An Artificially Designed Pore-Forming Protein with Anti-tumor Effects*

The tendency of amphipathic peptides to assemble in aqueous solution and of the $\beta$-turn to form a loop has been successfully employed to design coiled-coil proteins (1–3), various helix bundle proteins (4–9), and $\beta$-structural proteins (10, 11). De novo design of proteins with biological function, such as membrane binding, catalysis, or the formation of a membrane pore or channel, is perhaps the most challenging goal of peptide chemistry (12–19). Much has been done recently in terms of designing membrane proteins that are correctly incorporated into membranes. However, relatively few attempts have been made to design proteins capable of disrupting membranes and subsequently causing cell death in vivo (19, 20).

Small globular protein (SGP)\(^{\dagger}\) is a 69-amino acid, 4-helix bundle protein, composed of 3 amphipathic helices, which consist of Leu and Lys residues and surround a single hydrophobic helix consisting of Ala residues, which create a pocket-like structure (Fig. 1, A and B) (21, 22). SGP is monomeric in solution and denatures in a highly cooperative manner, characteristic of native globular-like proteins. SGP was conceived and designed based on the structure of the colicin family of bacteriocins (23–26). Although most naturally occurring, pore-forming proteins maintain their tertiary structure when disrupting membranes, the colicins undergo a spontaneous transition from a native folded state in solution to an open umbrella-like state in membranes. SGP was designed to mimic this membrane insertion mechanism, which was confirmed in synthetic bilayers, where SGP formed a uniform size pore (14pS) (21). It is still unknown whether or not SGP oligomerizes to form a channel.

Given that SGP forms pores in synthetic membranes, we asked whether it could disrupt biological membranes at the cellular level and whether it could be used successfully in vivo as an anti-tumor agent. We also investigated whether SGP would show any selectivity toward tumor cell lines in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

Reagents—SGP, SGP-L, and SGP-E were synthesized according to the Fmoc procedure starting from Fmoc-Leu-PEG (polyethylene glycol) resin using a Milligen automatic peptide synthesizer (Model 9050) to monitor the de-protection of the Fmoc group by UV absorbance (21). After cleavage from the resin by trifluoroacetic acid, the crude peptide obtained was purified by HPLC chromatography with an ODS column, 20 $\times$ 250 mm, with a gradient system of water/acetonitrile containing 0.1% trifluoroacetic acid. Amino acid analysis was performed after hydrolysis in 5.7 M HCl in a sealed tube at 110 °C for 24 h. Analytical data obtained were as follows: Gly, 6.2 (6); Ala, 9.5 (10); Leu, 26.5 (25); Asp, 3.0 (3); Pro, 2.9 (3); Tyr, 3.1 (3); Lys, 18.9 (18). Molecular weight was calculated by fast atom bombardment mass spectrometry using a JEOL JMX-HX100: base peak, 7555.1; calculated for C\(_{367}\)H\(_{639}\)O\(_{77}\)N\(_{91}\) mass spectroscopy using a JEOL JMX-HX100: base peak, 7555.1; calculated for C\(_{367}\)H\(_{639}\)O\(_{77}\)N\(_{91}\)

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The abbreviations used are: SGP, small globular protein; Fmoc, N-(9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.

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apoptosis-acridine orange positive) or with compromised plasma membranes (late apoptosis-ethidium bromide positive) were scored as not viable; 500 cells per time point were scored in each experiment. Percent viability was calculated relative to untreated cells.

**Human Tumor Xenografts**—MDA-MB-435-, KS1767-, PC-3-, and H358-derived human tumor xenografts were established in 5-month-old female or male (according to the tumor type), nude/nude Balb/c mice (Jackson Labs, Bar Harbor, ME) by administering $10^6$ tumor cells per mouse in a 200 $\mu$l volume of serum-free Dulbeco’s modified Eagle’s medium into the mammary fat pad or on the flank (29). The mice were anesthetized with Avertin as described (29). SGP was administered into the skin. The injected areas were monitored for 2 weeks.

**Skin Toxicity**—2-month-old female nude mice (Jackson Labs) were anesthetized with Avertin as described (29). SGP was administered intraperitoneally on the back (as described in Table 1).

**Cytotoxicity Assays**—Cell viability was determined by morphology (29, 30). KS1767 cells were incubated with SGP at 1 mM in the presence or absence of matrigel or polymeric fibronectin (sFN). The fibronectin polymer was produced as described (31). Briefly, cell culture medium was aspirated from adherent cells. Cells were then coated with matrigel (gently pipetted on each well to completely coat the entire cell layer), or the fibronectin polymer, and incubated at 37 °C for 10 min. SGP was added and the cells were viewed on an inverted microscope (Nikon TE 300). KS1767 cells were also exposed to doxorubicin (20 $\mu$g/well) or SGP in the presence or absence of matrigel for 24 h. Cell viability (%) was evaluated after no treatment (medium or matrigel alone), incubation with SGP or doxorubicin. Cell death was evaluated morphologically (29, 30), and cell viability was compared relative to untreated controls (no matrigel) or absence of SGP.

