Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy

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Abstract

Elastin-like polypeptides are biopolymers composed of the pentapeptide repeat Val-Pro-Gly-Xaa-Gly. Elastin-like polypeptides are soluble in aqueous solution below their transition temperature, but they hydrophobically collapse and aggregate when the temperature is raised above the transition temperature. Previous studies have suggested that the aggregation of these polypeptides in response to externally applied hyperthermia may be exploited in the use of elastin-like polypeptide for thermally targeted drug delivery. This work shows the application of elastin-like polypeptide as a delivery vehicle for a short peptide that can inhibit the transcriptional function of a specific oncogene. The coding sequence for elastin-like polypeptide was modified by the addition of the membrane translocating sequence penetratin and a peptide derived from helix 1 of the helix-loop-helix region of c-Myc (H1-S6A,F8A), known to inhibit c-Myc transcriptional function. The designed polypeptide (Pen-ELP-H1) was then expressed and purified from Escherichia coli. Cellular uptake of Pen-ELP-H1 is enhanced by both the penetratin sequence and by the hyperthermia-induced phase transition as shown by flow cytometry studies. Using immunofluorescence and reverse transcription-PCR, we show that Pen-ELP-H1 is able to disrupt the nuclear localization of c-Myc and inhibit transcriptional activation by c-Myc. Cell proliferation studies showed that Pen-ELP-H1 inhibits growth of MCF-7 cells. Furthermore, the use of hyperthermia increased the antiproliferative effect of a thermally responsive Pen-ELP-H1 ~2-fold compared with a nonthermally responsive control polypeptide. These studies show that genetically engineered elastin-like polypeptide carriers may provide a new way to thermally target specific oncogene inhibitors to solid tumors. [Mol Cancer Ther 2005;4(7):1076–85]

Introduction

A major limitation in cancer chemotherapy of solid tumors is the lack of selective delivery of anticancer agents to tumor cells. In most cases, only a small fraction of the administered dose of drug reaches this site, whereas the rest of the drug is distributed throughout the body, resulting in unacceptable damage to normal tissue when used in doses required to eradicate cancer cells. It has therefore been recognized that site-specific drug delivery vehicles are needed to make chemotherapy more effective and less toxic. In response to this need, numerous drug delivery systems have been employed. These include macromolecular carriers such as soluble synthetic and natural polymers (1), liposomes (2), microspheres, and nanospheres (3). Most of these carriers maximize localization of the drug to the tumor by optimizing whole-body pharmacokinetics (4). However, the use of thermally responsive polymeric carriers introduces the additional advantage of active targeting by application of focused hyperthermia.

In our study, we used the thermally responsive elastin-like polypeptide as a macromolecular therapeutic delivery vehicle. Elastin-like polypeptide is a biopolymer derived from a structural motif found in the mammalian elastin protein (5). It is composed of a Val-Pro-Gly-Xaa-Gly (VPGXG) pentapeptide repeated 120 times, where Xaa, the "guest residue," is any amino acid except Pro. These polypeptides have several attractive features for the delivery of cancer therapeutics to solid tumors. First, soluble polymeric carriers are intrinsically attractive for systemic drug delivery because polymer-drug conjugates preferentially accumulate in tumors due to the enhanced permeability and retention effect (6–9), and they also exhibit significantly lower systemic toxicity compared with free drug (10–12). In addition, studies have shown that water-soluble polymer carriers can overcome multidrug resistance (13–16). Second, elastin-like polypeptides are temperature-sensitive biopolymers that undergo a reversible phase transition in aqueous solution. They are soluble in aqueous solution below their transition temperature (Tt). However, when the temperature is raised above their Tt, they undergo a phase transition, become insoluble, and form aggregates. It has been hypothesized that i.v. delivered thermally responsive elastin-like polypeptides are likely to be cleared under physiologic conditions (T < Tt), and they will form aggregates and accumulate at targeted diseased sites where local hyperthermia will be applied (T > Tt). This hypothesis is supported by a previous in vivo study of elastin-like polypeptide delivery to human tumors implanted in nude mice (17). These studies showed a 2-fold higher accumulation of systemically injected thermally responsive elastin-like polypeptide in tumors treated with external local hyperthermia compared with

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the same polypeptide without hyperthermia. Application of hyperthermia to thermally target the elastin-like polypeptide therapeutic carriers may offer further advantages in the treatment of solid tumors. Specifically, hyperthermia preferentially increases the permeability of tumor vasculature compared with normal vasculature (18, 19), and therefore it may further enhance the delivery of drugs to tumors.

