Using Apoptosis for Targeted Cancer Therapy by a New Gonadotropin Releasing Hormone-DNA Fragmentation Factor 40 Chimeric Protein

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ABSTRACT

Purpose: GnRH-based chimeric proteins have been shown to specifically target and kill adenocarcinomas both in vitro and in vivo. The purpose of this study is to construct a new GnRH-based chimeric protein for the treatment of adenocarcinomas in humans.

Results: In this study, we constructed and characterized a new chimeric protein, GnRH-DFF40, composed of a new human killing moiety: the apoptotic DNase-DFF40 (DNA fragmentation factor), known also as caspase-activated DNase (CAD). GnRH-DFF40 exhibits Dnase activity in vitro. We found that this chimeric protein can target and kill adenocarcinoma cells. Such death occurs via apoptotic pathways, resulting in an increase in the sub-G1 population, DNA fragmentation, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)-positive cells, and morphology typical of apoptotic cells. These apoptotic events involve the mitochondria because we found cytochrome c depletion and caspase-9 and caspase-3 activation. Preliminary in vivo results showed that treatment of colon adenocarcinoma xenografts in nude mice with the new chimeric protein caused a reduction in tumor weight.

Conclusions: Because GnRH-DFF40 is a whole human-based chimeric protein when applied to humans, the nonspecific toxicity and immunogenicity seen with bacterial/plant-based chimeric proteins should be avoided. Thus, GnRH-DFF40 is a promising candidate for the treatment of adenocarcinomas in humans.

INTRODUCTION

We recently described chimeric proteins that can both target and kill adenocarcinoma cells (1–3). These chimeric proteins include GnRH, also known as luteinizing hormone releasing hormone, as the targeting domain. GnRH is a hypothalamic decapeptide that plays a role in the reproductive system (4). For the killing domain, we used various derivatives of PE (1). This bacterial toxin inhibits protein synthesis in eukaryotic cells, thereby causing cell death (5, 6). GnRH-PE chimeric proteins kill a wide variety of cancers arising from hormone-responsive tissues, as well as from nonresponsive ones such as ovarian, breast, endometrial, cervical, colon, lung, hepatic, and renal carcinomas, all confined to the adenocarcinoma type (1–3). PE and other bacterial or plant toxins are commonly used in chimeric proteins (7). Although several chimeric proteins are already in clinical use, they have several disadvantages that limit their clinical application. The major obstacle in the clinical application of such chimeric proteins is the human immune response they elicit, mainly toward the toxin moiety. Bacterial toxins such as PE are highly immunogenic and cannot be humanized by standard techniques. Moreover, each chimeric protein displays some degree of nonspecific toxicity (8, 9).

To overcome these obstacles, we propose the use of human apoptosis-inducing proteins as the killing moiety.

Apoptosis, or programmed cell death, is an intrinsic mechanism common to all cells. Initially found in Caenorhabditis elegans (10, 11), there are three protein families involved in promoting or inhibiting apoptosis: the Bcl-2 family (12, 13); Apaf-1 (14, 15); and the caspases (16–18). To overcome these obstacles, we propose the use of human apoptosis-inducing proteins as the killing moiety.

The first chimeric protein constructed with an apoptotic moiety as the trigger of selective cell death was IL-2-Bax (19). In this chimera, the killing domain is Bax, a proapoptotic protein of the Bcl-2 family. IL-2-Bax specifically targets and kills cells overexpressing the IL-2 receptor via apoptosis (19). Next, we constructed a number of GnRH-based chimeric proteins fused to proapoptotic proteins Bax, Bik, and Bak of the Bcl-2 family (20). These proteins are able to specifically target and kill adenocarcinoma cells, similar to the specific activity of GnRH-PE chimeric proteins (1–3).

However, as we didn’t know which apoptotic protein will serve as the most potent killing domain in the chimeric protein, we have chosen an executor of apoptosis, a nuclease that is a downstream component of the complexed apoptotic cascade and tested its potential as a novel killing domain of a GnRH-based chimeric protein.

Our present study focuses on the DFF40 apoptotic protein as the novel killing domain of a chimeric protein and examines

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2 The abbreviations used are: GnRH, gonadotropin releasing hormone; PE, Pseudomonas exotoxin A; IL, interleukin; DFF, DNA fragmentation factor; FACTS, fluorescence-activated cell sorting; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; DAPI, 4',6-diamidino-2-phenylindole; NLS, nuclear localization sequence.
its ability to cause cell death. In eukaryotic cells, DFF40, known as DFF or caspase-activated DNase (21–23), is bound to its inhibitor DFF45 (24). A physiological apoptotic signal activates caspase-3, resulting in an active cytosine protease. Two of the caspase-3 substrates are DFF45 and its splice variant DFF35 (25). Cleavage of DFF45 or DFF35 by caspase-3 releases DFF40 from inhibition, thus allowing it to enter the nucleus and degrade DNA. The outcome of this apoptotic cascade is one of the most common morphological features of apoptosis: DNA fragmentation (24, 26, 27). It was recently shown that in addition to caspase-3, also granzyme B could indirectly activate DFF40 (28).

In this study, we use natural cellular apoptotic pathways to cause cell death of adenocarcinoma. We describe the construction and action of the GnRH-DFF40 chimeric protein. We demonstrate that GnRH-DFF40 causes specific cell death of adenocarcinoma cells via apoptosis through a cascade of events that starts with DNA nicking. DNA damage recruits cellular apoptotic proteins such as caspase-3, which execute cell death. This pathway does not involve the p53 protein. In addition, preliminary results show that in a colon carcinoma xenograft model in mice, the partially purified GnRH-DFF40 chimeric protein is able to inhibit tumor growth. Because adenocarcinomas account for ~50% of cancer-related deaths, this new whole human-based chimeric protein is a promising candidate for the treatment of cancer patients.

