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A Novel Class of Peptides That Induce Apoptosis and Abrogate Tumorigenesis *in Vivo*

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In testing the ability of certain synthetic peptides of biological origin to bind to cell surfaces, we unexpectedly found that they could induce apoptosis and inhibit tumorigenesis in rodent and human tumor cell lines. In vitro pre-incubation with the peptides at concentrations as low as 10^{-12} M inhibited tumorigenesis in nude mice, in a dose-dependent fashion. The inhibition of tumorigenesis was reflected in the rapid induction of apoptosis of tumor cells, pre-incubated with the peptides, and tested under conditions of anchorage-independence. Induction of apoptosis was detectable even at concentrations of 10^{-12}–10^{-13} M (0.1-1.0 pM). Aspecific toxicity of the synthetic peptides was ruled out by the demonstration that single amino acid substitutions (in at least 4 peptides) completely abrogated the pro-apoptotic effect, even at a concentration of 10^{-5} M (10 μM).

Key Words: **apoptosis; tumorigenesis; peptides.**

While testing the ability of certain synthetic peptides of biological origin to bind to cell surfaces, we found that these peptides were directly cytotoxic, could induce apoptosis of tumor cells in anchorage-independence conditions, and markedly inhibited tumorigenesis in nude mice. Although the finding was totally unexpected, we think that it is worth reporting, because the active peptides are of biological origin and could induce apoptosis at concentrations as low as 0.1-1.0 pM. Furthermore, the fact that single amino acid substitutions abrogated the cytotoxicity of these peptides, even at concentrations of 10 μM (i.e. 8 orders of magnitude more concentrated) ruled out an aspecific toxic effect. These peptides were also found to cause cell death in a variety of tumor cell lines of both human and rodent origin.

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MATERIALS AND METHODS

Cell lines. C6 rat glioblastoma cells (1) and CaOV-3 human ovarian carcinoma cells (2) have been previously described in detail in these references. Both cell lines grow in serum-free medium, form colonies in soft agar, double in cell number in diffusion chambers (3) and are tumorigenic in nude mice. Colo-205 (human colorectal carcinoma), DU-145 (human prostate carcinoma) and NCI-N417 (human small cell lung carcinoma) were kindly provided by Apoptosis Technology Inc. T98G and U87MG human glioblastoma cells were obtained from the ATCC. OVCAR-3 and OVCAR-5 human ovarian carcinoma cells were a kind gift of Dr. Hamilton (Fox Chase Cancer Center).

Synthesis of peptides. The peptides were prepared by solid phase synthesis with Fmoc-strategy using an Applied Biosystems model 430A peptide and Perspective Biosystems 9050 Pepsynthesizer Plus. The synthetic procedure has been previously described (4). The crude peptides were purified by preparative RP-HPLC using Dynamax-300Å C18 25 cm × 21.4 mm I.D. column with flow rate of 9 ml/min. The fractions containing the peptide were pooled together and lyophilized. The purity of the final products was assessed by analytical RP-HPLC, capillary electrophoresis and MALD-TOF MS. The peptides were estimated to be 99% pure, and did not change the pH of the medium in which they were dissolved.

In vivo apoptosis assay. The test for apoptosis has been described in detail (3,5,6).

Tumorigenesis. For these experiments, C6 cells were pre-incubated in vitro with the chosen peptides for 24 hours in serum-free medium (Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin fraction V (Sigma) and 0.1 μM ferrous sulfate) and were then injected subcutaneously in nude mice (10^5 cells in 0.1 ml. of phosphate-buffered saline), as previously described in detail (5,6).

RESULTS

Inhibition of tumorigenesis in nude mice. In preliminary experiments, designed for different purposes, we had noticed that the 3 synthetic peptides described below caused cell death when incubated in vitro with C6 rat glioblastoma cells. The effect of the peptide YLRPGPVTA (see below) on tumorigenesis of C6 cells in nude mice was then analyzed. In some experiments, the cells were pre-incubated in vitro with the peptide at the indicated concentrations, and then injected sub-

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TABLE 1

Tumorigenesis in Nude Mice

Treatment	Palpable tumors (days)
None	4
YLEPG**A**VTA (10^{-5} M)	4
YL**R**PGPTA (10^{-12} M)	11
YL**R**PGPVTA (10^{-10} M	14
YL**R**PGPVTA (10^{-8} M)	15
YL**R**PGPVTA (10^{-5} M)	21