In the development of novel cancer therapies, considerable effort has been focused on targeting oncogenes. Recent progress in X-ray crystallography, nuclear magnetic resonance, and molecular modeling has provided structural information about many oncogenes and their effectors. Consequently, based on these target structures, peptides have been developed that selectively inhibit specific oncogene signaling pathways by tightly binding and sequestering pathway intermediates. A peptide derived from helix 1 (H1) of the helix-loop-helix region of c-Myc (H1-S6A,F8A), which inhibits the c-Myc signaling pathway, has been characterized by Draeger and Mullen (20). c-Myc is a transcriptional regulator which controls cell growth, proliferation, apoptosis, and tumorigenesis. It has been shown that deregulated expression of c-Myc is associated with numerous types of human cancers, confirming its strong oncogenic potential. For its oncogenic activity, c-Myc must dimerize with its partner protein Max. Consequently, inhibition of c-Myc-Max dimerization seems to be a potent method of inhibiting proliferation of cancer cells and represents an attractive target for tumor therapy. Giorello et al. (21) have shown that the H1-S6A,F8A peptide was able to block c-Myc activity as a transcription factor and inhibit cell proliferation in MCF-7 cells.

Here, we investigate the ability of elastin-like polypeptide to function as a thermally responsive macromolecular carrier for delivery of this peptide to cancer cells. Elastin-like polypeptides are genetically encoded, and synthesis of elastin-like polypeptides by recombinant DNA methods provides precise control over the elastin-like polypeptide sequence. We used this attractive property of elastin-like polypeptide and modified its original coding sequence by a c-Myc inhibitory peptide as well as the penetratin peptide (RQIKIWFQNRRMKWKK) to the NH₂ terminus and the c-Myc inhibitory peptide H1-S6A,F8A (NELKRAFAALRDQI) to the COOH terminus using the method described by Meyer and Chilkoti (25). The resulting sequence was then confirmed by DNA sequencing.

**Polypeptide Purification**

pET25b+ expression vectors containing the desired constructs were transformed into E. coli BLR(DE3) (Novagen, Madison, WI) for protein hyperexpression (26). One liter of Circle Grow (Q Biogene, Carlsbad, CA) media plus ampicillin (100 µg/mL) was inoculated with the expression strain and grown at 37°C, 240 rpm agitation, for 24 hours. Cells were harvested by centrifugation (3,000 × g, 10 minutes), resuspended in 70 mL PBS, lysed by sonication (Fisher Scientific 350 Sonic Dismembrator), and centrifuged to remove cell debris (13,000 × g, 45 minutes). Polyethylene imine (0.5% w/v) was added to the lysate to precipitate nucleic acids, which were removed by centrifugation at 4°C for 45 minutes. The polypeptide phase transition was induced in the soluble cell lysate by heating the cell lysate to 44°C and increasing the NaCl concentration to 2 mol/L. Polypeptides were then collected by centrifugation (11,000 × g, 10 minutes) and resuspended in PBS (Gibco, Carlsbad, CA). This process was repeated three to five times and purity was assessed by SDS-PAGE.

**Characterization of Transition Temperature**

The temperature-induced aggregation of the proteins was characterized by monitoring absorbance at 350 nm as a function of temperature. Solutions containing 18 µmol/L protein in phosphate buffer (1 mmol/L Na₂PO₄, 0.2 mmol/L KH₂PO₄, 0.27 mmol/L KCl, 13.7 mmol/L NaCl) were heated or cooled at a constant rate of 1°C/min in a temperature-controlled multicell holder in a UV-visible spectrophotometer (Cary 100, Varian instruments). The Tᵢ was defined as the temperature at which the A₃₅₀ reached 50% of the maximum turbidity.