MATERIALS AND METHODS

Construction of a Plasmid Encoding the GnRH-DFF40 or GnRH-DFF40ΔC Proteins. The plasmid T-GnRH-PE66 (3) was cleaved with EcoRI and HindIII to remove the sequence fragment encoding PE. This fragment was ligated to a synthetic short oligonucleotide that includes the three restriction sites EcoRI, HindIII, and ClaI. The resulting plasmid served as an intermediate plasmid for constructing both pAB02 (encoding the protein GnRH-DFF40) and pAB02ΔC (encoding the protein GnRH-DFF40ΔC).

Total RNA was isolated from fresh human lymphocytes with the TriPure Isolation reagent (Boehringer Mannheim, GmbH, Freiburg, Germany) and then reverse transcribed into first strand cDNA using a reverse transcription system (Promega, Madison, WI). The DFF40 or DFF40ΔC sequences (24), flanked by ClaI restriction sites, were generated by PCR, using synthetic oligonucleotide primers covering the entire coding region of each protein. Both the intermediate plasmid and the PCR fragments were cleaved with ClaI and ligated to generate pAB02, encoding the protein GnRH-DFF40 or pAB02ΔC, encoding the protein GnRH-DFF40ΔC.

Expression of the Chimeric Proteins, Partial Purification, and Characterization. The chimeric genes were expressed in Escherichia coli strain BL21 (DE3). Subfractionation of the expressing cells revealed high enrichment of both chimeric proteins in the insoluble fraction. The insoluble fraction was denatured and refolded as described previously (20). The partially purified proteins, enriched with GnRH-DFF40 or GnRH-DFF40ΔC, were used in all our experiments.

Cell Lines. Cells were maintained and grown as described previously (20). All media and supplements were acquired from Biological Industries (Beit Ha’emek, Israel).

Far-Western Analysis. Proteins separated on SDS/polyacrylamide gels (~7.5 μg protein/lane) were transferred to nitrocellulose filters, as described previously (29). The membranes, after denaturation and renaturation, were incubated with 7.5 μg of a lysate of untreated Colo205 cells containing endogenous DFF45 for 16 h at 4°C, followed by three washes of 5 min in PBS containing 0.01% Tween 20. The membranes were incubated with anti-DFF45 and analyzed, using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

In Vitro DNA Cleavage. A quantity of 10 μg of naked plasmid DNA was incubated with 1, 10, or 25 μg of partially purified GnRH-DFF40. The control was 4 ng of DNase I (Boehringer Mannheim) or a control chimeric protein (Fc-Bak) devoid of DNase activity. The reaction mix was incubated for 90 min at 37°C and then loaded onto a 1.5% agarose gel.

Cell Viability. Cells were incubated overnight in 24-well plates (5 × 10⁴ cells in 1 ml of culture medium/well). The chimeric proteins or PBS, as control, were then added, and the cultures were harvested and counted, monitoring for viability after 24, 48, or 72 h of incubation.

FACS Analysis. Cells were analyzed for DNA content as a function of cell number by FACSscan (Becton Dickinson, Immunocytometry System, San Jose, CA), using the Lysys II program, as described previously (19).

Detection of DNA Fragmentation. DNA was extracted from mouse 2B4 T cells exposed to dexamethasone (5 × 10⁻⁶ m) or from human Colo205 cells exposed to GnRH-DFF40 (5 μg/ml) for 24 or 48 h, as described previously (19, 30), and run on 1.5% agarose for detection of DNA ladders.

TUNEL Assay. Cells (0.5 × 10⁶/10 ml) were incubated for various periods of time with 5 μg/ml GnRH-DFF40. We used the Apoptosis Detection System, Fluorescein (Promega) according to the manufacturer’s instructions. The slides were screened with the aid of a Zeiss Axiosvert 135M inverted microscope equipped with 63/1.2 C-achromat water immersion lens to visualize the fluorescein 12-dUTP-labeled DNA.

Confocal Microscopy. Colo205 cells were treated with GnRH-DFF40 or GnRH-DFF40ΔC for 4 or 24 h. The cells were collected and allowed to adhere to cover slips pretreated with 10% polylysine A by incubating them for 10–15 min at 37°C. The slides were treated as described previously (31). Samples were analyzed using 488-nm excitation for green florescence, 543-nm for red excitation, and a 364 Innova Enterprise Ion Laser for DAPI staining (blue).

Subfractionation. Cells treated with GnRH-DFF40 for various time periods were collected and washed twice with cold PBS. The cells were centrifuged at 500 × g for 5 min at 4°C. A quantity of 10⁶ cells was resuspended on ice in 100 μl of buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTG] for 15 min. The solution was forced 30 times through a 26 × g needle. The lysate was then centrifuged at 1900 × g for 10 min. The supernatant contained the cytoplasmic proteins. The pellet was resuspended in 70 μl of buffer B [20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA,
lysate was inverted for 30 min at 4°C and centrifuged at 39,000 × g for 60 min. The supernatant contained the nuclear proteins. The subcellular fractions were loaded onto 15% SDS-PAGE and Western blotted with various antibodies as described below (32).