**RESULTS**

**SGP Effects on Cultured Cells**—To evaluate the effects of SGP on cell membranes we treated multiple human cell lines of different origins (Table I). These lines included the Kaposi’s sarcoma-derived cell line KS1767, the breast carcinoma-derived cell line MDA-MD-435, and the microvascular endothelial cell line dermal microvessel endothelial cells (27–29). Treatment of KS1767 cells with >10 $\mu$M SGP led to rapid non-necrotic, non-apoptotic cell death, characterized by 100% loss of viability within 60 s (Fig. 2A), as determined by Trypan Blue positivity. Such a rapid response suggests that the plasma membrane has been disrupted. Lowering the concentration of SGP to between 5 and 10 $\mu$M led to induction of necrosis (scored morphologically), resulting in almost 100% loss of KS1767 cell viability over 60 min (Fig. 2B). SGP levels below 5 $\mu$M led to the induction of apoptosis over a 24-hour period (Fig. 2C, which was confirmed by a caspase-3 activation assay. KS1767 cells were unaffected by a 24-h incubation in 100 $\mu$M of a control peptide (Fig. 2D). However, the classic morphological signs of apoptosis, such as nuclear condensation (Fig. 2E, short arrow) and plasma membrane bleeping (Fig. 2E, long arrow), were apparent in KS1767 cells after a 24-hour treatment with 3 $\mu$M SGP. Similar results were obtained using different cell lines, including several types of malignant cells (solid tumors and leukemic cell lines) and non-neoplastic cells (including endothelial cells and fibroblasts isolated from multiple organs and cells of glial origin, Table I). As negative controls, we used altered forms of SGP (SGP-L and SGP-E). In SGP-L, the central all alanine helix was replaced by an all leucine helix. In SGP-E, lysines have been replaced by glutamic acids, and we had previously determined that the ability of such analogs to
Tumor-bearing mice were given four weekly treatments treated with 100 cells (Table I). The LC50 was increased by at least 10-fold in all SGP-E were substantially less toxic to mammalian cultured disrupt synthetic membranes is diminished (22). SGP-L and SGP-E were substantially less toxic to mammalian cultured cells as well as normal cells at similar concentrations (~3 μM).

SGP Has Anti-tumor Activity in Vivo—Given the potent membrane-disrupting activity of SGP, we proceeded to evaluate SGP anti-tumor activity in nude mice bearing human tumor xenografts. We hypothesized that direct administration of SGP might reduce tumor volume and retard metastasis. In the first set of experiments, tumors were allowed to form after injection of a breast carcinoma cell line (MDA-MD-435) and then treated with local injections of SGP. We observed that tumor volume was significantly smaller in SGP-treated mice than in the PBS-treated control mice (Fig. 3A). Starting tumor volumes ranged from about 100 mm³ to large sizes of about 600 mm³. Tumor-bearing mice were given four weekly treatments of PBS, or 100 μM or 1 mM SGP (40 μl/treatment given in 5 μl increments). After a 4-week period without treatment, the tumor volumes were measured at 8 weeks. The average tumor volume at the end of the experiment in the SGP-treated groups was 5X less than the average volume seen in the PBS-treated group (Fig. 3A). There was no difference between the average

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**Fig. 2. SGP treatment of cultured tumor cells.** A, human Kaposi’s sarcoma-derived KS1767 cells treated with 10 μM SGP undergo extremely rapid non-necrotic, non-apoptotic cell death within 60 s (black bars), whereas those treated with 100 μM of negative control peptide DLSLARLATARLAI are unaffected (scheme bars) (p < 0.04). B, necrosis is observed in KS1767 cells treated with 10 μM SGP within 60 min (black bars), whereas those treated with 100 μM of negative control peptide are unaffected after 60 min (gray bars) (p < 0.03). C, apoptosis is observed after treatment with 3 μM SGP over 24 h, whereas cells treated with 100 μM of negative control peptide are unaffected after 24 h (gray bars) (p < 0.05). Hoffman contrast microscopy of KS1767 cells treated with 100 μM of negative control peptide (D) for 24 h or 3 μM SGP for 24 h (E). Cells with nuclei exhibiting margination and condensation of chromatin and/or nuclear fragmentation (early/mid apoptosis-acridine orange positive) or with compromised plasma membranes (late apoptosis-ethidium bromide positive) were scored as not viable (500 cells per time point were scored in each experiment). Percent viability was calculated relative to untreated cells under all experimental conditions. Classic morphological characteristics of cell death including condensed nuclei (short arrows) and plasma membrane blebbing (long arrows) are evident. Results were reproduced in more than three independent experiments.

