CHAPTER 8

UVEAL MELANOMA ASSOCIATED PEPTIDES IDENTIFIED WITH A PHAGE DISPLAY LIBRARY AND IN VITRO PANNING

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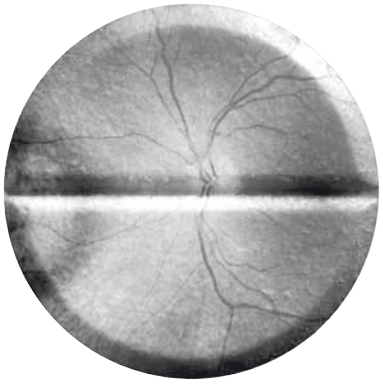

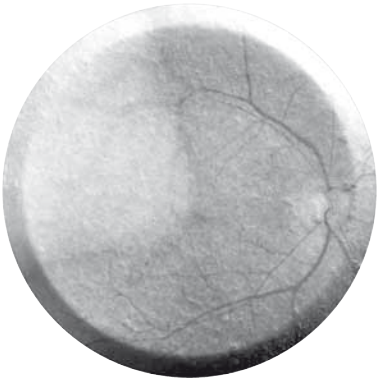

ABSTRACT

Introduction. Uveal melanoma (UM) is the most common primary intraocular tumor, in which still 50% of patients may die due to metastatic disease. Specific targeting of UM for imaging and delivery of drugs is urgently needed for improved identification and subsequent killing of tumor cells. Ligand-specific antigens that are preferentially expressed by primary or metastatic UM cells are ideal targets for tumor-targeting applications.

Material and Methods. UM cell lines were screened in vitro for tumor-specific peptides using a phage-displayed peptide library. Binding and internalization of peptides to cell lines was visualized using fluorescein and rhodamine-labeled synthetic peptides and confocal microscopy.

Results. Phage display experiments resulted in the identification of three UM-associated peptides (UMAPs). After labeling with fluorescein or rhodamine, internalization of these peptides in several UM cell lines was visualized by confocal fluorescent microscopy. One peptide, UMAP1, was identified multiple times in phages that had bound to metastatic cell lines, while UMAP2 showed homology to the insulin-like growth factor 1 receptor (IGF-1R).

Discussion. Three UMAPs were identified by screening phage peptide libraries and shown to successfully internalize in targeted UM cells. UMAP1 may have potential for future usage for UM-targeted treatment. UMAP2 is especially interesting because of its homology to the IGF-1R, which is a bad prognostic marker in UM.

INTRODUCTION

Isolation of tumor-specific ligands has been extensively researched in this last decade, as specific tumor agents can be used for in vivo imaging and thus for early detection of tumors and micrometastases. In case of uveal melanoma (UM), the most common intraocular tumor in adults, these micrometastases are believed to play an important role: in spite of an effective treatment of primary UMs by enucleation or radiotherapy, with a local tumor control rate of 97% of all treated cases [1-3], still half of all patients may die due to UM-related metastatic disease [4,5]. The occurrence of metastases has even been described 35-40 years after initial diagnosis and treatment of the primary UM tumor [5,6]. It has been proposed that dissemination occurs several years before identification of the primary tumor and its treatment [7]. Non-invasive detection of dormant micrometastases using specific peptides coupled to a fluorescent probe or a radionuclide and subsequent treatment with specific tumor ligands that are conjugated to an anticancer drug may improve survival. Specific peptide-based agents that achieve apoptosis, or block angiogenesis and proliferation of UM metastatic tissue, would be of great value in sparing healthy tissue.

For this purpose, various melanoma-derived autoantigens and cell surface receptors have been explored in uveal melanoma. Cutaneous melanoma markers such as MAGE are not widely distributed in ocular melanoma tissue [8]. For histopathologic diagnosis of uveal melanoma, antibodies such as S-100 (specific for a protein derived from bovine brain cross-reacting with melanoma and melanocytes), HBM45 (specific for gp100) and A103, (recognizes the Melan-A/Mart-1 protein) are used [9-11].

Unfortunately, most of these receptors are not UM specific and are also expressed by normal cells (melanocytes). Therefore, tartgeting these receptors may cause significant side-effects.