C6 cells were incubated with the peptides at the indicated concentrations for 24 hours in serum-free medium before injection (10^5 cells in 0.1 ml) into the subcutaneous tissue of 7-week-old male Balb/c nude mice. Three nude mice were used in each experimental condition. Animal care was in accordance with institutional guidelines. Upon the development of bulky tumors, the animals were sacrificed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

cutaneously in nude mice. The results (Table 1) show that there is a concentration-dependent inhibition of tumorigenesis in C6 cells pre-treated with the active peptide. The time of tumor appearance is delayed even at a concentration of 10^{-12} M, while an inactive peptide YLEPGAVTA (see below), with two aminoacid substitutions, was completely ineffective at a concentration of 10^{-5} M.

In another experiment, an all in vivo experiment, the active peptide (suspended in 0.1 ml) was injected subcutaneously in nude mice, simultaneously and adjacent to C6 cells (10^5 cells injected in 0.1 ml). Tumors appeared after 11 days with a peptide injection of 10^{-10} to 10^{-12} M, compared to 4 days with no peptide injection or injection with a control peptide. Thus, an active peptide can inhibit tumorigenesis even after a single injection into the subcutaneous tissue of nude mice.

Abrogation of tumorigenesis implies that the treatment may cause cell death, but this is difficult to demonstrate in vivo, since the dead cells are no longer recoverable from the subcutaneous tissue. We therefore decided to test these synthetic peptides in an in vivo assay, which allows to determine with precision the fraction of injected cells that die. We used for this purpose the diffusion chamber assay, previously described in detail (3,5,6), in which the cells are tested under conditions of anchorage-independence and behave precisely as they do when injected in the subcutaneous tissue of animals (3,5,6,7).

We will first present the results obtained with peptide YLEPGPVTA, which is the one we have most extensively studied. C6 cells were pre-incubated with decreasing concentrations of this peptide, and then tested for survival, as described in ref. 3. Recovery of viable cells after 24 hours showed a linear dependence on the concentration of the peptide, with only 18% recovery at a concentration as low as 10^{-12} M (Figure 1). The dose of peptide required to kill 50% of the cells (ED_{50}) was in the order of 10^{-13} M (0.1 pM), as shown in Figure 1. A control peptide with the same amino acid composition, but in scrambled order (control) was also tested. Recovery of C6 cells pre-treated with the control peptide (at a concentration of 10^{-5}M) was >200% (Figure 1).

Evidence for apoptosis. To test whether the loss of cells was due to apoptosis, the presence of apoptotic cells was determined at several intervals by FACS analysis (3, 6). A peak of apoptotic cells was observed in C6 cells pre-treated with the active peptide (YLEPGPVTA) for 24 hours and then incubated for 90 min. in vivo (Figure 2, left panel). Cells treated with an inactive peptide did not show any evidence of apoptosis (Figure 2, right panel). Similar results were obtained after 2, 4 and 6 hours in vivo and also with CaOV-3 cells (see below).

The induction of apoptosis is peptide-specific. In order to determine the specificity of the pro-apoptotic action of the synthetic peptide, several amino acid substitutions of the peptide YLEPGPVTA were carried out, and the resultant peptides tested on C6 cells. There are at least 4 peptides (the last 4 in Table 2) in which a single amino acid substitution completely abrogates their ability to induce apoptosis. It seems extremely unlikely that the effect of the original peptide can simply be due to aspecific toxicity, when 4 other peptides (differing from it by a single amino acid) are totally inactive. The results of Table 2 also indicate that some amino acid substitutions do not affect the ability of the peptide YLEPGPVTA to induce apoptosis. For instance, the peptide YLRPGPVTA, used for the in vivo