**Conjugation of Polypeptides with Fluorescent Probes**

In a typical conjugation reaction, proteins were diluted to 200 to 400 µmol/L in PBS, and tris-(2-carboxyethyl)phosphine was added to a 10-fold molar excess. Thiol-reactive probes (5-iodoacetomido fluorescein or tetramethylrhodamine-5-iodoacetamide dihydroiodide; Molecular Probes, Eugene, OR) were slowly added while mixing to a final 2-fold molar excess and incubated with continuous stirring for 2 hours at room temperature. The reaction was then halted by adding an excess of β-mercaptoethanol, and the
unreacted label was removed by extensive dialysis into PBS followed by one thermal cycle. Efficiency of labeling on the single cysteine residue was assessed by UV-visible spectrophotometry (UV-1600 Shimadzu). The typical molar label to protein ratio was 0.3 to 0.5.

Cell Culture and Polypeptide Treatment
MCF-7 breast carcinoma cells (American Type Culture Collection, Manassas, VA) were grown as a monolayer in 75 cm² tissue culture flasks and passaged every 3 to 5 days. MCF-7 cells were grown in MEM supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, Basal Medium Eagle amino acids, 5 µg/mL insulin (Sigma, St. Louis, MO), 100 units/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B (Invitrogen, Carlsbad, CA). Cultures were maintained at 37°C in a humidified atmosphere + 5% CO₂. For experiments, cells were removed from tissue culture flasks by brief treatment with 0.05% v/v trypsin-EDTA (Invitrogen), plated in six-well plates (100,000 cells/well for flow cytometry; 20,000 cells/well for proliferation), and allowed to grow for 24 hours. Cells were treated with media containing polypeptides for 1 hour, rinsed, and replaced with fresh media.

Polypeptide Uptake
Cells were treated with 18 µmol/L fluorescein-labeled Pen-ELP1-H1, Pen-ELP2-H1, or control polypeptides lacking the penetratin sequence as described above. Duplicate plates were treated at 37°C and 42°C. Cells were washed thrice with PBS and harvested at different time points with nonenzymatic cell dissociation buffer (Invitrogen), centrifuged for 2 minutes, and resuspended in 1 mL PBS. Total uptake of the fluorescein-labeled polypeptides was measured using a fluorescence-activated cell scanner (Becton Dickinson, San Jose, CA). Fluorescence data were corrected for labeling efficiency and normalized to fluorescent bead standards (Bangs Laboratories, Fishers, IN).

Cell Proliferation
Cells were plated and treated for 1 hour as described above with Pen-ELP1-H1, Pen-ELP2-H1, and control polypeptides lacking the penetratin or the c-Myc H1 sequence. Media was changed every 3 days. Daily cell counts for growth curves were done using a Coulter counter. Proliferation experiments were harvested on day 11 as described above. Viable cells were counted using the trypan blue dye exclusion assay and expressed as a percentage of untreated cells. Data represent an average of at least three experiments.

Laser Scanning Confocal Fluorescence Microscopy
MCF-7 cells were plated on chamber slides at ~50% confluence and treated as described above with rhodamine-conjugated Pen-ELP1-H1 and Pen-ELP2-H1. Cells were rinsed with PBS at the indicated times, fixed with paraformaldehyde (2% v/v), and visualized using a TCS SP2 laser scanning confocal microscope with a 100× oil immersion objective (Leica, Wetzlar, Germany). PMT voltages were adjusted during image acquisition to maximize image resolution and intensity. Therefore, the image intensity does not represent the quantitative amount of polypeptide in the cells. For immunofluorescence of endogenous c-Myc and Max, cells were treated with 18 µmol/L unlabeled polypeptides, rinsed with PBS, and allowed to grow for 24 hours. Cells were fixed with 2% paraformaldehyde, rinsed thrice with PBS, and permeabilized with PBS containing 0.25% Triton X-100 for 5 minutes. Slides were blocked for 1 hour at 37°C with PBS + 1% bovine serum albumin in a humidity chamber and immunostained with mouse c-Myc monoclonal antibody 9E10 and rabbit Max polyclonal antibody C-17 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. After washing in PBS, cells were incubated with goat anti-mouse fluorescein-conjugated immunoglobulin G (Coulter, Miami, FL) and goat anti-rabbit Cy5-conjugated immunoglobulin M (Molecular Probes) for 45 minutes in the dark. Coverslips were visualized with anti-fade reagent, and cells were visualized using a Leica TCS SP2 laser scanning confocal microscope.

