Isolation of a Peptide for Targeted Drug Delivery into Human Head and Neck Solid Tumors

Frank D. Hong and Gary L. Clayman

The Departments of Head and Neck Surgery (F. D. H., G. L. C.) and Cancer Biology (G. L. C.), The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Lack of tumor specificity remains a major problem with chemotherapies in that side effects prevent the delivery of dosages of drugs that are required to eliminate tumors. In this report, we describe the isolation of a 12-mer peptide (HN-1), with \( \sim 1\% \) of the mass of typical antibodies, that meets several criteria for targeted drug delivery into a solid tumor. First, internalization of HN-1 by human head and neck squamous cell cancer (HNSCC) cells suggests that HN-1 is capable of translocating drugs across cell membranes. Second, HN-1 appears to be HNSCC-specific, given its reduced uptake by nonmalignant human oral keratinocytes and other types of human cells, its preferential binding to primary HNSCC, and its localization to HNSCC-derived xenografts. Third, the presence of HN-1 within HNSCC xenografts suggests that it is capable of penetrating tumor tissues. Our results establish the utility of tumor-specific peptides for targeted drug delivery into solid tumors.

Introduction

Over the past three decades, systemic chemotherapy has evolved as a major therapeutic modality in HNSCC including approaches or organ preservation, management of recurrent disease, and concomitant chemotherapy and radiotherapy approaches (1). However despite response rates of approximating 80% or greater, the lack of tumor specificity in these approaches produces dose-limiting toxicity that has impeded the development of curative systemic approaches as well as significant improvement in survival (2).

For solid malignancies, which comprise more than 90% of human cancers, antibodies recognizing tumor-specific antigens have provided little utility for drug delivery because the immunoconjugates cannot penetrate tumor tissue (3, 4). The development of diverse peptide libraries over the past decade has ushered in the opportunity to identify small peptides that may not be as limited as the larger antibody predecessors.

We believe that promising new therapies for HNSCC will require tumor-targeted approaches that afford tumor specificity and limited toxicity. Here we describe the isolation and identification of a novel peptide, HN-1, a 12-mer peptide with \( \sim 1\% \) of the mass of typical antibodies, which may provide the foundation for such an intervention strategy.

Materials and Methods

Cell Lines. All HNSCC cell lines used in this study were established at M. D. Anderson Cancer Center. MDA Tu167 and MDA Tu177 are both moderately differentiated squamous carcinoma cell lines derived from primary tumors of the floor of the mouth and the larynx, respectively. Both are tumorigenic in nude mice. DU148 and SW480 cell lines were obtained from American Type Culture Collection (Manassas, VA), and normal human fibroblast AG04354 was obtained from Coriell Cell Culture Facility (Bethesda, MD). The above cell lines were maintained in DMEM/F-12 medium with 10% fetal bovine serum, 2 mm l-glutamine, and antibiotics at 37°C in 5% CO₂.

Immortalized human oral keratinocytes HOK16B was maintained in keratinocyte-serum-free media 17005-042 (Life Technologies, Inc.; Bethesda, MD) supplemented with epidermal growth factor and bovine pituitary extract. Peptide-Display Library Screening. Plaque-forming units (2.5 \( \times 10^7 \)) of M13 phage peptide library PhD-12 (New England BioLabs, Beverly, MA) were incubated with 5 \( \times 10^7 \) MDA167Tu cells in growing medium at 37°C in 5% CO₂ for 3 h. Internalized phages were recovered by lysing with Triton X-100 (1%) detergent and amplified. Although Triton X-100 could not lyse the nuclei, ionic detergents capable of disrupting nuclear membrane were avoided because they inactivate phages. To eliminate phages that became internalized after interacting with constitutively expressed molecules, the isolated phages were subtracted using NHFs. Recovered phages were subjected to five rounds of MDA167Tu selection successively. These selections were then followed by three rounds of NHF subtraction in succession. DNA sequencing of 12 finally selected phages revealed that they encoded an identical peptide. MDA167Tu cells exhibited 10.3-fold greater internalization potential for TSPLNIHNGQLK-phage than NHFs. Basic alignment search tool (BLAST) search revealed no homology with existing libraries.