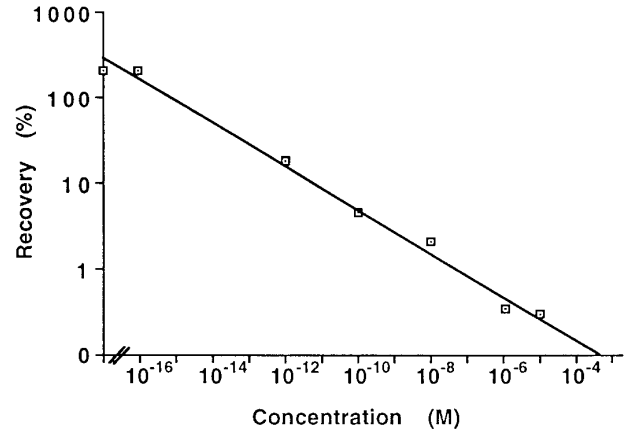

FIG. 1. Dose Response of Peptide-induced Apoptosis of C6 Cells in vivo. C6 wild-type cells, incubated for 24 hours with peptide YLRPGPVTA (see Table 2) at the concentrations given on the abscissa, were tested for survival under conditions of anchorage-independence. The ordinate shows the percentage of cells recovered after 24 hours in vivo, expressed as percentage of the original inoculum (3,5). C6 cells incubated with a control peptide at 10^{-5} M doubled in number (recovery slightly more than 200%, symbol at the ordinate).

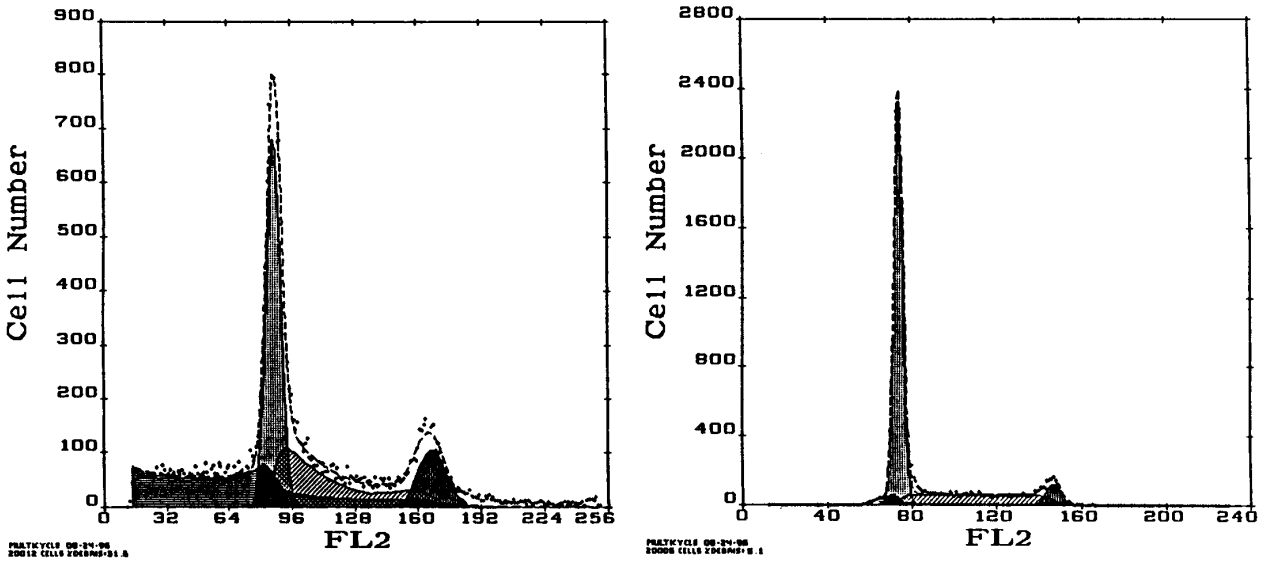

FIG. 2. FACS Analysis of C6 Cells Treated with Active and Inactive Peptides. C6 cells were pre-incubated with the peptides for 24 hours in serum-free medium before being placed in diffusion chambers. The chambers were implanted for 90 mins. in vivo. The cells were then examined for apoptosis, as previously described (3). Left panel shows cells treated with the active peptide YLEPGPVTA; right panel shows cells treated with an inactive peptide, both at a concentration of 10^{-5} M.

experiments described above is as effective as the original peptide in inducing apoptosis. However, mutations at tyrosine in position 1, or the two prolines in position 4 and 6 effectively inactivate this peptide. The only exception is when the prolines are replaced by an N-methyl alanine, whose conformation mimics that of proline. Mutations at E3 or T8 have little effect, but when both residues are mutated, cell recovery increases to 77%. In addition, the inverted D-amino acid version (ATVPGPELY) is as effective as its L-amino acid counterpart. The last peptide, YLEPGAVTA (proline 6 replaced by alanine), which is totally inactive, was the one used as control in tumorigenesis experiments (see above).