**Fig. 3. SGP treatment of nude mice bearing human breast cancer-derived xenografts.** Data are shown for human MDA-MB-435-derived breast carcinomas. Mice had tumor volumes ranging from 100 mm³ to 600 mm³ and were divided in similar groups based on matched tumor volumes at the start of the experiment (open circles). A, SGP-treated tumors are smaller than controls (PBS-treated or SGP-treated tumor volumes at the end of the experiment are represented as closed circles). Differences in tumor volumes at 8 weeks are shown (t test, p < 0.05). A total of 10 mice received SGP. B, representative pictures of tumors after 4 weekly treatments with SGP at 40 μl/week, n = 5 for each experimental group. The volume of the PBS-treated tumor is 400 mm³ (left), whereas 100 μM SGP (middle) and 1 mM SGP (right) treated tumors have flattened and virtually disappeared. These three tumors began at volumes of 100 mm³. C, lack of skin toxicity of SGP. Subcutaneous injection (40 μl) of 100 μM SGP (left injection site, arrow) and of PBS (right injection site, arrow) demonstrates that SGP is relatively non-toxic to normal skin. Results represented in C were reproduced in eight independent experiments.
tumor volumes of the 2 SGP treatment groups. Mice treated with SGP remained tumor-free for up to 4 months after tumor implantation, before being euthanized for histological evaluation. These observations indicate that both primary tumor growth (Fig. 4) and metastases were inhibited. Surgical examination of the tumor sites revealed no sign of tumor cells. Similar results were obtained when xenografts were produced by injection of prostate (Fig. 5A) and lung carcinoma (Fig. 5, B and C) cell lines. By successfully treating a large number of mice and testing the effects of SGP on several different tumor xenograft models (including carcinomas, sarcomas, and melanomas), we firmly established the therapeutic properties of SGP. Our data also show that the anti-tumor effects of SGP are not limited to a specific tumor type. We also determined whether SGP produced adverse side effects such as necrosis when injected under normal skin. Strikingly, in all mice tested, SGP did not produce any surface effect when injected intradermally or sub-cutaneously (Fig. 3C) when compared with mice that did not receive the active form of SGP.

Histopathological analysis of SGP-treated MDA-MD-435 human breast carcinoma xenografts showed widespread cell death (Fig. 4, upper right panel), as compared with PBS-treated tumors (Fig. 4, upper left panel). Many condensed nuclei were apparent (Fig. 4, upper left panel, short arrows), and there was no effect on the extracellular matrix (Fig. 4B, long arrows). Apoptosis was confirmed by a caspase-3 activation assay (data not shown). It is noteworthy that whereas 100 μM SGP induced almost immediate cell death in vitro that was apparently neither apoptotic nor necrotic, 100 μM SGP induced apoptosis in vivo. Work is underway to evaluate lower concentrations. SGP-treated human KS1767 Kaposi’s sarcoma-derived xenografts showed similar effects (Fig. 4, left and right panels). Histological analysis of the major organs of SGP-treated mice showed no overt pathology, confirming that SGP treatments do not affect sites other than the injected tumor area (data not shown). Thus, SGP has anti-tumor specific effects, without showing any tumor cell-specific effects.

**Mechanism of SGP Action and Selectivity toward Cell Membranes**—To determine the mechanisms responsible for selective anti-tumor activity of SGP in vivo, we designed a matrigel

**FIG. 4.** SGP-treated tumors undergo widespread cell death. Histopathological tissue sections of human tumor xenografts harvested at 8 weeks after treatment initiation are shown. Tissue sections from human MDA-MB-435-derived breast carcinoma xenografts from nude mice treated with PBS-treated tumor tissue but with 100 μM SGP, show extensive apoptosis with many evident condensed nuclei (short arrows) and an intact extra-cellular matrix (long arrows); n = 7 for each experimental group. Tissue sections from human KS1767-derived Kaposi’s sarcoma xenografts in nude mice had a similar outcome, a representative image of a PBS-treated tumor, and a tumor treated with SGP are shown.

