Induction of Apoptosis of Integrin-expressing Human Prostate Cancer Cells by Cyclic Arg-Gly-Asp Peptides

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ABSTRACT
Prostate cancer is the second most common cause of cancer deaths among men in the United States. We have investigated the effect of cyclo-(Arg-Gly-Asp-D-Phe-Val; cRGDfV), Arg-Gly-Asp, or Arg-Gly-Asp-Ser, on survival of human prostate cancer (LNCaP and PC-3) and normal (HEL) cells in vitro. Addition of cRGDfV (20 μg/ml) but not the linear Arg-Gly-Asp or Arg-Gly-Asp-Ser peptide induced significant (~84%) killing of LNCaP cells expressing αvβ3 integrins on their surfaces. In contrast, none of these peptides had any major effect on the growth of PC-3 or HEL cells, which express little αvβ3 integrin on their surfaces. Treatment of LNCaP but not of PC-3 or HEL cells with cRGDfV resulted in cleavage of focal adhesion kinase, a key player in integrin-mediated signal transduction pathway. The evidence we present here suggests that the killing of LNCaP cells after cRGDfV treatment was attributable to apoptosis or programmed cell death. This is evidenced by activation of at least two caspases (caspase-3 and caspase-9) as detected by cleavage of poly(ADP-ribose) polymerase and partial blocking of apoptosis by a selective inhibitor of caspase-9. Our results suggest that cRGDfV may be an effective treatment for some human prostate cancers by inducing apoptosis through interference with the regulation of integrin/focal adhesion kinase-mediated signal transduction pathway necessary for cell survival.

INTRODUCTION
Prostate cancer is the most commonly diagnosed malignancy in men and the second leading cause of cancer deaths among men in the United States (1).

Beside surgery and radiation therapy, hormonal or antiandrogen therapy is the only treatment available for localized prostate cancer. However, hormonal therapy often leads to severe toxicity. Moreover, progressive, androgen-independent, and clinically aggressive metastatic prostate cancer is difficult to treat and essentially incurable (2, 3). Thus, it is important to develop novel therapeutics that will be effective for the treatment of this life-threatening disease. One possible target for these novel therapeutics may be the integrins several investigators have reported on the surfaces of different human prostate cancer cell lines and human prostate tumor tissues (4–7). Integrins are heterodimeric transmembrane glycoproteins composed of α and β subunits occurring in 24 different combinations (8–11). Members of the integrin family are responsible for cell-cell adhesion as well as adhesion to a large number of extracellular matrices such as fibronectin, laminins, von Willbrand factor, vitronectin, collagen, tenascin, and others (9, 12–14). Integrins have been shown to be involved in several important cellular processes, including angiogenesis, apoptosis, cell migration, and tumor growth (8, 15–17).

Recently, we have shown that cRGDfV2 peptide induces direct killing of human glioblastoma cells that express αvβ3 integrins on their cell surfaces (18). Furthermore, treatment of intracranial tumors in scid mice with this cyclic peptide significantly prolonged their survival (18). In a recent report, Romanov and Goligorsky (5) described the effect of a similar peptide, cyclo-(Arg-Gly-Asp-D-Phe-Lys), on the detachment of human prostate cancer cells including DU145 and PC-3. These and other reports, including ours, prompted us to study the effect of cRGDfV on the survival of human prostate cancer cells in vitro. In this communication, we report that cRGDfV induces apoptosis or PCD of prostate cancer cells that express αvβ3 integrins on their surfaces. Our results suggest that this peptide induces killing of prostate cancer cells through interference with an integrin/FAK-mediated signal transduction pathway, and cRGDfV therapy may be an effective and nontoxic treatment for some human prostate cancers.

MATERIALS AND METHODS
Cell Cultures. Human prostate carcinoma and adenocarcinoma cells (LNCaP and PC-3) and human embryonic lung cells (HEL 299) were purchased from the American Type Culture Collection (Rockville, MD). LNCaP and PC-3 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories Inc., Logan, UT), sodium pyruvate (55 mg/liter), sodium bicarbonate (1.2 g/liter), HEPES (1.2 g/liter), and gentamicin (50 μg/ml). HEL cells were grown in DMEM supplemented with 10% fetal calf serum, sodium bicarbonate (1.2 g/liter), and gentamicin (50 μg/ml).