Gel Electrophoresis. Cell lysates suspended in Sample buffer [0.1 M Tris (pH 6.8), 2% SDS, 20% glycerol, and 10% 3-mercaptoethanol] were separated by 15% SDS-PAGE and viewed using UV light (5). The images were captured digitally using Kodak Digital Science 1D software.

Peptide Synthesis. Peptides were synthesized and purified by reverse-phase high-performance liquid chromatography to >95% purity (Research Genetics, Huntsville, AL). A fluorescent label was added at the NH₂ terminus, and the COOH-terminus was capped with an amide group. Mass spectrometry confirmed the predicted mass. Peptides were further purified by gel electrophoresis, excised, dialyzed, lyophilized (in the dark), resuspended in PBS, and filter sterilized.

Fluorescence Microscopy. Cells were fixed with 3% formaldehyde, were mounted using Anti-Fade (Molecular Probe; Eugene, OR), and viewed using a Nikon fluorescence microscope Eclipse E400. Images were captured digitally and analyzed using Metamorph version 3.6a software.

To determine fluorescence intensity, the extent of autofluorescence was subtracted from the observed intensity.

Competition Assay. MDA177Tu cells were incubated with FITC-HN-1 in the presence of 200X molar excess of unlabeled HN-1, a specific competitor, or unlabeled irrelevant peptide (GIGKFLHSAKKFGKAFVGEIMNS), a non-specific competitor.

Subcellular Fractionation Study. Subcellular fractionation was performed as described previously (6). Isolation of nuclear, cytoplasmic, and cell membrane fractions was confirmed by Western blot analysis using antibodies specific for human retinoblastoma protein, glutathione transferase, and GLUT1 glucose transporter protein, respectively (not shown). Individual fractions were electrophoresed and viewed. An equivalent amount of each fraction was loaded.

Received 7/21/00; accepted 10/17/00.
Protease Protection Assay. Peptide-incubated cells were rinsed with PBS, scraped, pelleted by centrifuging at 2000 rpm, and resuspended in 100 μl of PBS. To lyse, cells were freeze-thawed 10 times using dry ice. After treating with chymotrypsin (10 units) for 5 min at 25°C, the enzyme was inactivated with SDS (1%). Samples were suspended in Sample buffer, electrophoresed, and viewed. No peptide was detected in cell extract when incubated with chymotrypsin-pretreated peptide (not shown).

Primary Tissue Analysis. A biopsy of human invasive squamous cell cancer was rapidly frozen in optimum cutting temperature compound blocks and 4-μm-thick cryostat sections were prepared. No fixative or embedding material was used to avoid modifying molecules that may interact with HN-1. As H&E dyes fluoresced under the wavelength used for viewing fluorescein, an untreated adjacent section was incubated with FITC-HN-1. After incubating with FITC-HN-1 (2.6 μM) in PBS-GLY (PBS containing 10 mM glycine and 0.01% BSA) for 12 h at 25°C in a sealed environment, slides were rinsed in PBS-GLY for 48 h with frequent changes. Samples were mounted and viewed as described above.

Animal Experiment. Five week-old female nude mice (Harlan Sprague Dawley) purchased from Parke-Davis (Morris Plains, NJ) were injected s.c. with 5 × 10^6 tumor cells suspended in PBS. Mice harboring tumors (~0.5 cm in diameter) were randomized into separate groups (five per group), and peptides or other indicated agents (2.6 × 10^{-8} mole), suspended in 100 μl of PBS, were injected at the tail vein. All of the mice were maintained under identical conditions. After 48 h, mice were euthanized, and their tissues were recovered to prepare cryostat sections. Autofluorescence was suppressed with Eriochrome Black T (1.3%). For peptide extraction, specimens (harvested after perfusion with PBS) of equivalent mass were frozen in liquid nitrogen, pulverized, and resuspended in Lysis buffer [50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.5% NP40, and protease inhibitors]. After centrifuging to remove nuclei and other cell debris, the supernatant was electrophoresed and viewed as described above. For the extract analysis, mice were injected with 2.6 × 10^{-7} mole of FITC-HN-1. All animal protocols were reviewed and approved by the institution’s Animal Care and Use Committee.