**Western Blot Analysis.** Cells were solubilized using radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 1% SDS, and 50 mM Tris (pH 8.0)]. Whole cell extracts were run on 4–15% gradient SDS/polyacrylamide gels (~7.5 μg protein/lane) and then transferred onto nitrocellulose membranes. The membranes were incubated with the primary antibody and with peroxidase-conjugated second antibody and analyzed by the Amersham enhanced chemiluminescence system (Amersham). When noted, the membranes were stripped and reincubated with anti-β-Actin or anti-αTubulin as control for protein loading. Antibodies were purchased as follows: anti-DFF40 and anti-DFF45 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-2l-2 from Dako (Glostrup, Denmark); anti-Bid from Biogenesis (San Diego, CA); anti-αTubulin from Serotec (Raleigh, NC); anti-β-Actin from Sigma (Rehovot, Israel); anti-p73 from Oncogene (Boston, MA); and anti-p53 was a kind gift from Igal Haupt (The Hebrew University).

**Measurement of Endogenous Cytochrome c Levels by FACS.** Cells (1–2 × 10^6) were treated as described in Ref. 20, using anti-cytochrome c (PharMingen International). The cells were then washed twice and incubated with FITC-conjugated goat antimouse IgG (Jackson ImmunoResearch Lab, West Grove, PA). The cells were washed twice, resuspended in 0.5 ml of PBS, and analyzed by a FACScan.

**In Vivo Treatment of Nude Mice with the GnRH-DFF40 Chimeric Protein.** A quantity of 2.5 × 10^6 colon adenocarcinoma Colo205 cells in 100 μl of PBS were injected s.c. into 6-week-old female nude mice. Treatment started 96 h later, as in our previous study (2). All mice bore measurable tumors. A quantity of 5 μg (total protein concentration) of a partially purified GnRH-DFF40 chimeric protein preparation was injected i.v. into each of 11 mice. The control groups received PBS or 0.17 g/mouse/day GnRH-trp^6 (an analogue of GnRH that serves as the targeting moiety of the chimeric protein) in equal volumes. All injections were administered in a volume of 100 μl. In all experiments, tumors were measured every 2 days, and tumor size was calculated according to the formula length × width^2 × 0.4 (2). Upon termination of the experiment on day 11, the mice were sacrificed, and the tumors were removed and weighed.

**Statistical Analysis.** Significance levels of group differences between control and treated groups were assessed with planned comparisons using a two-tailed t test for independent samples. Differences were considered significant for Ps < 0.01. Bars indicate the SE of the mean.

**RESULTS**

**Construction, Expression, Partial Purification, and Characterization of GnRH-DFF40 and GnRH-DFF40ΔC.** cDNA fragments encoding DFF40 or DFF40 devoid of the COOH terminus (DFF40ΔC; 24) were generated by PCR amplification. These fragments were ligated downstream of a DNA sequence encoding an analogue of GnRH (trp replacing gly as the sixth amino acid) to generate pAB02, encoding the chimeric protein GnRH-DFF40 or pAB02ΔC, encoding the control truncated chimeric protein GnRH-DFF40ΔC. A schematic diagram of pAB02 is presented in Fig. 1A and of the chimeric proteins in Fig. 1B.

After transformation of E. coli BL21 (ADE3) with one of the two plasmids, expression of the fusion genes was controlled by the bacteriophage T7 late promoter, as described previously (1). SDS-PAGE and Western blotting using anti-DFF40 antibodies revealed that both chimeric proteins accumulated mainly in the insoluble subcellular fraction (Fig. 1, C and D). These partially purified insoluble protein preparations, after denaturation and refolding, were used in all experiments. It should be pointed out that we were able to express and partially purify GnRH-DFF40 in a bacterial system (Fig. 1, C and D), although previous studies indicated that DFF40 requires the presence of DFF45 for expression and proper folding (24).

**In Vitro Characterization of GnRH-DFF40.** We first characterized the GnRH-DFF40 chimeric protein. Native DFF45 binds to DFF40 via both N’ termini (24, 33, 34). Because in our chimeric protein the N’ terminus of DFF40 is blocked by the GnRH sequence, we examined whether DFF40 in the form of the chimeric protein still retains its ability to bind DFF45. To this end, Far-Western analysis was performed. Cell lysates of Colo205, E. coli extracts enriched with GnRH-DFF40, and E. coli extracts enriched with an irrelevant GnRH-based chimeric protein (carrying an irrelevant killing domain, different from DFF40) were run on SDS-PAGE, blotted onto a nitrocellulose filter, probed with Colo205 lysate containing endogenous DFF45, and characterized with antibodies against DFF45. As demonstrated in Fig. 2, in the Colo205 lysate two bands were detected corresponding to endogenous DFF45 and DFF45 bound to DFF40 (Fig. 2A, Lane 1). In E. coli extracts expressing GnRH-DFF40, a bandshift appeared at the M_r 40,000 (Fig. 2A, Lane 2), lower than the endogenous DFF45, and similar in size to that of DFF45 bound to the endogenous DFF40 in Colo205 cells (Fig. 2A, Lane 1). Using a control E. coli extract, expressing an irrelevant protein, as expected, no band was detected (Fig. 2A, Lane 3). These results indicate that GnRH-DFF40 can specifically bind endogenous DFF45. However, it is not clear whether the affinity is similar to that of native DFF40 for DFF45.

It has been shown that DFF40 possesses DNase activity (24, 35), although this activity is very low when cleaving naked DNA (36). Therefore, we next examined whether our chimeric protein GnRH-DFF40 shows DNase activity in vitro. As can be seen in Fig. 2B, when tested on purified plasmid DNA in a cell free system, the partially purified insoluble GnRH-DFF40 preparation exhibited dose-dependent DNase activity. In contrast, neither the soluble fraction of E. coli-expressing cells (Fig. 2B, Lane b) nor an irrelevant control chimeric protein devoid of DNase activity was able to nick naked DNA (Fig. 2B, Lane d). As a positive control, we used DNase I, which completely digested the plasmid DNA (Fig. 2B, Lane c).

