2/3; 18/19; 11/16</td>
</tr>
<tr>
<td>4A clones 4, 5, and 8</td>
<td>8/18; 10/14; 20/20</td>
</tr>
<tr>
<td>93/94 clones 1, 13, and 15</td>
<td>92/98; 92/97; 73/69</td>
</tr>
</tbody>
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**Fig. 9** Bcl-2 protects T98G/MyCF cells from apoptosis. T98G cells and the derived cell lines (indicated on the abscissa) were tested for survival in poly(HEMA) plates (see "Materials and Methods"). A, the percentage of surviving cells (from initial seeding) was determined after 24 h. B, the level of expression of Bcl-2 in the T98G-MyCF, T-MyCF 93/94, T-MyCF Bcl2.10, and T-MyCF Bcl2.13 cell lines.
finding that a two-amino acid mutation (at residues 1293 and 1294) inactivates the inhibitory and proapoptotic activities of MyCF seems to rule out a non-specific effect of this membrane-directed COOH terminus sequence. This is further confirmed by the fact that certain activities (the induction of apoptosis and the inhibition of tumorigenesis) are confined to the MyCF sequence, with its myristylation signal.

Two of the constructs carried a myristylation signal for the purpose of localizing them to the membrane, because substantial evidence has shown that the component molecules of the ras transduction pathways are activated when they are localized to the membrane (54). Other artificial constructs have been shown to be activated by myristylation. For instance, Klippel et al. (55) reported that the myristylated p110 catalytic subunit of PI3k localized to the membrane, where it was constitutively activated in the absence of the p85 regulatory subunit. There are several other instances in which membrane targeting results in the activation of a signal-transducing molecule, as reported by Marais et al. (56), who also give several appropriate references. We speculate that myristylation of the IGF-IR COOH terminus by targeting it to the membrane could activate it, generating a proapoptotic signal. In fact, the localization of the myristylated MyCF to the membrane has been documented by Liu et al. (31).

The mechanism by which MyCF inhibits tumorigenesis and induces apoptosis in vivo is not clear, nor is it easy to investigate. The first obvious explanation would be competition with the endogenous IGF-IRs. However, this explanation does not seem compatible with the results we have obtained. In the first place, neither autophosphorylation of the IGF-IR nor tyrosyl phosphorylation of IRS-1 is affected. Serine phosphorylation of IRS-1 in excess of normal could not be detected either (data not shown). Direct competition with the IGF-IR is also incompatible with the finding that even with a very high expression of the IGF-IR, the proapoptotic effect of MyCF is unchanged.

The absence of protection by the IGF-IR also seems to be incompatible with an interference of its pathway: evidence is accumulating that PI3k and Akt are at least partially involved in the antiapoptotic action of the activated IGF-IR (25, 57); however, in our case, because the IGF-IR offers no protection, this pathway may not be affected. Indeed, preliminary studies from our laboratory indicate that PI3k activity in suspension cultures is the same in T98G/MyCF cells and in the parental cell line.\(^5\)

We have therefore made a few steps in elucidating the mechanism(s) by which the independently expressed COOH terminus of the IGF-IR, when myristylated, can induce large-scale apoptosis in tumor cells under anchorage-independent conditions. We know that the IGF-IR does not protect MyCF cells, but that Bcl-2 does. The possibility that caspases may be activated is being investigated. We tentatively suggest that all of these constructs inhibit the transforming signal that originates from the COOH terminus of the IGF-IR (30) by partial competition, but that the induction of apoptosis is due to a proapoptotic signal that the MyCF peptide (but not the other two peptides) sends by interaction with other molecules at the membrane.

The fact that a mutation at residues 1293 and 1294 (this study) completely abrogates this property of the MyCF plasmid gives us a clue to follow in identifying the pathway.

REFERENCES


\(^5\) G. Yumet el al., unpublished observations.
TUMORIGENESIS AND APOPTOSIS IN HUMAN TUMOR CELLS


Inhibition of Tumorigenesis and Induction of Apoptosis in Human Tumor Cells by the Stable Expression of a Myristylated COOH Terminus of the Insulin-like Growth Factor I Receptor

Atsushi Hongo, Gladys Yumet, Mariana Resnicoff, et al.