Reverse Transcription-PCR
MCF-7 cells were grown to 70% confluence in 75 cm² flasks and treated as described above with Pen-ELP1-H1 or control polypeptides lacking the penetratin or the H1 sequence. After 48 hours, cells were collected by trypsinization, and total RNA was purified using a Micro to Midi Total RNA Purification System (Invitrogen) according to the protocol of the manufacturer. RNA quality was assessed by monitoring the 28S and 18S rRNA ratios by capillary electrophoresis in a Bioanalyzer (Agilent Technologies, Palo Alto, CA) using an RNA 6000 Nano Assay. One microgram of total RNA was reverse transcribed using oligo dT primers and Reflectase reverse transcriptase (Active Motif, Carlsbad, CA) according to the protocol of the manufacturer. The reverse transcription reaction was diluted 24-fold, and 1 µL was used in a PCR reaction with 16 µmol/L of each primer, 1× FailSafe buffer C or D (Epigenetix, Madison, WI), and 1× Titanium Taq DNA polymerase (Clontech, Palo Alto, CA). PCR primers for ornithine decarboxylase (ODC; sense, 5'-GAGGACATCCTCACAACCAAGGT; antisense, 5'-TCCAGAGTCTGACGGAAAAGTA); lactate dehydrogenase-A (LDH-A; sense, 5'-TGAAGATGCTTATGAGGTGA; antisense, 5'-CAAAGGTGTATCTGCACTCT), and glyceraldehyde phosphate dehydrogenase (GAPDH; sense, 5'-GGTACATCCTGACCGAAGC; antisense, 5'-TGCCTGTGCATACCCAGGAATTG) were used in a 6-µL reaction to amplify fragments of 374, 268, and 295 bp, respectively. PCR was carried out in a PTC-200 thermal cycler with a temperature gradient block (MJ Research, Waltham, MA) using 35 cycles consisting of 95°C for 30 seconds, 57°C (ODC), 54°C (LDH-A), or 58.3°C (GAPDH) annealing for 1 minute, and 72°C extension for 1 minute. The end point of the PCR reaction was analyzed by capillary electrophoresis in a Bioanalyzer using a DNA 1000 Labchip. Data were converted to the tif file format using Agilent software, and band intensities were normalized to control.

Results
Design and Thermal Properties of Polypeptides
The thermally responsive polypeptides were designed as schematically represented in Fig. 1A. The Pen-ELP1-H1 polypeptide, designed to inhibit c-Myc transcriptional...
of polypeptide aggregates (29, 33). Polypeptide aggregates undergo a hydrophilic to hydrophobic phase transition which results in the formation of polypeptide–based aggregates at temperatures greater than the hyperthermia temperature \(T_h\) because of polypeptide aggregation. The inverse transition of each polypeptide was reversible (data not shown), which is consistent with previously reported elastin-like polypeptide thermal properties (29).

**Cellular Uptake of Polypeptides**

To reach their intracellular molecular site of action, the polypeptides have to be efficiently internalized by target cells. It has been shown that efficient internalization of relatively large molecules across the cell membrane can be mediated by the penetratin peptide (34, 35). To test whether penetratin can work as an internalization vector for the 51 kDa Pen-ELP-H1 polypeptide, we compared the cellular uptake of fluorescein-labeled Pen-ELP-H1 with that of the control polypeptide ELP-H1 lacking the penetratin sequence. Cells were incubated with fluorescein-labeled polypeptides for 1 hour, then rinsed and collected. Their fluorescence intensity was measured by flow cytometry. Figure 2 shows that the fluorescence intensity of cells scatter light, causing an increase in turbidity of the polypeptide solution. To characterize the phase transition behavior of these polypeptides, the turbidity of polypeptide solutions was monitored as a function of temperature. As shown in Fig. 1B, a solution of Pen-ELP1-H1 is clear below \(T_t\) but becomes turbid on heating above its \(T_t\) because of polypeptide aggregation. The sharp increase in the turbidity on reaching \(T_t\) shows that polypeptide aggregation is initially very rapid with respect to temperature, with 75% of the polypeptide aggregated at the hyperthermia temperature \(T_h = 42^\circ C\). In contrast to Pen-ELP1-H1, the turbidity profile of Pen-ELP2-H1 shows that the polypeptide solution is clear even above the hyperthermia temperature. It becomes turbid only at a temperature significantly higher than \(T_h \) \(T_t = 65^\circ C\), indicating that Pen-ELP2-H1 does not undergo a phase transition when heated to 42°C. Pen-ELP2-H1 is a useful control for the effect of hyperthermia because it remains soluble at temperatures significantly higher than the hyperthermia temperature. All constructs containing ELP1 displayed similar transition curves, as did all ELP2-containing polypeptides. The inverse transition of each polypeptide was reversible (data not shown), which is consistent with previously reported elastin-like polypeptide thermal properties (29).