**Intracellular Pathway of GnRH-DFF40.** To exert its activity within the target cells, GnRH-DFF40 has to be internalized and reach the nucleus where its substrate is located. We,
therefore, followed the intracellular fate of the chimeric protein’s pathway in Colo205 cells using confocal microscopy and specific antibodies. In the control cells, DFF40 (green fluorescence) was present throughout the cell and was abundant in the nucleus (Fig. 3A). We also followed the fate of its inhibitor, DFF45 (red fluorescence), and found that DFF45 was also present throughout the nontreated cells, including the nucleus (Fig. 3A). An overlay of the two antibodies shows that DFF40 colocalizes with DFF45 throughout the cell (indicated by yellow staining). It should be pointed out that although the two proteins colocalized, the cells maintained a reddish tinge, pointing to an excess of DFF45 in the untreated control cells (Fig. 3A).

At 4 h after treatment with GnRH-DFF40, the amount of DFF40 increased both in the cytoplasm and in the nucleus seen by the colocalization between DFF40 and DAPI (white staining). However, after a 24-h treatment, DFF40 increased mainly in the nucleus, detected by the white staining of the nucleus (Fig. 3A). These results show that GnRH-DFF40 enters the target cells and reaches the nucleus. It should be pointed out that anti-DFF40 antibodies recognize both GnRH-DFF40 and endogenous DFF40.

Monitoring of the intracellular fate of the truncated chimeric protein, GnRH-DFF40ΔC, in a similar manner, revealed that the mutated protein entered the cell. However, it did not penetrate the nucleus, even 24 h after treatment, seen by the green ring around the blue DAPI staining (Fig. 3B). These results are consistent with the lack of the chimeric protein’s nuclear localization signal.

Fig. 1 Construction of the plasmids encoding GnRH-DFF40 and GnRH-DFF40ΔC and characterization of the chimeric proteins. A, schematic presentation of the plasmid encoding GnRH-DFF40. B, schematic presentation of the two chimeric proteins GnRH-DFF40 and GnRH-DFF40ΔC. C, 12% SDS-PAGE stained with Coomassie Blue. D, immunoblot using αDFF40: Lanes 1–3, GnRH-DFF40; Lanes 4–6, GnRH-DFF40ΔC. Lanes 1 and 4, whole cell extract; Lanes 2 and 5, soluble fraction; Lanes 3 and 6, insoluble fraction. For details, see “Materials and Methods.” Arrow points to the chimeric proteins.

Fig. 2 In vitro characterization of GnRH-DFF40 activity. A, ability of GnRH-DFF40 to bind endogenous DFF45 as determined by Far-Western analysis. Whole cell extracts from Colo205 cells containing endogenous DFF45 were run on SDS/PAGE and Western blotted, using anti-DFF45 antibodies (1). Whole cell E. coli extracts containing GnRH-DFF40 were run on SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with a lysate of Colo205 control cells containing endogenous DFF45 (2) and as control, E Coli extracts, expressing on irrelevant protein (3) (as described in “Materials and Methods”) and analyzed using anti-DFF45 antibodies. B, in vitro DNA cleavage by GnRH-DFF40. Three concentrations of different chimeric proteins were added to 10 µg of naked plasmid DNA. Cleavage activity was seen in a, insoluble fraction of GnRH-DFF40, but not in b, soluble fraction of GnRH-DFF40. (1) 1 µg, (2) 10 µg, and (3) 25 µg total protein concentration of the partially purified E. coli extract containing GnRH-DFF40 were used in a and b. c, a total of 4 µg of DNase I was added to the plasmid DNA. d, a control chimeric protein (Fc-BAK) devoid of DNase activity was added to the purified DNA. M = marker.
Subfractionation Supports Confocal Results. Subfractionation, using anti-DFF40 antibodies, revealed an increase in GnRH-DFF40 throughout the treatment (Fig. 3C), first in the cytosol (Fig. 3C), and later in the nucleus. This was followed by a decrease in the total amount of DFF40 (Fig. 3C and data not shown). We used anti-α-Tubulin to verify the purity of the cytoplasmic fraction as well as a control for protein loading (Fig. 3C) and anti-poly(ADP-ribose) polymerase antibodies to verify the nuclear fraction (data not shown). Subfractionation of cells treated with GnRH-DFF40 enabled us to follow changes in the protein level in the cytoplasm and nucleus and, therefore, to validate our confocal results. Using anti-DFF45 antibodies, we found that similarly to the results obtained in the confocal microscopic studies, DFF45 was present in both compartments of the control cells (Fig. 3C). After treatment with GnRH-DFF40, there was an increase in the amount of DFF45 in the cytoplasm after 3 h. The amount of DFF45 and its subcellular distribution reverted to that of the control cells, 24 h after treatment.

GnRH-DFF40 Specifically Inhibits the Growth of Adenocarcinoma Cell Lines. Because DFF40 is an apoptotic protein (24) and exhibited DNase activity in vitro in a cell-free system (Fig. 2B), we examined its effect on target cell lines. To this end, we closely monitored the viability of cultured cells exposed to GnRH-DFF40 for 72 h. GnRH-DFF40 efficiently inhibited the growth of both the 293 renal (Fig. 4A) and the Colo205 colon (Fig. 4B) adenocarcinoma cells, eventually leading to cell death. The inhibition was dose and time dependent, indicating that the chimeric protein affects cell viability (Fig. 4). Whereas the control untreated cells continued to proliferate, the number of treated cells barely increased, even 72 h after a single treatment. The inhibition was apparent not only in 293 and Colo205 cells but also in a wide range of adenocarcinoma cell lines tested (Table 1). It should be pointed out that these results

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**Fig. 3** Intracellular localization of GnRH-DFF40 and GnRH-DFF40ΔC. A, localization of DFF40 in GnRH-DFF40-treated Colo205 cells studied by confocal microscopy. Anti-DFF40 antibodies were visualized with FITC (green), whereas anti-DFF45 was visualized with Cy5 (red). DAPI was used to stain the nucleus. B, localization of DFF40 in GnRH-DFF40ΔC-treated Colo205 cells by confocal microscopy. The antibodies were the same as those used in A. C, subcellular localization of DFF40 and DFF45 by subfractionation. α-Tubulin was used as a control to verify the purity of the cytoplasmic subfraction and for protein loading. C = cytoplasm, N = nucleus.
are consistent with our previous results, showing that GnRH-based chimeric proteins specifically target and kill a variety of adenocarcinoma cells (1, 3, 20).