Chemicals and Antibodies. Peptides, cRGDfV, and RGD were obtained from BIOMOL Research Laboratories Inc. (Plym-
outh Meeting, PA), and RGDS was obtained from Calbiochem (San Diego, CA). Stock solutions were prepared in sterile PBS (pH 7.2; Life Technologies, Inc., Grand Island, NY). Monoclonal antibody (mouse) LM609 against human integrin αvβ3 was purchased from Chemicon International, Inc. (Temecula, CA). Fluorescein-conjugated antimouse IgG + IgM and horseradish peroxidase-conjugated antimouse IgG and antirabbit IgG were also purchased from Chemicon International, Inc. Monoclonal antibody (mouse) to PARP was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Polyclonal antibody (rabbit) to FAK was purchased from NeoMarkers, Inc. (Fremont, CA). Polyclonal antibodies (rabbit) against Akt and phospho-Akt were obtained from New England BioLabs, Inc. (Beverly, MA). Inhibitor of caspase-9 (LEHD-FMK) was purchased from BioVision Inc. (Palo Alto, CA). Substrate 4-chloro-1-naphthol was purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Surface Immunofluorescence Assay.** To determine the expression of αvβ3 integrin on the membranes of LNCaP and HEL cells, a cell surface immunofluorescence assay was performed as described previously (18, 19).

**PAGE and Immunoblotting.** Untreated and peptide-treated cell lysates were subjected to SDS-PAGE (10%) using a Novex X Cell Minicell (Novex, San Diego, CA) gel electrophoresis system (~1.5 × 10⁶ cell equivalents/lane). Fractionated proteins were electrophoretically transferred to Fluoro Trans PVDF membrane (Pall Corporation, Port Washington, NY) and immediately processed for immunoblotting (20–22). Transfer membranes were first incubated at room temperature with primary antibodies followed by incubation with the appropriate antimouse or antirabbit peroxidase-labeled secondary antibodies. Resolved proteins were visualized by staining with 4-chloro-1-naphthol.

**Treatment of Cells with RGD-containing Peptides.** In brief, semiconfluent monolayers of LNCaP, PC-3, and HEL cells were incubated (18 h, 37°C) with different concentrations of cRGDfV, RGD, or RGDS in an incubator with 5% CO₂. One set of cells was incubated with growth medium only and served as untreated control. Untreated and treated cells were then harvested, and the number of viable cells were counted by the trypan blue dye (Life Technologies, Inc.) exclusion method using a hemacytometer (Hausser Scientific, Horsham, PA). The mean and SE for each group were determined and used to calculate the percentage reduction of cell survival.

**RESULTS**

**Expression of αvβ3 Integrin on the Surface of LNCaP, PC-3, and HEL Cells.** Cell surface immunofluorescence was used with mouse monoclonal antibody LM609 and fluorescein-conjugated antimouse IgG to determine the expression of αvβ3 integrin on the surface of LNCaP, PC-3, and HEL cells. The result of this experiment (Fig. 1) demonstrated expression of αvβ3 integrins on the surface of LNCaP cells but little on the surface of HEL cells. The surface of PC-3 cells also showed little expression of αvβ3 integrin when compared with the surface immunofluorescence of LNCaP cells (data not shown).

**Sensitivity of Human Prostate Cancer and Normal Cells to RGD-containing Peptides.** The capacity of cRGDfV, RGD, or RGDS to inhibit the growth of LNCaP, PC-3, and HEL cells separately is shown in Table 1. It is evident that 10 µg/ml of cRGDfV significantly (~70%) reduced the number of LNCaP cells recovered from tissue culture after 18 h (Table 1; Fig. 2). By 18 h after treatment, most of the treated LNCaP cells had rounded up. Given the slow growth rate of LNCaP cells and the relatively short exposure time to cRGDfV, any reduction of viable cells in excess of 30% must be attributable to direct killing of treated cells. In contrast, cRGDfV had no major effect on the growth and morphology of HEL (Table 1; Fig. 2) or PC-3 cells in parallel experiments (Table 1). Linear RGD had little effect on the growth of any of these cells in tissue culture (Table 1). However, linear RGDS caused a partial (47%) reduction of the number of recovered LNCaP cells at a concentration of 20 µg/ml. By comparison, HEL and PC-3 cells were largely unaffected at this concentration (Table 1). This data, together with the results described above, indicated that cyclic RGDFV produced a significant cytotoxic effect on αvβ3 integrin-expressing human prostate cancer cells as evidenced by dose-dependent reductions in cell survival after 18 h exposure.