TABLE 2

Effect of Amino Acid Substitutions on the Proapoptotic Activity of Synthetic Peptides

Peptides	% viable cells (24 hrs)
YLEPGPVTA	3.0 ± 0.1
YL**R**PGPVTA	3.5 ± 0.2
ATVPGPELY (D-amino acids)	6.0 ± 0.2
YLE**X**G**X**VTA (X: N-Methyl-A)	6.5 ± 0.2
YL**A**PGPVTA	7.4 ± 0.4
YLEPGPV**A**A	9.3 ± 0.9
YLEPGP**A**TA	11.0 ± 0.2
YLEPGPVK**A**	12.0 ± 0.5
YLEP**A**PVTA	70.0 ± 3.0
YL**R**PGPV**R**A	77.0 ± 1.4
Y**A**EPGPVTA	186.0 ± 4.6
YLE**A**GPVTA	221.0 ± 3.9
ALEPGPVTA	224.0 ± 3.2
YLEPG**A**VTA	260.0 ± 9.0

C6 cells were pre-treated with the indicated peptides at 10^{-5} M for 24 hours in serum-free medium and tested for apoptosis as described (3,5,6). Results are given in percentage recovery of viable cells. Amino acid substitutions in the original peptide (the first one in the list) are indicated in bold characters. The peptides were obtained as coded samples and the sequences were revealed at the end of the experiment. Each value represents the mean of 4 independent determinations.

Other peptides can induce apoptosis in vivo. Thus far, we have limited ourselves to the results obtained with one peptide, YLEPGPVTA, or its active derivative YLRPGPVTA. But we tried two other peptides (the choice of these peptides will be explained in the Discussion), LLDGTATLRL and FECNTAQPG, using the same methodology as described in Fig. 1 and Table 2. Both of them were very active, causing apoptosis in vivo (cell recovery determined after 24 hours varied from 5.0 to 7.5% of the original inoculum). In addition, the D-amino acid version of the former peptide LRLTATGDLL was as active as its L-amino acid counterpart (cell recovery after 24 hours was 4.9% of the original cell number). Indeed, the D-amino acid LRLTATGDLL, was still effective at 10^{-13} M (0.1 pM) with only 30% recovery of C6 cells. In all of these experiments, the control peptides, like the last one listed in Table 2, had no effect whatsoever, cell recovery being in excess of 200% of original inoculum.

The cytotoxic effect of the peptides is not tumor or species-specific. In order to show that the synthetic peptides also induce apoptosis of human tumor cells, CaOV-3 human ovarian carcinoma cells (2) were tested

TABLE 3

Selected Peptides Induce Apoptosis of Human Ovarian Carcinoma Cells *in Vivo*

Peptide	% viable cells (24 hs)
YL**R**PGPVTA	3.0 ± 0.1
YLEPGPVTA	0.2 ± 0.01
LRLTATGDLL	6.6 ± 0.2
YLEPG**A**VTA (control)	280.0 ± 8.4

The synthetic peptides are described in Table 2. CaOV-3, human ovarian carcinoma cells (5×10^5 cells) were implanted in diffusion chambers in the subcutaneous tissue of mice. The peptides were injected subcutaneously (0.2 ml of a 5×10^{-5} M solution) simultaneous and adjacent to the implanted diffusion chambers. The peptides were used as coded samples and the sequences were revealed at the end of the experiment. Three mice were used in each experimental condition.

as described in Table 3. The results of Table 3 show that active peptides can induce apoptosis of human tumor cells, even when they are injected into mice. The control peptide, YLEPGAVTA had no effect, and the number of cells more than doubled. CaOV-3 also underwent apoptosis, as determined by FACS analysis (not shown).

The pro-apoptotic effect of these peptides was also verified in a variety of human tumor cell lines. The results presented in Table 4 indicate that human colorectal carcinoma, prostate carcinoma, small cell lung carcinoma and even cis-platinum resistant ovarian carcinoma cells were all sensitive to peptide-induced apoptosis, therefore ruling out the possibility that the effects observed with C6 and CaOV-3 cells are due to a peculiarity of these two cell lines. A control peptide YLEPGAVTA had no effect and the percentage recovery was the same as for untreated controls.