**Figure 2.** Cellular uptake of polypeptides. The effect of penetratin and hyperthermia on polypeptide uptake was determined by flow cytometry. Cells were treated for 1 h at 37°C or 42°C with fluorescein-labeled polypeptides (18 amol/L) and immediately analyzed after treatment. Results are expressed as fluorescence relative to standard beads and corrected for labeling efficiency. Columns, mean of three to five experiments (n = 5,000 cells); bars, SE.
immediately after incubation at 37°C with Pen-ELP1-H1-fluorescein or Pen-ELP2-H1-fluorescein is at least 3-fold greater than that of cells incubated with ELP1-H1-fluorescein or ELP2-H1-fluorescein. The higher fluorescence intensity of Pen-ELP1-H1-fluorescein–treated cells as compared with Pen-ELP2-H1-fluorescein–treated cells at 37°C is most likely due to the initial Pen-ELP1-H1 phase transition.

Previous studies have shown that cellular uptake of thermally responsive elastin-like polypeptide was significantly enhanced by the thermally triggered phase transition of the polypeptide (36). The magnitude of the increase in cellular uptake of elastin-like polypeptides in response to hyperthermia was also shown to be cell line dependent. To investigate whether hyperthermia enhances the uptake of Pen-ELP-H1 in MCF-7 cells, we measured the fluorescence intensity of Pen-ELP-H1-fluorescein–treated cells as a function of solution temperature. As shown in Fig. 2, the uptake of Pen-ELP-H1 increased 13-fold when the cells were heated to T > T_{c}, as compared with cells at 37°C. Hyperthermia itself may affect cellular processes including cellular uptake (37). Therefore, we wanted to discriminate the effect of heat in stimulating the cellular uptake from that of the thermally triggered phase transition of Pen-ELP-H1. In a control experiment, we therefore determined the uptake of a fluorescein-labeled thermally nonresponsive control polypeptide, Pen-ELP2-H1. As shown in Fig. 2, the uptake of Pen-ELP2-H1 in cells heated to 42°C was similar to uptake in nonheated cells, indicating that hyperthermia itself does not affect uptake of these polypeptides in cultured cells. In summary, these results show that the enhanced cellular uptake of the thermally responsive Pen-ELP-H1 in heated cells is not the result of nonspecific effects of hyperthermia, but rather it is attributable to its hyperthermia-triggered phase transition.

**Internalization and Intracellular Localization of Polypeptides**

In an effort to elucidate the basis of the enhanced uptake of thermally responsive polypeptides, we examined the cell fluorescence intensity and cellular localization of the polypeptides with time after a 1-hour treatment in heated and nonheated cells. Figure 3A shows that the mean fluorescence intensity obtained by flow cytometry of cells treated with Pen-ELP1-H1 at 37°C increases slightly just hours after treatment, then undergoes a slow decline over 24 hours. Cells treated with the same concentration of ELP1-H1 at 37°C showed at least 8-fold lower fluorescence intensity at all time points. However, regardless of the difference in the amount of polypeptide associated with the cells, the patterns of cell staining by ELP1-H1 and Pen-ELP1-H1 were remarkably similar (Fig. 3B). Both ELP1-H1 (top left) and Pen-ELP1-H1 (bottom left) seemed to be associated with the plasma membrane immediately after the 1-hour treatment. Twenty-four hours later, both ELP1-H1 (top right) and Pen-ELP1-H1 (bottom right) displayed a punctate, cytoplasmic staining pattern. This data implies that elastin-like polypeptides with or without the penetratin sequence are initially attached to the cell surface and consequently internalized by the cells. However, as evidenced by flow cytometry, the presence of penetratin greatly enhances the initial attachment of the elastin-like polypeptide to the cell surface, and consequently leads to the uptake of greater quantities of the elastin-like polypeptide polymer.