Phage display is a powerful technique for the isolation of peptides that bind to a particular target with high affinity and specificity. In contrast to larger molecules, such as proteins and antibodies, small peptides can efficiently penetrate tissues and are relatively easy to synthesise. Several studies describe the successful detection of organ and tumor-specific peptides using phage peptide libraries, for example in bladder cancer, brain and kidney tissue [12,13]. Using phage display, Howell et al. [14] have reported the identification of heptapeptides that specifically bind to human tumor melanin, as demonstrated in human metastatic melanoma tumor-bearing nude mice. These heptapeptides may be used as a tool in targeted therapy for (metastatic) melanoma [14]. In addition, phages alone are also known to be highly immunogenic. Treatment of B16-F10 melanoma in mice with tumor specific (selected with phage display) phages induced a massive infiltration of neutrophils, which delayed tumor growth and increased survival [15].

Using in vitro biopanning on (metastatic and primary) UM cells, we report the identification of UM associated peptides (UMAPs) selected from phage display libraries. Additionally, we demonstrate the potent binding and internalisation of fluorescein and rhodamine-conjugated synthetic peptides in UM cells.

MATERIAL AND METHODS

Cell Lines

Four UM cell lines were used as targets in biopanning experiments. Mel270 and 92.1 are primary tumor-derived cell lines, while OMM1 and OMM2.5 are cell lines derived from metastases. Mel270 and OMM2.5 were provided by dr. B. Ksander (Schepens Eye Research Institute, Boston, MA), OMM1 by dr. G.P.M. Luyten (Erasmus University Medical Center, Rotterdam, the Netherlands) and 92.1 was established in our laboratory [16-20]. All cell lines were cultured in complete RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin/streptomycin (GIBCO, Life Technologies, Paisley, UK) at 37°C and 5% CO_2 (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). The cells were passaged once or twice a week using trypsin (0.05%).

Mel1, a normal melanocyte culture established in our own laboratory, was used for pre-adsorption (diminishing tumor aspecificity in subsequent incubation rounds) and as a control for internalisation experiments. In addition, HUVECs (human umbilical vein endothelial cells) and SAOS-2 (primary osteosarcoma cells) served as controls in internalisation assays.

Biopanning

M13 phage display libraries containing random hepta- and dodecapeptides fused to the N-terminus of minor coat protein pIII (Ph.D.-C7C and Ph.D.12 Phage Display Peptide Library, New England Biolabs, Ipswich, MA, USA) were used for in vitro experiments.

Biopanning was performed for three to four rounds, starting with an equal mixture of 10^{11} pfu of each library, with and without pre-adsorption on normal melanocytes (Mel1). In case of pre-adsorption, the mixed Ph.D.-C7C and Ph.D.12 libraries were first incubated with each of the four UM cell lines. The selected phages were amplified and subjected to pre-adsorption on a Mel1 cell monolayer, grown in a T25 tissue culture flask (Corning, NY), incubated with phages ($2x10^{-11}$ pfu) for 1 h at 37°C for melanocyte-specific phage depletion. Subsequently, the medium, including phages, was transferred to 90-100% confluent UM monolayers in T75 flasks and incubated again for 1 h at 37°C. After incubation, cells were washed five times with 10 ml of phosphate-buffered saline (PBS) and treated with trypsin (0.05%) for 10 min. at 37°C to inactivate non-internalized phages. Subsequently, 10 ml of RPMI was added and the cells were harvested in a 15 ml tube and spun down. Cell pellets were washed another

3-5 times with PBS. Internalized phages were recovered by lysing the cells in 1 ml of cell culture lysis buffer (Promega, Benelux) for 15 min. Phages were precipitated by addition of 250 µl of a 20% PEG8000/ 2.5 M NaCl solution and overnight incubation at 4°C. Pellets were resuspended in 100 µl TBS. The internalized phages were amplified and subjected to another round (round 3) of biopanning on their respective cell lines. Round 3 phages were subjected to an additional round of pre-adsorption and biopanning (round 4) on their respective cell lines. Phages from round 3 and 4 were sequenced.

Phage titering, amplification, and sequencing

Phage titers were determined after each round of selection and amplification by mixing appropriate dilutions of the phage suspension with 100 µl of an overnight culture of E.coli XL-1 Blue F^+ cells. After five minutes of incubation at room temperature, three ml of topagar was added and the mixture was plated onto LB agar plates containing kanamycin. Plaques were counted after overnight incubation at 37°C. Five to ten plaques were picked after each round, amplified and phage DNA was isolated as described by the manufacturer (New England Biolabs, Ipswich, MA, USA). Sequencing of phage DNA was performed by automated dideoxy sequencing with chain terminator dyes (LGTC, Leiden).