DISCUSSION

The induction of apoptosis and inhibition of tumorigenesis in several tumor cell lines by the synthetic peptides cannot be attributed to an aspecific toxicity for the following reasons: 1) the peptides are active at very low concentrations: 0.1-1.0 pM; 2) there are 4 peptides in which a single amino acid substitution completely abrogates their ability to induce apoptosis. Two other peptides have a markedly decreased activity. This makes it extremely unlikely that the original peptide(s) are simply toxic; 3) the peptides induce programmed cell death, not just cell death; and 4) the peptides are active also when injected subcutaneously. None of the peptides caused a visible change in pH of the medium in which they were dissolved, and the presence of toxic impurities is again rendered highly unlikely by the inactive peptides with a single amino acid substitution (Table 2).

Focusing on peptide YLEPGPVTA, which was the most studied, we can say that it inhibits tumorigenesis in nude mice and causes apoptosis, when the tumor

TABLE 4

Selected Peptides Induce Apoptosis in a Variety of Human Tumor Cell Lines

Condition	Recovery (%)
Colo-205 (human colorectal carcinoma); no treatment	218.0
+ YL**R**PGPVTA	30.0
+ YLEPG**A**VTA	206.0
DU-145 (human prostate carcinoma); no treatment	304.0
+ YL**R**PGPVTA	55.0
+YLEPG**A**VTA	300.0
NCI-N417 (human small cell lung Ca); no treatment	271.0
+ YL**R**PGPVTA	31.0
+ YLEPG**A**VTA	280.0
T98G (human glioblastoma); no treatment	200.0
+ YL**R**PGPVTA	5.6
+ YLEPG**A**VTA	190.0
U87MG (human glioblastoma); no treatment	200.0
+ YL**R**PGPVTA	5.3
+ YLEPG**A**VTA	200.0
OVCAR-3 (35S, cisPt.resistant human ovarian Ca.); n/t	200.0
+ YL**R**PGPVTA	15.4
+ YLEPG**A**VTA	198.0
OVCAR-5 (101S, cisPt.resistant human ovarian Ca.); n/t	298.0
+ YL**R**PGPVTA	38.0
+ YLEPG**A**VTA	300.0

The cells were pre-treated with the indicated peptides at 10^{-5} M for 24 hours before being tested as described (3,5,6). The results represent the mean from three independent determinations. n/t means no treatment.

cells are tested under conditions of anchorage-independence. The effect of peptide YLEPGPVTA on cells in monolayers is much more modest (it induces 25% inhibition of cell growth in monolayer), but this should not surprise. It has become increasingly clear in recent times that tumor cells are much more susceptible to apoptosis when forced to grow under anchorage-independence conditions (3,8-11). Tumor cells transplanted into the subcutaneous tissue of animals are asked to grow in anchorage-independence, and are therefore more susceptible to injurious agents (see also reviews in ref. 7 and 12). Finally, it does not seem unreasonable to assume that results obtained in the intact animal reflect what goes on in an animal more than results obtained in a petri dish.

The effects described in C6 and CaOV-3 cells were also verified in a variety of human tumor cell lines. Human ovarian carcinoma, small cell lung carcinoma, colon carcinoma, glioblastoma and prostate carcinoma cell lines were all sensitive to peptide-induced apoptosis (Table 4). The extent of apoptosis measured after 24 hours in vivo ranged from 45 to 95%, depending on the cell line (Table 4).

Two other peptides of biological origin also induce apoptosis, which brings us to the reasons for choosing these synthetic peptides. While our findings were, admittedly, serendipitous, our choice of peptides was not wholly fortuitous. The 3 peptides chosen are known from the literature: 1) YLEPGPVTA is recognized by melanoma-specific human CTL lines (13); 2) LLDGTATLRL, is derived from gp100 and involved in regression of human melanoma (14); and 3) FECNTAQPG, is derived from connexin 37, that induces CTL responses against murine lung carcinoma (15). The original purpose of our studies was to investigate whether these peptides could bind and associate with the IGF-I receptor, and the various mutants of this receptor that we have described (16). Whether the apoptosis-inducing ability of these peptides has anything to do with their role in immunological phenomenona is a question which we would like to leave unresolved for the moment. The observations that the synthetic peptides induce apoptosis, in naive animals and that they were equally active on rat and on human tumor cells, seem to militate against an immunological interpretation.

However, our experiments do demonstrate that some synthetic peptides of biological origin can induce apoptosis of tumor cells in vivo, and tumorigenesis in nude mice at remarkably low concentrations, and specifically, as they are inactivated by single amino acid substitutions. Our future experiments are directed toward an elucidation of the mechanism of this intriguing phenomenon. Although, admittedly, several questions remain unanswered, the observation is so startling that we believe it merits being brought to the attention of other investigators.

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