**Expression of FAK in cRGDfV-treated LNCaP, PC-3, and HEL Cells.** Because most integrins activate the FAK pathway by mechanisms that are incompletely understood, we determined whether blocking αvβ3 integrins with cRGDFV could affect
the activation of FAK in LNCaP cells. In brief, LNCaP, PC-3, and HEL cells were treated with cRGDfV (20 μg/ml) as before. Cell lysates were collected and subsequently processed for immunoblotting using an antibody to FAK. A band of ~M₆ 120,000, representing FAK, was detected in untreated LNCaP, PC-3, and HEL cells (Fig. 3). In addition, a major cleavage product of ~M₆ 80,000 was also observed in cRGDfV-treated LNCaP cells (Fig. 3A). Peptide-treated HEL and PC-3 cells displayed no specific cleavage product (Fig. 3, B and C). These data indicate that cRGDfV treatment resulted in the cleavage of FAK in LNCaP cells presumably as a consequence of ligating (and blocking) αvβ3 integrins on their membranes.

Table 1  Effect of RGD-containing peptides on the growth of human prostate cancer and normal cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Concentration (μg/ml)</th>
<th>Viable cells (×10⁵/ml)</th>
<th>% Reduction</th>
<th>Viable cells (×10⁵/ml)</th>
<th>% Reduction</th>
<th>Viable cells (×10⁵/ml)</th>
<th>% Reduction</th>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>LNCaP</td>
<td>0</td>
<td>1.40 ± 0.10</td>
<td>—</td>
<td>3.05 ± 1.75</td>
<td>—</td>
<td>2.65 ± 0.15</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.46 ± 0.04</td>
<td>67.2</td>
<td>2.85 ± 1.75</td>
<td>6.6</td>
<td>1.95 ± 0.05</td>
<td>26.4</td>
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<tr>
<td></td>
<td>20</td>
<td>0.23 ± 0.13</td>
<td>83.6</td>
<td>2.60 ± 1.40</td>
<td>14.8</td>
<td>1.40 ± 0.20</td>
<td>47.2</td>
</tr>
<tr>
<td>PC-3</td>
<td>0</td>
<td>6.25 ± 0.55</td>
<td>—</td>
<td>5.75 ± 0.25</td>
<td>—</td>
<td>5.05 ± 2.45</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.85 ± 0.85</td>
<td>6.4</td>
<td>5.65 ± 0.05</td>
<td>1.8</td>
<td>4.80 ± 2.30</td>
<td>4.9</td>
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<tr>
<td></td>
<td>20</td>
<td>5.50 ± 0.40</td>
<td>12.0</td>
<td>5.25 ± 0.05</td>
<td>8.7</td>
<td>4.55 ± 2.35</td>
<td>9.9</td>
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<tr>
<td>HEL</td>
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<td>—</td>
<td>3.55 ± 0.05</td>
<td>—</td>
<td>2.85 ± 0.05</td>
<td>—</td>
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<tr>
<td></td>
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<td>7.2</td>
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</tr>
<tr>
<td></td>
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<td>2.70 ± 0.10</td>
<td>5.3</td>
</tr>
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</table>

Fig. 2  Effect of cRGDfV on the growth of human prostate cancer and normal cells. A, LNCaP cells, untreated control. B, LNCaP cells treated with cRGDfV. C, HEL cells, untreated control. D, HEL cells treated with cRGDfV.
Recently it has been reported that Akt can directly phosphorylate one of the caspases (25–27) or cysteine proteases, known as caspase-9 (on serine 196), and prevent its protease activity (28, 29).