Phages were amplified essentially as described in the manual (New England Biolabs, Ipswich, MA, USA).except that XL-1 cells were used.

Synthetic peptides

UMAP 1 was synthesized using F-moc chemistry on a peptide synthesizer (LIC, Leiden). The lysine-residue was labeled with fluorescein using FITC (Sigma-Aldrich). The peptide was purified by HPLC, and analyzed by MALDI-TOF mass spectrometry. Rhodamine-labeled UMAP2 and 3, and fluorescein-labeled UMAP3 were purchased from the peptide synthesis facility at the LUMC (Leiden, The Netherlands).

Internalisation assay and Confocal imaging

For internalisation assays, cells were cultured on eight-chamber slides (Nalgen Nunc Int.) to ~70% confluency. Prior to addition of peptides, medium was refreshed (200 µl RPMI 1640) and 50 µl of a 0.2mM solution of peptide in 50% ethanol was added. After 30 min incubation, cells were washed 2 times with 200 µl of medium to remove unbound peptides. Images were recorded on a Leica SP1 confocal microscope equipped with an Argon laser (488 excitation and emission 500-540 nm).

RESULTS

Selection of UM cell-binding peptides

Two primary and two metastatic UM cell lines were used to select melanoma-specific phages from 7- and 12-mer random peptide phage libraries. To assess whether cell-binding phages were enriched during the panning experiments, phages eluted after each round of selection were amplified and their DNA sequenced.

After three rounds of biopanning, four peptide sequences dominated in the selected phage pool in specific cell lines as shown in Table 1. One peptide that was repeatedly amplified (9 of 10 plaques) from phages targeting the metastatic UM cell lines OMM 1 and OMM2.5, consisted of the following sequence: DLNYFTLSSKRE and was named uveal melanoma associated peptide 1 (UMAP1). This peptide was identified during biopanning without pre-adsorption onto normal melanocytes, and may thus carry melanocyte-lineage and not melanoma-specific characteristics. UMAP1 has also been reported in a liver cancer phage display experiment as Titanium Oxide binding peptide (www.freshpatents.com).

Two other potentially-interesting peptides, found after pre-adsorption by normal melanocytes, were UMAP2 (YITPYAHLRGGN) from metastatic cell line OMM2.5, and UMAP3 (ELQVMPIHIAAS) which were selected from both primary UM cell lines 92.1 and Mel270.

The sequence of UMAP2 was identified to be a homologue of the rat insulin-like growth factor receptor [21]. UMAP3 could not be linked to any known receptor or protein.

TABLE 1. Identified peptide sequences after biopanning in UM primary and metastatic cell lines.

Peptide	Sequence *	Label	Cell line of origin	Remarks
UMAP 1	DLNYFTLSSKRE *** (9/10)	FITC	OMM2.5, OMM1	Without counterselection on melanocytes
UMAP 2	YITPYAHLRGGN**(9/9)	Rhodamine	OMM2.5	Present in round 3 and 4
UMAP 3	ELQVMPIHIAAS (4/17)	FITC and Rhodamine	Mel270, 92.1	Present in round 3 and 4
	CLSYLPHKC (3/5), CFSSHPHVC*1/5) CLSYLPHKC (2/5),	n/a	OMM1, Mel270	Round 3, lost in round 4 (no clear consensus sequence in round 4)
	HYSRYNPGPHPL (3/17)	n/a	92.1	Round 3 only
	SPITISSWFPMP (3/17)	n/a		3 & 4 suspicious
	SVSVGMKPSPRP (2/17)	n./a		3 & 4 aspecific

Umap= uveal melanoma associated peptide , n/a = not applicable.
* (x/y) x plaques of total y plaques sequenced in rounds 3 and 4 have this sequence for the indicated cell lines.
homologous to rat IGF-1 receptor. * homologous to Titanium Oxide binding peptide SSWSSPITTAAV.
minor sequences NFMESLPRLGMH (92.1), CNHLNHHLC (92.1), ITSTSPMFATPP (omm1), NADNQMTWRHVL (OMM1),

TABLE 2. Internalisation capacities of synthetic labelled peptides.