Control nontarget cell lines, lacking GnRH-binding site expression, were not affected by the treatment with the GnRH-DFF40 chimeric protein (Table 1). Other positive control chimeric proteins bearing GnRH as the targeting moiety and fused to additional apoptosis-inducing killing moieties (20) inhibited the growth of 293 renal adenocarcinoma cells. In contrast, nonrelevant chimeric proteins composed of various moieties as the targeting domain had no effect on 293 cell growth (Table 2). Thus, GnRH-DFF40 shows a high specificity for adenocarcinoma cells expressing the GnRH-binding site (2, 3).

Nagata (24) demonstrated that DFF40 possesses a NLS in its C-terminus. Therefore, removal of the sequence should prevent the protein from entering the nucleus and causing cell death. After finding that the truncated GnRH-DFF40, although able to enter target cells, is unable to reach the nucleus (Fig. 3B), we examined the effect of the truncated chimera on Colo205 target cells. As can be seen in Fig. 4D, GnRH-DFF40ΔC had no effect on Colo205 cell growth. These results indicate that the NLS sequence at the C-terminus of DFF40 is crucial for GnRH-DFF40 activity.

We showed previously that an excess amount of GnRH

![Fig. 4](image-url) Partially purified GnRH-DFF40 inhibits proliferation of Colo205 and 293 cells; GnRH-DFF40ΔC has no effect. Cells were incubated for various periods of time with the chimeric proteins or PBS as control, stained with trypan blue and counted, and representative results are shown. A, time-dependent effect of GnRH-DFF40 on 293 cells. ■, control; ◆, treated cells. B, time-dependent effect of GnRH-DFF40 on Colo205 cells. ■, control, ◆, treated cells. C, dose-dependent effect of GnRH-DFF40 on 293 cells after 72-h treatment, and (D) effect of GnRH-DFF40ΔC on Colo205 cells after 72-h treatment. All experiments were performed with partially purified GnRH-DFF40 or GnRH-DFF40ΔC. Data shows one representative experiment for each cell line.

### Table 1  Effect of GnRH-DFF40 on various human cell lines

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<th>Cell type</th>
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<tr>
<td>Target cell lines</td>
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<tr>
<td>293</td>
<td>Renal cell adenocarcinoma</td>
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<tr>
<td>Colo205</td>
<td>Colon adenocarcinoma</td>
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<tr>
<td>A204</td>
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* □ indicates the SD of three to four experiments, performed using two to three different chimeric protein preparations. We used partially purified GnRH-DFF40 preparations in all experiments. Controls received PBS in equal volumes.

### Table 2  Effect of various chimeric proteins on 293 carcinoma cells

<table>
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<tr>
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<tr>
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<tr>
<td>GnRH-DFF40</td>
<td>Adenocarcinoma</td>
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<tr>
<td>L-GnRH-PE66</td>
<td>Adenocarcinoma</td>
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<tr>
<td>GnRH-BIK</td>
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<tr>
<td>Negative control</td>
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<tr>
<td>IL-2-BAX</td>
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<td>BPP-BAX</td>
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<tr>
<td>Fc-BAX</td>
<td>Mast cells</td>
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* L-GnRH-PE66 was tested using a highly purified protein (1); the other chimeric proteins were only partially purified as described in “Materials and Methods.”
inhibits the cytotoxic effect of additional GnRH-based chimeric proteins (1, 3, 20). In consistency with these results, we found that the cytotoxic effect of GnRH-DFF40 was inhibited by an excess amount of GnRH in both Colo205 and 293 cell lines (data not shown). These results indicate that the effect of GnRH-DFF40 toward target cells is a specific receptor-mediated response and, therefore, confirming the specificity of GnRH-DFF40 on adenocarcinoma cells.

GnRH-DFF40 Causes Cell Death of Target Cells via Apoptosis. To determine whether cells treated with GnRH-DFF40 are dying because of apoptosis, we measured the induction of apoptosis in target cells by a number of characteristic assays. GnRH-DFF40 augmented the population of apoptotic cells, as assessed by cell cycle staining of the cells with propidium iodide (FACS analysis). An increase in the sub-G1 population of a number of adenocarcinoma cell lines was observed (Fig. 5A). This increase is statistically significant ($P < 0.01$). In addition, a reduction in the S-phase population was observed after a 24-h treatment with GnRH-DFF40 (data not shown), both indicating augmentation of the apoptotic population. This phenomenon was detectable as early as 6 h after treatment. No effect was observed in nontarget cell lines lacking the expression of the GnRH-binding site (data not shown).

Because DFF40 exhibited DNase activity and cleaved naked DNA in vitro (Fig. 2), we tested its ability to induce DNA fragmentation in the 293 renal adenocarcinoma cell line. The cells were treated with GnRH-DFF40 for various periods of time, and DNA was extracted and analyzed. As can be seen in Fig. 5B, DNA cleavage was seen after 24 h (Lane 2), and DNA fragmentation was apparent after a 48-h treatment (Fig. 5B, Lane 4). DNA fragmentation was time dependent, peaking at 48 h after treatment. Similar DNA fragmentation was observed when control mouse T cells, the 2B4 cells, were treated with dexamethazone (Fig. 5B, Lanes 5 and 6), a known inducer of apoptosis in many cells (19).