Results

The ability to translocate across the cell membrane is critical for drug delivery, and, thus, peptides that could be internalized by cells were sought. An M13 single-stranded phage-based random peptide-display library was screened using the human HNSCC cell line MDA167Tu at 37°C to allow endocytosis to occur (see “Materials and Methods” for details). The screening of the library was performed in growth medium to ensure that the peptide isolated would not be degraded in the presence of serum during drug delivery. It led to the isolation of the novel peptide TSPLNIHNGQKL (HN-1). Its NGQ sequence resembles the NGR cell adhesion motif, but the possibility of the two motifs interacting with a common receptor appears unlikely.

![Fig. 1. Internalization of HN-1.](image)
because of the single amino acid change from asparagine to glutamine. This type of conservative substitution was similarly observed in the case of the RGD (fibronectin-binding) motif (RGD→RGE), resulting in abolished binding properties (7–9).

To mimic drug delivery, synthetic TSPLNHNGQKL peptide (HN-1) was conjugated to fluorescein, a complex organic molecule with ~44% of the molecular mass of paclitaxel (Taxol; Ref. 10). After incubation in FITC-HN-1 for 48 h, the human HNSCC cell lines that we examined (MDA138Tu, MDA159Tu, MDA167Tu, MDA686Tu, MDA1986Tu, and MDA177Tu) exhibited internal fluorescence. In contrast, little fluorescence was observed with similarly incubated human papilloma virus-immortalized normal human oral keratinocytes (Fig. 1, a and b). The fluorescence intensity was time- and dose-dependent. The distribution of cells with respect to fluorescence intensity for each cell line is shown in Fig. 1b. Internal fluorescence was also observed when the cells were not fixed, excluding the possibility of the peptide being artifactually internalized during fixing. The viability of fluorescing cells was confirmed by trypan blue
exclusion (not shown). None of the cell lines exhibited autofluorescence (see Fig. 1a for untreated MDA177Tu cells; others not shown). When the lysate of FITC-HN-1-incubated MDA177Tu cells was electrophoretically separated and viewed with UV light, intact peptide was detected (Lane 4 of Fig. 1d; Lane 3 of Fig. 1e). Degradation by an externally applied protease occurred only with prior cell lysis, confirming the internalization of FITC-HN-1 (Fig. 1d). The fact that fluorescein did not get internalized (Fig. 1a), that little dissociation of fluorescein from the peptide was detected when the medium of FITC-HN-1-incubated cells was analyzed (not shown), and that little labeling occurred after incubating with fluorescein and HN-1 separately (Fig. 1a) suggests that the possibility of the observed fluorescence being attributable to the uptake of dissociated fluorescein is unlikely.

Both the fluorescence microscopy data (Fig. 1, a–c) and the subcellular fractionation data (Fig. 1e) indicate that the internalized HN-1 was present mainly in the cytoplasm, which is consistent with the fact that HN-1-displaying phages were isolated from the cytoplasm during the screening (see “Materials and Methods”). Under higher magnification, a punctate fluorescence pattern was observed, which suggests that HN-1 may be compartmentalized after entry into the cell (Fig. 1c). The punctate pattern is similar to that previously observed with internalized epidermal growth factor, which enters via receptor-mediated endocytosis (11).