We also examined the effect of hyperthermia on the internalization of fluorescein-labeled Pen-ELP1-H1 and Pen-ELP2-H1 polypeptides. First, we compared the mean fluorescence intensity of cells incubated with Pen-ELP1-H1 for 1 hour at 37°C with that of cells incubated at 42°C. As shown by the mean cell fluorescence, the hyperthermia caused a large increase in the amount of polypeptide associated with cells (Fig. 3C). The increased cell fluorescence seen at 42°C can be attributed to polypeptide aggregates (Fig. 3D, bottom). Initially after treatment, all polypeptides were localized to the cell plasma membrane (Fig. 3D, top left: 37°C, bottom left: 42°C). After 24 hours, all polypeptides, including the large, hyperthermia-induced aggregates, are internalized into the cytoplasm (Fig. 3D, top right: 37°C, bottom right: 42°C).

We also conducted control experiments with the thermally nonresponsive Pen-ELP2-H1 to discriminate hyperthermia effects from effects of the phase transition. As shown in Fig. 3E, cells treated with Pen-ELP2-H1 showed a similar level of fluorescence at 37°C and 42°C with a slow decay over 24 hours. At both temperatures tested, Pen-ELP2-H1 showed a similar staining pattern to Pen-ELP1-H1 at 37°C (Fig. 3F, top: 37°C, bottom: 42°C). Hyperthermia did not have any visible effect on the internalization or distribution of this nonthermally responsive control polypeptide.

Finally, the polypeptide staining pattern was observed 11 days after treatment, which marks the end point for cell proliferation studies. At this time point, cells still show the punctate, cytoplasmic staining seen at 24 hours. However, no large aggregates were observed 11 days later (Fig. 3G).

**Effect of Polypeptides on Cell Proliferation**

To examine the antiproliferative effects of these polypeptides, MCF-7 cells were exposed to polypeptides for 1 hour at 37°C or 42°C. The polypeptides were washed away, and the cells were allowed to grow until day 11, when they were counted using the trypan blue dye exclusion assay. Figure 4A shows the effect of Pen-ELP1-H1 concentration on cell proliferation 11 days after a 1-hour polypeptide exposure at 37°C. A concentration-dependent decrease in cell number was observed. Because cells were treated at 37°C and we expected an additional effect of hyperthermia, we chose 18 μmol/L as a sufficient concentration for more detailed studies of polypeptide-mediated inhibition of cell growth. Figure 4B shows growth curves of the MCF-7 cells following a 1-hour exposure at 37°C to Pen-ELP1-H1 and of control cells. Control cells made ~6 doublings at day 11. After the same length of time, Pen-ELP1-H1–treated cells had only doubled ~5 times. Finally, cell proliferation in response to all control polypeptides with and without hyperthermia was examined 11 days after a 1-hour treatment. Figure 4C shows no inhibition of cell growth by polypeptides lacking either the penetratin or the H1 sequences. The
thermally sensitive polypeptide Pen-ELP1-H1 inhibited cell proliferation by 35% when cells were treated at 37°C. However, when cells were treated at 42°C, cell growth was inhibited by 70%. The antiproliferative effect of the nonthermally responsive polypeptide Pen-ELP2-H1 was temperature independent, and it inhibited cell growth by about 25%. These results suggest that the polypeptides exhibit an antiproliferative effect in MCF-7 cells that can be further enhanced by hyperthermia.