	UMAP 1	UMAP 2	UMAP 3R	UMAP 3F
Mel270	+	+	+/-	+/-
OMM2.5	+	++	+	+/-
92.1	+	+	+	+/-
OMM1	+	-	+/-	-

- no internalisation,+/- moderate internalisation, + internalisation, ++ excessive internalisation
UMAP= uveal melanoma associated peptide
R= rhodamine
F= FITC

Two peptides from the PhD-C7C library with the related sequences CLSYLPHKC and CFSSHPHVC, named UMAP4 and 5, were isolated from Mel270 and OMM1 cells during round 3 only. In general, peptides from the PhD-C7C library were highly underrepresented. A peptide with the sequence HYSRYNPGPHPL dominated in the 92.1 pool during round 3, but was not retrieved in round 4. On a few occasions, peptides with the sequences SPITISSWFPMP and SVSVGMKPSPRP were selected. These peptides were considered aspecific because of their homology to a titanium-oxide binding peptide (SSWSSPITTAAV) or to the well-known target unrelated peptide (SVSVGMKPSPRP) [22]

A total of three peptides were synthesized and either labelled with fluorescein (UMAP 1 and 3) or rhodamine (UMAP 2 and 3).

Internalization of selected peptides by UM and other cells

The biopanning method is expected to favor the selection of internalizing phages. We therefore investigated whether the synthesized peptides demonstrated internalization in UM cells in vitro. UMAP1 showed a fast and efficient penetration in all UM cell lines both in the cytoplasm as well as in the nucleus, with high concentrations in the nucleolus (Table 2, Figure 1). As expected, also normal melanocytes were penetrated by UMAP1, since no negative selection against these cells had taken place. UMAP1 was not internalized by SAOS-2 and HUVEC cells.

All UM cell lines showed a similar mottled distribution of UMAP 2 and 3, which were primarily concentrated in the cytoplasm. UMAP 2 was especially internalized in the OMM2.5 cell line, from which the peptide was initially selected (Figure 2), and showed less penetration in the other UM cells. UMAP 3 only moderately penetrated cell lines Mel270, 92.1 and OMM2.5, and was not internalized by SAOS-2 and HUVEC cells. Moreover, labelling did not influence staining patterns since also fluorescein-labelled UMAP 3 showed a moderate mottled internalisation (Figure 3).

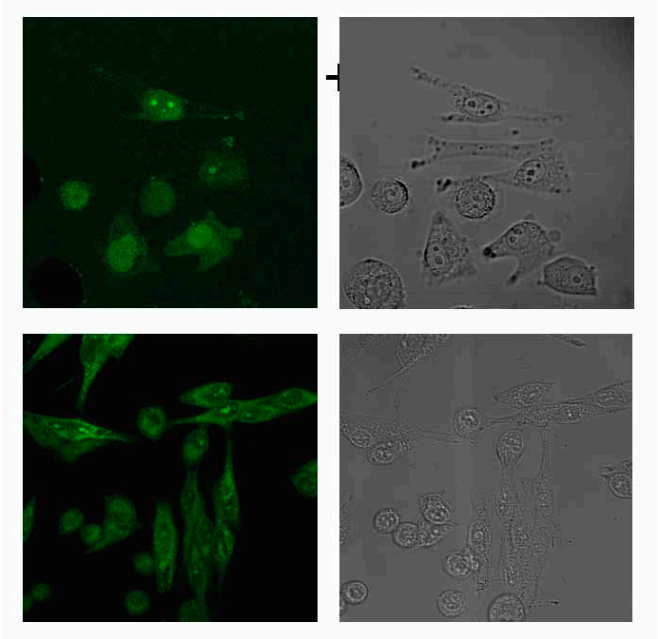

FIGURE 1. Internalization of UMAP 1.
UMAP1 showed penetration of all UM cell lines both in the cytoplasm as well as in the nucleus, with high concentrations in the nucleolus (OMM2.5 (top) and Mel 270 (bottom)). UMAP1 was not internalized in control cell lines SAOS-2 and HUVEC cells (not shown).

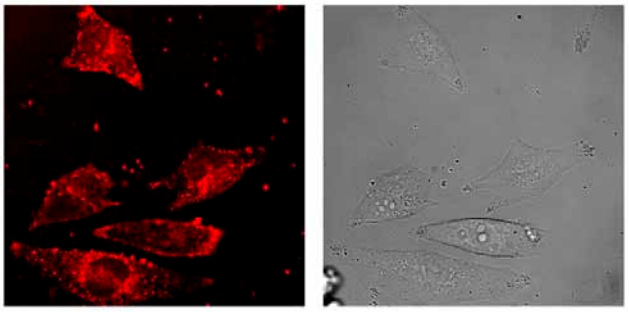

FIGURE 2. Internalization of UMAP 2.
UMAP 2 shows a mottled distribution primarily concentrated in the cytoplasm. UMAP 2 was especially internalized by OMM2.5 from which the peptide was initially selected.