Internalization of HN-1 was also observed after it was conjugated to Texas Red (Fig. 1a). Because fluorescein (Fig. 1a) and Texas Red (not shown) are impermeable, the dyes themselves could not have mediated the internalization of HN-1. Shifting the relative position of HN-1 with respect to the peptide [GGGTSPNLIHGQKLGGGS (HN-2) or GSRRASVTSPNLIHGQKL (HN-3)] did not inhibit its internalization (not shown), but jumbling the sequence did [NQHS-KNTLLIGP (HN-J); Fig. 1a, panel 3], which suggests that HN-1 internalization is “position-independent” but “sequence-dependent.” The uptake of FITC-conjugated HN-2 or HN-3 excludes the possibility that the ability of HN-1 to enter cells is a property acquired through its juxtaposition with fluorescein.

To determine whether HN-1 internalization occurs specifically, a competition assay was performed. Whereas the internalization of FITC-HN-1 was blocked by unlabeled HN-1 when provided in excess, no such inhibition occurred with an irrelevant peptide (Fig. 1f). This suggests that HN-1 uptake may require a specific interaction with a heterologous molecule, which may be a cell-associated molecule or a molecule present in the growth medium. The latter possibility, however, appears unlikely given that HN-1 internalization also occurred in PBS (not shown).

Intriguingly, DU145 human prostate, SW480 human colon, or U373 MG human astrocytoma cells displayed little fluorescence (Fig. 1b), even after a prolonged (96–120-h) incubation with FITC-HN-1,

Fig. 3. HN-1 localizes to HNSCC-derived xenograft. a, fluorescence microscopy. Cryostat section of MDA177Tu- or DU145-derived xenograft resected from a mouse treated with the indicated agent. H&E staining of a representative tumor is shown. b, electrophoretic analysis. Tumor or normal tissue extract from a mouse harboring MDA167Tu-derived xenograft that was treated as indicated. c, fluorescence microscopy. Various normal tissues from a mouse with MDA177Tu-derived xenograft that was treated with FITC-HN-1. H&E staining of the corresponding views are shown. In all of the experiments, animals were treated with the indicated agent for 48 h. Bar (μm), 92 (a); 48 (c).
which indicated that HN-1 uptake does not occur ubiquitously. That the lack of its uptake was not attributable to the degradation of peptide in the medium was independently confirmed (not shown). The results also suggest that not all actively dividing cells can internalize HN-1.

An in situ peptide-binding assay performed on cryostat sections prepared from a biopsy sample of human invasive HNSCC, which contained invasive malignant cells as well as adjacent nonmalignant squamous epithelium, showed the preferential binding of FITC-HN-1 to invasive cancer cells (Fig. 2). The inability of fluorescein or FITC-HN-J to bind (Fig. 2) suggests that the binding of FITC-HN-1 was mediated by HN-1.

To determine whether HN-1 localizes to tumor, nude mice harboring s.c.-established MDA177Tu-derived tumor xenografts were injected i.v. with FITC-HN-1. Cryostat sections of the tumors resected after 48 h showed fluorescing tumor cells (Figs. 3a and 4b). As in in vitro, little labeling of the nuclei was observed in vivo. The untreated tumor cells did not autofluoresce (Fig. 3a). The presence of intact peptide was confirmed by the electrophoretic analysis of tumor extract (Fig. 3b). Inasmuch as the latter was prepared from a FITC-HN-1-injected mouse harboring MDA167Tu-derived xenograft, it suggested that HN-1 can be internalized by xenografts derived from multiple HNSCC cell lines. Little labeling of tumor cells was observed after injecting equimolar concentrations of fluorescein, FITC-HN-J, or fluorescein and unlabeled HN-1 separately into mice with size-matched tumors (Fig. 3a). FITC-HN-1 failed to label xenografts derived from prostate cancer cell line DU145 cells (Fig. 3a), which poorly internalizes the peptide in vitro (Fig. 1b). Importantly, brain, heart, lung, kidney, and liver from FITC-HN-1-injected tumor-bearing mice showed little labeling (Fig. 3, b and c). A similar result was also obtained with FITC-HN-1-injected tumor-free mice (not shown), which suggested that the inefficient labeling was not attributable to peptide depletion by the tumor. The alternate possibility of its being attributable to rapid peptide degradation in these tissues or attributable to the inability of the peptide to recognize the murine homologue of its cognate receptor cannot be excluded.