This prompted us to investigate whether treatment of LNCaP cells with cRGDFV caused any opposing effect such as induction of caspase-9 leading to PCD, because we know that cRGDFV reduced the expression of phosphorylated Akt/PKB in treated LNCaP cells. In brief, semiconfluent monolayers of LNCaP and HEL cells were treated with LEHD-FMK (15 μM), an inhibitor of caspase-9, for 90 min at 37°C. One set of cells without any inhibitor served as the untreated control. Cells were then washed and incubated in the presence or absence of cRGDFV (20 μg/ml) at 37°C for 18 h. The number of viable cells, both in control and treated groups, was counted as before and the percentage inhibition was determined. The results displayed in Fig. 5 reveal that LEHD-FMK reduced the inhibitory effect of cRGDFV by ~50% in treated LNCaP cells, suggesting that the cRGDFV-mediated cell killing was attributable to an induction of caspase-9 (presumably because of dephosphorylation of Akt/PKB). As expected, there was no major effect of this inhibitor on the growth of cRGDFV-treated or untreated HEL cells (data not shown). However, this inhibitor did not completely prevent the killing of LNCaP cells, indicating that other caspases or caspase-independent mechanisms are also involved in peptide-induced killing of human prostate cancer cells.

**Cleavage of PARP in cRGDFV-treated LNCaP Cells.**

It is known (25–27) that activation of caspase-9 in turn activates caspase-3, which cleaves PARP, a cellular enzyme implicated in DNA repair (30, 31). Cleavage of PARP by activated caspase-3 is one of the hallmarks of apoptosis. Because we detected activation of caspase-9 in cRGDFV-treated LNCaP cells, we next investigated whether there was cleavage of PARP. In brief, LNCaP, PC-3, and HEL cells were treated with cRGDFV for 18 h as before. Cell lysates were then processed for PAGE and subsequent immunoblotting using an antibody to PARP ("Materials and Methods"). A band of ~M, 115,000 representing full-length PARP was seen in lysates from untreated LNCaP cells (Fig. 6A). In contrast, two major cleavage products of ~M, 85,000 and 65,000 were detected in cRGDFV-treated LNCaP cells suggesting an induction of caspase-3 (Fig. 6A). The faster migrating band of ~M, 65,000 presumably represents the final cleavage product of PARP in LNCaP cells. As expected, these cleavage products were not detected in peptide-treated HEL or PC-3 cells (Fig. 6, B and C).
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Fig. 6 Expression of PARP and its cleavage products in cRGDV-treated and untreated LNCaP (A), PC-3 (B), and HEL (C) cells. Lane 1, untreated control; Lane 2, cRGDV-treated. Arrows, positions of the cleavage products in treated LNCaP cells. The faster migrating bands (*) in both treated and untreated HEL cells (C) possibly represent a degradation product.

DISCUSSION

The results we presented here clearly suggest that cRGDV induces apoptosis of αβ3 integrin-expressing LNCaP cells. Independent reports from several laboratories have documented the expression of different types of integrins in various prostate cancer cell lines and human prostate tumor tissues (4–7) including the expression of αβ3 integrin on the surface of LNCaP cells (7). However, our observation that LNCaP cells express αβ3 integrin on their surfaces is at variance with a report by Zheng et al. (4), in which they demonstrated that LNCaP cells did not express αβ3 integrins. The difference between these two observations could be attributable to the clonal variation as a result of long-term culture and/or differences in culture conditions. In fact, in the case of human glioma cells, numerous sublines have been established by single cell cloning, some of which lacked the expression of αβ3 integrins on their surfaces in subsequent studies (18, 32). In parallel, we also examined the effect of cRGDV on the growth of another prostate cancer cell line, DU 145. These cells partially expressed αβ3 integrins on their membranes as determined by an indirect immunofluorescence assay (data not shown). As expected, cRGDV (20 μg/ml) caused a partial (35–40%) reduction in the number of recovered DU 145 cells. In contrast to the results we observed in LNCaP and DU 145 cells, cRGDV had no major effect on the growth of HEL or PC-3 cells, which did not express αβ3 integrins on their surfaces in appreciable quantity. This preferential affinity of cRGDV peptide for αβ3 integrins in tumor cells has been documented by several laboratories including ours (15, 16, 18, 33). Using a similar cyclic peptide, cyclo-(Arg-Gly-Asp-D-Phe-Lys), Romanov and Goligorsky (5) also observed ~40% and 15% detachment of peptide-treated (20 μg/ml) DU 145 and PC-3 cells, respectively. These cells express lower levels of αβ3 integrins than the other members of the integrin family (5). However, it was unknown whether the detachment of the prostate cancer cells was attributable to apoptosis or to an activation of a signal transduction pathway in the treated cells. Normal control cells were largely unaffected by the treatment of this peptide (5) as was also observed for HEL cells in our experiments. Our data indicated that linear RGD peptide had no significant effect on the growth of any of these cells, although a 47% reduction of the number of recovered LNCaP cells was noticed after they were treated with linear RGDS peptide (20 μg/ml). Recently Buckley et al. (34) reported that linear peptides containing RGD sequences (including RGD and RGDS) induced PCD of lymphocytes within 24 h. The difference between our observations could be because of the use of different cell lines in these studies. In our experiments, cRGDfV was more effective in induction of apoptosis of LNCaP cells than the two linear RGD peptides we used.