Effect of Pen-ELP-H1 on c-Myc Localization and Transcriptional Function

To investigate the mechanism of inhibition of cell proliferation by Pen-ELP1-H1, we examined the cellular localization of c-Myc and its dimerization partner Max by confocal immunofluorescence microscopy in Pen-ELP1-H1–treated and untreated cells. Figure 5A (top) shows that, in untreated cells, Max displays primarily a nuclear distribution exclusive of nucleoli (FITC channel, green). A similar distribution is also observed for c-Myc (Cy5 channel, red), which is consistent with previous studies (38, 39). The overlay of images of Max and c-Myc (top, third figure) shows nuclear colocalization of these two proteins, as indicated by the yellow color. The localization of c-Myc and Max is unchanged when cells are treated with Pen-ELP1 (middle). Treatment of cells with Pen-ELP1-H1 did not change the intracellular distribution of Max (bottom, first figure). However, Pen-ELP1-H1 treatment caused redistribution of c-Myc from predominantly nuclear to cytoplasmic localization, thus preventing colocalization of Max and c-Myc (bottom, second and third figures).
Because c-Myc-Max heterodimerization is required for c-Myc transcriptional activity, blocking the c-Myc-Max interaction may be an effective mode of inhibiting transcription of c-Myc responsive genes. The ability of Pen-ELP1-H1 to inhibit transcriptional activity of c-Myc was evaluated by assaying the mRNA expression of genes known to be direct targets of c-Myc. c-Myc responsive genes displaying c-Myc-Max binding sites in their promoters include ODC (40) and LDH-A (41). Expression levels of ODC and LDH-A mRNA from treated and untreated cells were analyzed by reverse transcription-PCR and compared with the expression of GAPDH, a gene not regulated by c-Myc. The control polypeptide Pen-ELP1 did not show any inhibition of c-Myc transcriptional activity (Fig. 5B, lane 2). The control polypeptide ELP1-H1 also showed no significant effect (lane 3). In contrast, Pen-ELP1-H1 led to a strong decrease in mRNA expression of ODC and LDH-A (lane 4). GAPDH mRNA levels were unaffected by the polypeptide treatments.

Discussion

Previous in vivo studies have suggested that elastin-like polypeptides are potentially useful as macromolecular drug carriers for thermally targeted delivery. This approach may be applied to a variety of therapeutic molecules that can be conjugated to these polypeptides. By exploiting the phase transition–induced aggregation of these polypeptides, this method potentially provides a new modality to target polymer-drug conjugates to solid tumors. Classic approaches rely on chemical synthesis to attach therapeutic molecules to polymer carriers (4, 42). However, in this study, we take advantage of the fact that the elastin-like polypeptide polymer is genetically encoded, and we introduce a therapeutic peptide using simple molecular biology techniques. This approach allows expression of the polypeptide in bacteria and purification of large quantities of the molecule by simple thermal cycling (25). In the present study, the coding sequence for a peptide inhibitor of c-Myc was incorporated into the elastin-like polypeptide gene. We report here the design and synthesis of this novel class of thermally responsive elastin-like polypeptides, which is capable of inhibiting the transcriptional function of c-Myc and the proliferation of cancer cells.

The plasma membrane of eukaryotic cells is generally impermeable to therapeutic macromolecules, such as oligonucleotides and proteins, due to the large size and inherently poor penetration capabilities of these molecules. To overcome these limitations and allow easy, noninvasive delivery of polypeptides to their appropriate intracellular molecular target, we fused the coding sequence for penetratin to the polypeptide encoding sequence. Penetratin has been used as a “leading edge” to carry enzymes and proteins into cells (24). As reported in Results, the penetratin sequence markedly enhanced cellular uptake of the thermally responsive polypeptides, thus confirming what had been already reported for different penetratin fusion proteins.

Confocal fluorescence microscopy confirmed a similar localization pattern for elastin-like polypeptides both with and without the penetratin sequence. Both proteins were initially bound at the plasma membrane, and 24 hours after treatment showed punctate, cytoplasmic staining. Taken together, the flow cytometry and confocal microscopy suggest that the effect of the penetratin sequence is to significantly enhance the amount of protein delivered into the cells by increasing the initial attachment of elastin-like polypeptides to the cell surface.

We have shown that cellular uptake of the thermally sensitive Pen-ELP1-H1 is enhanced in heated cells compared with the uptake of the same polypeptide without heat or compared with the thermally nonresponsive control Pen-ELP2-H1. The enhanced uptake of Pen-ELP1-H1 by heated cells is most likely the result of increased uptake of Pen-ELP1-H1 aggregates, formed due to its hyperthermia-induced phase transition. This hypothesis is supported by the observation that fluorescent aggregates of labeled polypeptides were present on the cell surface of heated cells immediately after incubation of the cells with the
polypeptides (observed by confocal microscopy analysis of the cell surface; data not shown). Similar aggregates were observed within the cytoplasm 24 hours later. Aggregation of Pen-ELP1-H1 is reversible, even within the cells, because large fluorescent aggregates were not observed 11 days later. We have also shown that cells observed 11 days after polypeptide treatment appeared to have a normal morphology (Fig. 3). This is consistent with the observed mechanism of inhibition by the H1 peptide, in which the growth rate is slowed.