To determine whether HN-1 did infiltrate the tumor tissue, histological sections prepared from the center of the xenograft of FITC-HN-1-injected mice shown in Fig. 3a, panel 6, were examined. H&E staining showed that the lower half was comprised of dispersed tumor cells, whereas the remainder contained tumor cells compartmentalized (Fig. 4b) that resembled well-differentiated human HNSCC (11). An adjacent section from the treated mice showed fluorescing tumor cells (Fig. 4b). [The fluorescence was attributable to tumor cells, not keratins (compare panels 2 and 3 of Fig. 4b).] Fluorescence appeared ubiquitous, because the tumor cells that were located centrally as well as peripherally fluoresced (Fig. 4b). Labeling of tumor cells located at the interior suggests that FITC-HN-1 is capable of penetrating tumor tissues. Histological sections prepared from other points throughout the tumors from the treated mice also showed diffuse and homogeneous fluorescing tumor cells (not shown).
Discussion

We used a “panning” technique to screen a large peptide library to identify a 12-mer peptide (HN-1) capable of systemically localizing and internalizing into HNSCC cell lines in vitro and within a xenograft mouse model. The utility of this approach in this setting suggests the potential application of this approach toward other malignancies as well.

This study lacks data regarding in vitro internalization and in situ binding assays for squamous cell carcinoma derived from other organ sites including the lung, cervix, esophagus, or skin. The potential of HN-1 to internalize in other squamous malignancies will be further investigated to determine whether this approach may have other far-reaching considerations.

We believe that HN-1 represents a novel peptide and that multiple data suggest that its internalization may be receptor mediated. First, internalization of labeled HN-1 is specific as demonstrated by competition assay. Second, HN-1 entry also occurs in serum free conditions (PBS), which suggests that the interacting molecule of HN-1 is not present in growth medium. Lastly, shifting the position of individual amino acids of HN-1 with respect to the overall peptide did not affect its internalization. All of the above data suggest that HN-1 may require specific interaction with a cognate cellular receptor. The exact sequence that mediates this interaction is currently not known and will require additional analysis.

Previously, it was shown that drugs conjugated to tumor vasculature-specific peptides could eliminate tumors indirectly by destroying endothelial vessels (7). However, because tumors smaller than 1 mm³ can persist through nutrients obtained from adjacent normal blood vessels, the task of eliminating the remaining tumor still remains (12). Hopefully, our isolation of HN-1 may allow physicians to provide the necessary dose of a drug to destroy tumors without being restricted by the occurrence of harmful side effects to other cells. The potential of HN-1 as a shuttle is further strengthened by the fact that it is nontoxic (no histological evidence of organotoxicity was observed in HN-1-injected mice), nonimmunogenic in mice (data not shown), stable in vivo, protects its “cargo” during transit, and accumulates efficiently within the tumor in 48 h. As a result, efforts to conjugate paclitaxel, the most potent chemotherapeutic for treating HNSCC, to HN-1 are currently in progress. If the internalized HN-1 is compartmentalized in endosomes, the release of conjugated drugs to cytosol may need to rely on the endosomal degradation of the peptide (13). Other potential uses of HN-1 may include tumor diagnosis, imaging, or radioablation. It may also provide tumor-specificity to gene transfer approaches (14); this is supported by our observation that HN-1 can enhance the transfer of liposome-DNA complexes into HNSCC cells.⁴

Acknowledgments

We thank A. El-Naggar for pathological core support and K. Briggs and P. Holton for multiple laboratory efforts. We also express our sincere appreciation to Guang P. Xu for his technical assistance and expertise in the animal studies including tail-vein injections.

References

4. Unpublished data.

⁴ Unpublished data.
Isolation of a Peptide for Targeted Drug Delivery into Human Head and Neck Solid Tumors

Frank D. Hong and Gary L. Clayman


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/23/6551

Cited articles
This article cites 13 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/23/6551.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/23/6551.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.