An additional tool for detecting cells undergoing apoptosis is the TUNEL method, which discerns DNA cleavage. Fig. 5C shows TUNEL-positive cells, indicating that the cells are indeed undergoing apoptosis. This effect was apparent in a small number of the cells as early as 6 h after treatment, whereas the

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**Fig. 5** GnRH-DFF40 causes cell death via apoptosis. A, FACS analysis. GnRH-DFF40 was added at a concentration of 4.6 $\mu$g/ml for 24 h. Samples were FACS analyzed for DNA content as a function of cell number, as described in “Materials and Methods.” Bars indicate the sub-G1 phase, i.e., the apoptotic cells. Error bars denote the SE. Black bars indicate treated cells, and gray bars indicate control untreated cells. B, DNA fragmentation. Human renal carcinoma cells 293 were seeded at $1 \times 10^6$ cells/well, and 4.6 $\mu$g/ml (total protein concentration) of GnRH-DFF40 were added for 24 or 48 h. Dexamethazone ($10^{-7}$ M) was added to 2B4 cells for 24 h. The cells were then lysed, and DNA was extracted and loaded on a 1.5% agarose gel. 293 cells: Lane 1, control cells; Lanes 2 and 3, cells treated with GnRH-DFF40 for 24 h; Lane 4, cells treated with GnRH-DFF40 for 48 h. 2B4 cells: Lane 5, control; Lane 6, cells treated with dexamethazone for 24 h. C, TUNEL-positive Colo205 cells after incubation with GnRH-DFF40. Control cells (left) and treated cells (right; $\times 1000$) exposed to GnRH-DFF40. Red stained cells are normal cells; yellow-green stained cells are apoptotic cells. D, GnRH-DFF40 causes morphological changes in the target cells. Renal adenocarcinoma 293 or Nalm-6 B cells were treated with GnRH-DFF40 (5 $\mu$g/ml total protein concentration) for 72 h and photographed under a light microscope ($\times 100$).
control untreated cells remained unaffected (Fig. 5C). These two detection methods (DNA fragmentation and TUNEL) show that 293 renal adenocarcinoma cells undergo DNA cleavage after treatment with GnRH-DFF40.

Apoptosis was also evident when cells were examined for morphological changes under the light microscope. When 293 cells were treated with GnRH-DFF40, they exhibited morphological changes (Fig. 5D) typically seen in apoptotic cells. These included nuclear condensation and fragmentation, chromatin condensation, and blebbing (37). Nonadenocarcinoma cells such as Nalm-6 (of B-cell origin) were not affected (Fig. 5D).

Using a number of common apoptotic detection assays, we show that upon treatment with GnRH-DFF40 cells undergo a typical apoptotic cell death.

**Mechanism of GnRH-DFF40 Action.** There are major check points when following apoptotic pathways. We, therefore, determined changes in the levels of leading proteins involved in apoptotic pathways after treatment with GnRH-DFF40. Bid is a Bcl-2 family member that undergoes cleavage after activation of surface death receptors, giving rise to tBid (38, 39). To rule out an apoptotic pathway initiated by the chimeric protein via a surface receptor, we checked whether there were changes in the protein level of Bid and its cleaved product. We found that treatment of target cells with GnRH-DFF40 caused no difference in Bid levels (Fig. 6A). Moreover, we could not detect its truncated fragment (tBid). These results were obtained using a number of different target cell lines (data not shown).

p53 is a cellular protein that discerns DNA damage. When the damage is minor, p53 inhibits cell growth. However when there is extensive damage, p53 causes cell death via apoptosis (40–42). As DFF40 damages the DNA (Fig. 6A), we examined the level of p53 after treatment with GnRH-DFF40. There was no difference in p53 protein level after short-time treatment or long-term treatment (Fig. 6A). To examine whether these cells can accumulate p53 in response to DNA damage, we treated Colo205 cells with doxorubicin (3 µg/ml), a known inducer of p53. In contrast to treatment with GnRH-DFF40, when cells were treated with doxorubicin, there was an increase in the p53 level, which peaked after 45 min (Fig. 6A). As control for protein loading, we used anti-β-Actin antibodies on the same membrane (Fig. 6A).

Recently two homologous of p53 have been identified and characterized. The p73 is very similar to p53 both in sequence and in activation. The p73, like p53, undergoes activation after DNA damage, resulting in the transcription of many genes that lead to either cell arrest or apoptosis (43). Because p53 was not activated in our cells, we tested for changes in p73 levels after treatment with GnRH-DFF40. We found an increase in the protein levels both in Colo205 cells (Fig. 6B) and in 293 cells (data not shown). As control for protein loading, we used anti-α-Tubulin antibodies on the same membrane (Fig. 6B).

We next focused on mitochondrial involvement in promoting apoptosis upon cell treatment with GnRH-DFF40. We first examined differences in protein levels of a member of the Bcl-2 family known to promote apoptosis, Bax (12, 44). Bax is expressed and translocated to the mitochondria upon activation of apoptotic pathways. Therefore, differences in its total concentration could shed light on the GnRH-DFF40 mode of action. As shown in Fig. 7A, the amount of Bax did not change throughout the treatment, indicating that Bax does not trigger the death signal. The late increase in Bax levels observed at 24 h after treatment is a phenomenon that is at present unclear.