To better understand the mode of action of Pen-ELP-H1, we examined the cellular localization of c-Myc and Max by confocal immunofluorescence microscopy in treated and untreated cells. c-Myc and Max are nuclear proteins that have been shown to associate in vivo (43), and the resulting heterodimer is an important transcription factor for cell growth and proliferation. Using immunoprecipitation experiments, Giorello et al. (21) have shown that the 14-amino-acid peptide H1-S6A,F8A is capable of blocking the interaction between purified c-Myc and Max fragments in vitro. Here, in cell culture experiments, we have shown that the H1-S6A,F8A peptide fused to Pen-ELP prevents the colocalization and consequently the heterodimerization of c-Myc and Max. Furthermore, we show that this inhibition leads to reduction in the mRNA levels of the c-Myc-Max–controlled genes ODC and LDH-A, which is consistent with the results of Giorello et al. (21).

The redistribution of c-Myc to the cytoplasm and the down-regulation of c-Myc–controlled genes led us to propose the model illustrated in Fig. 6. Mitogen-induced cell proliferation stimulates production of c-Myc mRNA. c-Myc protein is translated in the cytoplasm, but before it is imported into the nucleus, it is sequestered by the cytoplasmically localized Pen-ELP-H1. The nascent c-Myc cannot enter the nucleus, therefore leaving Max without a binding partner. The result is the down-regulation of c-Myc-Max–controlled genes. The c-Myc protein has a high turnover rate (43), which explains why c-Myc staining is exclusively in the cytoplasm only 24 hours after treatment. Without a functional c-Myc-Max heterodimer, the processes of cell growth and cell proliferation are inhibited.

It has been shown that the c-Myc expression level is linked to proliferation of MCF-7 cells (44), making them an ideal cell line for this study. Because c-Myc heterodimerization with Max activates transcription of target genes involved in cell cycle progression and cellular transformation (45, 46), inhibition of c-Myc transcriptional activity seems to be a powerful method of inhibiting MCF-7 cell growth. Inhibition of cell growth by the H1 peptide in MCF-7 cells was originally reported by Giorello et al. (21). Although Giorello et al. treated cells continuously for 11 days with frequent addition of fresh H1 peptide, we achieved similar inhibition of cell growth with a single 1-hour exposure to the ELP-fused H1 peptide. Furthermore,
here we have shown that the antiproliferative effect of Pen-ELP1-H1 can be enhanced by hyperthermia. We believe that cell proliferation can be inhibited even more efficiently by different treatment schedules and/or systemic exposure to polypeptide combined with hyperthermia treatments. Current studies are under way to optimize conditions for more efficient inhibition of cell growth in an animal model.

We have also observed the ability of Pen-ELP1-H1 to slow the growth rate of HeLa and OVCAR-3 cells, demonstrating that inhibition of cancer cell growth by the H1 peptide is not limited to MCF-7 breast carcinoma cells, but it may be applied to other cancer cell types.

In summary, the results presented here show that genetically engineered elastin-like thermally sensitive polypeptides can inhibit the transcriptional function of a specific oncogene. This polypeptide has a transition temperature of 39°C, making it a very good candidate for thermal targeting. Therefore, the effect of hyperthermia combined with the therapeutic effect of a polymeric drug carrier might offer further synergistic advantages in treatment of localized tumors. Finally, the polypeptide inhibited proliferation of cultured cancer cell lines, indicating that it could have antineoplastic properties in cancer diseases aberrantly expressing c-Myc or in diseases in which c-Myc activity as a transcription factor is important for cell proliferation.

Although we have chosen c-Myc as a target in this study, this approach is not limited to inhibition of c-Myc transcriptional activity. A similar approach may be applied in the design and synthesis of polypeptides with different intracellular targets, such as other cellular oncogenes and their effectors. During the past several years, small synthetic peptides have been developed that selectively inhibit various oncogenes. Because it is genetically encoded, elastin-like polypeptide may be easily applied as a targeted macromolecular carrier for any of these new inhibitors. Such thermally responsive biopolymers with antiproliferative activity would have a great potential in cancer therapy for the thermally targeted treatment of solid tumors.

References


Molecular Cancer Therapeutics

Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy

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