**FIG. 5.** SGP treatment of nude mice bearing human prostate and lung cancer xenografts. Data are shown for human PC3-derived prostate carcinoma and H358 lung carcinoma. Tumor cells were implanted on the flank at the start of the experiments. Mice were divided in similar groups based on matched tumor volumes at the start of the experiment (open circles). A, SGP-treated PC-3 tumors are smaller than control PBS-treated tumors. Differences in tumor volumes at 10 weeks are shown (t test, p < 0.05). B, SGP-treated H358 tumors are smaller than control PBS-treated tumors. Differences in tumor volumes at 9 weeks are shown (t test, p < 0.05). C, representative pictures of tumors after 6 weekly treatments at 40 μl/week (see “Experimental Procedures”; n = 7 for each experimental group. SGP-treated tumors, as indicated, have disappeared. A, SGP-treated tumors are smaller than controls (PBS-treated or SGP-treated tumor volumes at the end of the experiment are represented as closed circles).
assay (to mimic extracellular matrix). In the absence of matrigel, SGP led to severe disruption of cell membranes, resulting in almost 100% loss of viability over 10 min (Fig. 6B). In contrast, in the presence of matrigel, KS1767 cells were unaffected by incubation with 1 mM SGP (Fig. 6D). This loss of membrane disrupting ability in the presence of a thin matrigel layer could account for the lack of SGP toxicity seen in vivo. Ethanol, as shown in Fig. 7A, or cytotoxic drugs such as doxorubicin (Fig. 7B) damaged the cell layer under similar conditions, regardless of the presence of matrigel, which fails to provide protection from the other toxic agents because these other agents more readily diffuse through the matrix. When matrigel was replaced by polymeric fibronectin (sFN) (31), another form of matrix, SGP was also ineffective and did not interfere with cell viability (Fig. 7A), whereas ethanol induced massive cell death. Fibronectin alone did not prevent SGP activity and was used as a control.

The observations in this model are consistent with the lack of skin toxicity seen with SGP. We propose that the discrepancy between in vitro and in vivo SGP effects (anti-tumor cell activity versus selective anti-tumor activity) results from the potent membrane-disrupting activity of SGP, which is inactivated in the presence of extracellular matrix and connective tissue.

**DISCUSSION**

SGP represents a novel class of anti-cancer proteins whose therapeutic effects can be optimized by amino acid substitution and by altering helical domain length and hydrophobicity (32).
Although SGP is a nonspecific membrane-disrupting agent, it is selective in the sense that the disruption is limited in vivo. Unlike detergents, which solubilize membranes, SGP physically disrupts membrane architecture, leading to cell lysis. This explains the lack of SGP toxicity when the protein is injected subcutaneously or intradermally. Recently published data (22) also suggest that the lipid membrane-disruption properties of SGP are responsible for the anti-tumor activity of the agent.

We report one of the first examples of a pore-forming peptide or protein, natural or synthetic, being applied successfully to treat established human tumor xenografts. It is important to emphasize that SGP is not a bacterial toxin, although such agents (or their natural or recombinant form) have been extensively explored as anti-cancer therapies (33, 34). Several pore-forming peptides and proteins have been shown to have moderate efficacy in killing tumor cells in vitro, yet very limited anti-tumor effects were seen in vivo. The anti-bacterial peptides magainin (and synthetic derivatives) (35), cecropin (and synthetic derivatives) (36), granulysin (37), and NK-lysins (38) are toxic to tumor cells in culture. The pore-forming protein verotoxin 1 (a colicin) has also been shown to have a toxic effect on tumor cells in vitro (39). Magainin, cecropin, and verotoxin 1 also had limited efficacy in vivo in mice bearing murine tumors (35, 36, 39).

Cytotoxic agents developed within the past few decades have been based on naturally existing compounds, synthetic peptides, or protein fragments representing active membrane-disrupting domains. In contrast to such compounds, SGP is a protein that was artificially created to perform a pre-determined biological function. Moreover, therapeutically significant cell membrane disrupting activity was observed in vivo.

SGP activity appears to be restricted to the presence of lipid bilayers in vitro, whereas in vivo its activity appears to be limited to tumors in vivo due to the protective effect of extra-cellular matrix components. In vitro, SGP shows no selectivity toward normal or malignant cells under the experimental conditions tested. We show here that SGP is potentially a valid anti-cancer agent; applications include Kaposi’s sarcoma, malignant melanoma of the skin, or palliation for unresectable or metastatic tumors in anatomical sites difficult to treat with other modalities. Moreover, SGP variants in which residues critical for helical structure are altered are inactive, suggesting that the structure of the protein is intrinsically linked to its ability to damage cell membranes. Although the de novo design of proteins with biological function is in its early stages, novel therapeutic strategies may emerge from the activity of designed proteins such as SGP.

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