It has been shown that a normal apoptotic signal causes cytochrome c to be secreted from the mitochondria and to bind to procaspase-9 and Apaf-1, resulting in the activation of caspase-9 and, later, caspase-3 (16). We, therefore, followed these proteins using the appropriate antibodies. We found that upon treatment with GnRH-DFF40, cytochrome c is released from the mitochondria (Fig. 7B). When examining differences in the caspase-9, we found that caspase-9 was cleaved in a time-dependent manner (Fig. 7A). The M<sub>1</sub> 18,000 fragment, indicating activation of caspase-9, was present as early as 3 h after treatment. Moreover, the amount of the M<sub>1</sub> 18,000 cleaved fragment remained constant throughout the treatment.

To follow additional activation of caspase-9, we examined its substrate, caspase-3. The level of caspase-3 increased after treatment with GnRH-DFF40 and underwent activation, as indicated by the appearance of its cleaved products (Fig. 7A). The

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**Fig. 6** Changes in p53/p73 levels after treatment with GnRH-DFF40. Proteins were extracted from Colo205 cells after treatment with GnRH-DFF40 or doxorubicin, as described in “Materials and Methods,” and run on SDS-PAGE. Using specific antibodies various proteins were identified at different time points after treatment. A, anti-p53. Left panel: proteins extracted from GnRH-DFF40-treated cells. Right panel: proteins extracted from doxorubicin-treated cells. B, anti-p73. Anti-β-Actin and anti-α-Tubulin antibodies were used to control protein loading (for details see “Materials and Methods”).
cleaved products were detected 3 h after treatment began, its level increasing 6 h after treatment. This time course lags behind the activation of caspase-9 (Fig. 7A). When added concomitantly with the chimeric protein, Z-VAD-Fmk, a wide-range inhibitor of caspases, including caspase-3 (45), inhibited the cytotoxic effect of GnRH-DFF40 on Colo205 cells, whereas Z-VAD-Fmk alone was ineffective (results not shown). Our findings indicate that mitochondria and caspases are involved in the apoptotic pathways evoked upon treatment with GnRH-DFF40.

Activated of caspase-3 cleaves Bcl-2, an antiapoptotic protein (46–48). Therefore, we examined differences in Bcl-2 protein level after treatment with GnRH-DFF40. We found that Bcl-2 undergoes cleavage as treatment progresses. At 24 h after treatment, Bcl-2 was almost completely cleaved (Fig. 7A). It has been reported (46) that the cleavage results in the inhibition of Bcl-2’s antiapoptotic activity, and the cleaved Bcl-2 protein product was shown to exhibit proapoptotic activity.

Effect of Chimeric Protein Treatment on Tumor Xenografts in Nude Mice: A Preliminary Study. In an attempt to determine the ability of the new chimeric protein to target and kill adenocarcinoma cells in vivo, Colo205 colon carcinoma cells were injected s.c. into nude mice to induce solid tumors. Four days later, 5 μg/day (total protein concentration) of the partially purified GnRH-DFF40 were administered i.v. for a period of 11 days. The dosage used was found to be safe because no mortality was recorded throughout the treatment. An equal volume of either PBS or GnRH-trpα (an analogue of GnRH that serves as the targeting moiety of the chimeric protein) was injected into the controls. Tumor size was measured every 2 days. We found that the treated tumors were 3.5 times smaller in size than those in both control groups (Fig. 8A). Upon termination of treatment, on day 11, the tumors were removed and weighed after the mice were sacrificed. Tumors in the treated group weighed 2.5 times less than those in the control groups (Fig. 8B). This difference, both in size and in weight, is statistically significant (P < 0.01). Similar results were obtained in three sets of independent experiments preformed with various GnRH-DFF40 protein preparations (data not shown).

DISCUSSION

We recently developed a new class of chimeric proteins in which proapoptotic proteins are the killing moiety (19, 20), instead of the previously used plant or bacterial toxins (1, 3). These new chimeric proteins are most probably less immunogenic and should exhibit less nonspecific toxicity because they include a human protein as the killing moiety. There are a large number of apoptotic proteins that can serve as the killing moiety of the chimeric protein. A number of them, members of the Bcl-2 family, were recently tested by us and found to target and kill specific cell populations (19, 20). In this study, we describe the construction and action of a new apoptotic-based chimeric protein: GnRH-DFF40.

We constructed and partially purified GnRH-DFF40. We found that this chimeric protein can target and kill adenocarcinoma...
GnRH-DFF40 Kills Adenocarcinoma Cells by Apoptosis

Effect of GnRH-DFF40 on tumor growth in nude mice. GnRH-DFF40 was administered i.v. for 11 days at a concentration of 5 μg/mouse/day (total protein concentration). Day 0 indicates the start of treatment (day 4 after induction of tumors). In all experiments, tumors were measured every 2 days. Upon termination of the experiment, the mice were sacrificed, and the tumors were removed and weighed. A, tumor volume. , GnRH-DFF40-treated mice; , control, PBS-treated mice. B, tumor weight. Error bars show the SE.

Fig. 8

The tumor growth was measured every 2 days. Upon termination of the experiment, the mice were sacrificed, and the tumors were removed and weighed.

One could speculate that the GnRH-DFF40 would not be an active chimeric protein within target cells because any GnRH-DFF40 molecule entering the cell would be recognized by the endogenous DFF45 and bound to it, thus, preventing it from acting. However, we found that the chimeric protein was able to cause specific cell death. There are two conceivable explanations for GnRH-DFF40’s action: (a) GnRH-DFF40 cannot bind to endogenous DFF45 because GnRH is fused to DFF40 at its N’ terminus, and DFF40 is known to bind DFF45 via both their N’ terminus (33, 34). (b) An excess of GnRH-DFF40 enters the cell, and some DFF45 binds to the DFF40. However, there is a large amount of DFF40 that does not bind to DFF45, which can enter the nucleus and cause DNA damage.