In this report, cRGDV-induced PCD of LNCaP cells is documented by changes in cell morphology, loss of attachment of cells, and activation of at least two caspases (caspase-3 and caspase-9) as detected by cleavage of PARP and partial blocking of apoptosis by a caspase-9-selective antagonist. However, the involvement of other caspases, responsible for the apoptosis of cRGDV-treated LNCaP cells, cannot be ruled out at this time.

It is well known that integrins activate several protein kinases including FAK (9) by mechanisms that are not completely understood. FAK, which binds to the cytoplasmic domains of the β subunit of integrins, is a key player in integrin-mediated signal transduction pathway and responsible for cell spreading and survival (9, 35). Recent reports from Zheng et al. (4) conclusively demonstrated that the αβ3 integrin regulates migration of human prostate cancer cells, and this response is mediated by a FAK signaling pathway. We have shown here that treatment of LNCaP cells with cRGDV resulted in the cleavage of FAK, and, thus, blocking the normal function of this kinase, which ultimately leads to PCD. It is thought that activated FAK induces PKB, a serine-threonine protein kinase, via PI3k, which stimulates cell survival (23, 24). Specifically, it has been shown that activated PKB directly phosphorylated Bad and caspase-9 on serine 196 and inhibited their activities (28, 29).

Thus, the cleavage of FAK in cRGDV-treated LNCaP cells could interfere with this pathway and induce PCD as evidenced by dephosphorylation of Akt and induction of caspases in treated cells. However, recently, Carson et al. (36) have identified a novel Akt/PKB-independent survival pathway in LNCaP cells, which blocks PCD at a level before caspase-3 activation. Thus, in LNCaP cells several distinct pathways may exist for cell survival. It is interesting to note that the tumor suppressor PTEN (also called PTEN/MMAC-1) can suppress the growth of glioblastoma cells by dephosphorylation of FAK leading to the inhibition of the PI3k/Akt cell survival pathway (37, 38). Furthermore, Wu et al. (39) have also shown that PTEN inhibits the PI3k/Akt pathway in prostate cancer cells, although it is unclear whether this inhibition is mediated via dephosphorylation of FAK or whether it leads to the induction of apoptosis. Thus, at least glioblastoma and prostate cancer cells display similar PI3k/Akt survival pathway influenced by PTEN.

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer deaths among men in the United States (1). Although treatments such as hormonal therapies or antiandrogen administration may be beneficial for the localized disease, clinically aggressive, androgen-independent, and metastatic prostate cancers are extremely difficult to treat (2, 3). In this report, we demonstrated that cRGDV induces apoptosis of prostate cancer cells that express αβ3 integrin on their surfaces possibly by blocking integrin/FAK-mediated signal transduction pathway. Our results suggest that cRGDV therapy, either alone or in conjunction with other
treatments, may be an effective and nontoxic treatment for some human prostate cancers.

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