To distinguish between the two possibilities, we first examined whether GnRH-DFF40 can bind endogenous DFF45. To do so, we resorted to Far-Western blotting. As shown by the bandshift (Fig. 2A), the two proteins can bind to each other. These results suggest that there is, most probably, an excess of DFF40 in the cell after GnRH-DFF40 is internalized into the target cells, and it can then enter the nucleus and cause the initial DNA cleavage. An additional possibility is that DFF45 binds to GnRH-DFF40, although with much lower affinity, allowing GnRH-DFF40 to dissociate readily and enter the nucleus.

It is conceivable that binding of the chimeric protein to cellular death receptors results in all of the effects observed in the cells after treatment with GnRH-DFF40. Moreover, it was found that cells responding to a GnRH analogue, used for treatment of cancer, have an elevated level of Fas receptors (49).

We show that the chimeric protein does not act via a surface cellular death receptor on at least two accounts. First, when surface cellular death receptors are activated, Bid is usually cleaved to tBid, which acts as a proapoptotic protein and causes cell death (38, 39). We did not detect differences in Bid levels nor the appearance of its truncated form (Fig. 7A). Second, removal of the C’ terminus of DFF40, which contains an NLS sequence, prevents the truncated molecule from entering the nucleus and did not cause cell death. We found that although the truncated chimeric protein GnRH-DFF40ΔC was able to enter the cell, it did not induce cell death, indicating that it did not bind to a death receptor (Figs. 3B and 4D).

As GnRH-DFF40 causes DNA damage, we examined if there were changes in the amount of p53 present during treatment with this chimeric protein. We did not find any differences in p53 protein level (Fig. 6A). A possible explanation is that >50% of tumors carry mutations in the p53 gene (40, 42), and in many cases, p53 is present in the cell in large amounts in its mutated form. Hence, we examined whether our target cells respond to p53 activation by a known inducer of DNA damage, doxorubicin, and found transient activation, peaking after 45 min (Fig. 6A). Therefore, the cells can undergo p53 activation but do not when treated with GnRH-DFF40. In contrast, we found that the cells responded to treatment of GnRH-DFF40 by an increase in p73 levels (Fig. 6B). We present a model of GnRH-DFF40 action involving two stages: first, GnRH-DFF40 enters the cell and causes initial DNA damage. Second, these nuclear changes are detected by the cell independently of p53, leading to cytoplasmic changes that include the release of cytochrome c from the mitochondria to the cytoplasm and activation of caspases. This cascade is similar to apoptotic pathways that begin with DNA damage such as γ-ray and UV radiation, except that in our case there is no p53 activation. It is possible that other DNases are recruited for the final execution of the cell because GnRH-DFF40 is barely detectable in the cells at this time (data not shown). A likely candidate, responsible for the DNA fragmentation, could be the recently characterized endonuclease G (50).

Because caspase-3 is activated by the initial DNA damage caused by GnRH-DFF40, it cleaves DFF45, thereby freeing endogenous DFF40 and/or other nucleases. These events aug-
ment the death signals transmitted throughout the cell, forming an amplification loop. A similar amplification loop caused by DFF40 was described recently by Bouloures et al. (51).

In this study and in our previous ones (1–3, 20), we have shown that the GnRH-based chimeric proteins are highly specific in targeting and killing adenocarcinomas. This action of the GnRH-based chimeric protein is achieved via a specific GnRH-binding site. We have data (data not shown) suggesting that binding of the chimeric protein to these cells is not via the known human hypotalamic GnRH receptor type I (52) but through an unknown GnRH or GnRH-like receptor. A similar receptor was described previously as a low affinity-high capacity receptor (53–55). Recently, a human GnRH type II receptor has been cloned (56, 57). However, its biological activity is yet unknown.

Theoretically, there are a number of ribonucleases that can serve as the killing moiety of a chimeric protein. Indeed, one such chimeric protein has been constructed. Human placental alkaline phosphatase fused to the Bovine pancreatic DNaI was recently used and showed the ability to cause cell death in a number of cell lines (58). However, this chimeric protein has a number of problems such as weak activity on the cells. Furthermore, this chimera is composed of a Bovine protein and, therefore, might cause an immune response when used repetitively. Moreover, it should be pointed out that the DNaI enzyme does not contain a translocation domain or any organelle-targeting sequence, mainly lacking the well known and recognized NLS. DFF40, on the other hand, contains a COOH-terminal NLS sequence; the NLS sequence being crucial for the chimaera’s activity and mechanism of action of GnRH-DFF40 was proven, leading to apoptotic death. Thus our DFF40-based chimera, potentially, may have a greater advantage over other DNases such as DNaI.

GnRH-DFF40 is the first whole human chimeric protein to be tested in vivo, showing promising results in preliminary experiments. Many cancer cells bear mutations in many apoptotic key point genes and cannot undergo apoptosis, although most apoptotic pathways are unaffected and, with proper signals, can cause cell death (59). The use of an apoptotic protein as the killing domain of the chimeric protein could overcome these limitations and allow or persuade the cell to undergo apoptosis. There is a great advantage in using such chimeric proteins for treatment of human patients, for this method both overrides mutations commonly found in apoptotic processes in tumors and involves the use of human, nonimmunogenetic toxins. Thus, apoptosis-inducing chimeric proteins are promising molecules for targeted cancer therapy in humans.

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REFERENCES


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GnRH-DFF40 Kills Adenocarcinoma Cells by Apoptosis

Using Apoptosis for Targeted Cancer Therapy by a New Gonadotropin Releasing Hormone-DNA Fragmentation Factor 40 Chimeric Protein

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