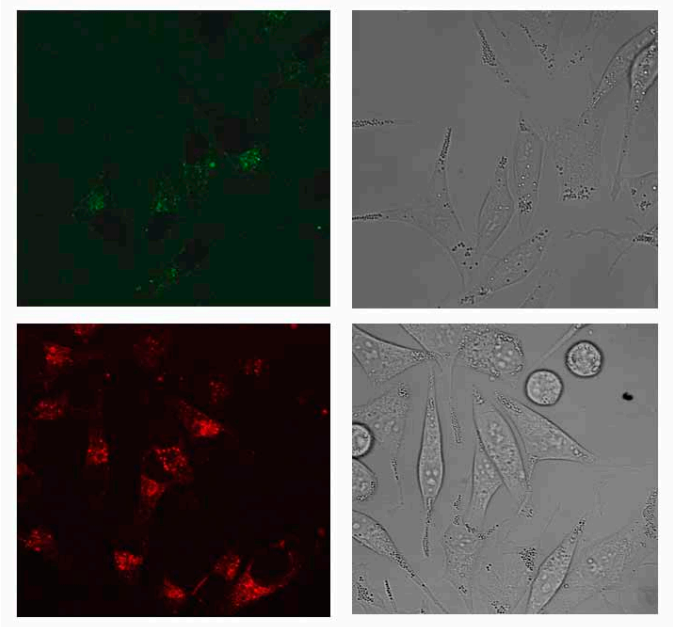

FIGURE 3. Internalization of UMAP 3 in UM cell line 92.1
Shows a mottled moderate staining with both FITC (top)and rhodamine label (bottom).

DISCUSSION

In cancer research, phage display libraries have been used widely for the identification of tumor-targeting peptides and antibodies. [23] Although UM is the most common intraocular tumor in adults, it remains a rare disease and very few reports exist of phage display experiments involving UM. We set out to identify specific UM peptides with good binding and internalization capacities and that can be used for in vivo imaging of (micro) metastases and as a tool for targeted treatment of UM primary and metastatic disease.

Four peptides were identified and subsequently amplified after several in vitro panning rounds. UMAP1 proved to be the most potent peptide, demonstrating excellent internalisation ability in primary and metastatic UM cell lines. This peptide was also internalized by normal melanocytes (due to the lack of counter-selection during biopanning). This is an unfavourable event in regard to the effect of targeted therapies on healthy tissue. However, the current most widely used treatment for UM related metastatic disease, chemotherapy, demonstrates no specificity at all [24]. In addition, UMAP1 was not internalized by SAOS-2 and HUVEC cells, demonstrating partial selectivity.

The low specificity of UMAP1 is also evident from its presence in some patents as a titanium oxide and antibody-binding peptide (www.frespatents.com). UMAP1 has also previously been selected by phage display on human embryonic stem cells [25] and heptocarcinoma cells [26]

UMAP2 showed a mild preference for metastatic cell line OMM2.5, from which the peptide was initially selected. Although internalisation was mottled, large amounts of peptide were visible in the cytoplasm of almost all cells. The sequence of UMAP2 has been identified as a homologue of the rat insulin-like growth factor receptor [21]. Interestingly, Economou et al described the expression of insulin-like growth factor-1 (IGF-1) in uveal melanoma to be associated with a bad prognosis [27,28]. IGF-1 binds to the IGF-1 receptor leading to phosphorylation of IGF-1R, which then activates key signal molecules involved in tumor transformation, maintenance of malignant phenotype, promotion of cell growth, and prevention of apoptosis [29]. IGF-1 is mainly produced by the liver, of which the metastatic cell line OMM2.5 originated (liver metastases). Since UM almost exclusively disseminate to the liver, the expression of IGFR may therefore explain the preferential liver-homing of UM cells [5]. One would expect to detect a peptide which shows homology with IGF rather than its receptor and to have human similarity. However, one should realize that the Ph.D.-12 phage library does not cover the entire landscape of all possible dodecapeptides (which is ~4x10e15) so exact homologues to human peptides will be extremely rare in the starting quantity of 1x10e11 pfu. Besides, the degree of amino acid homology between rat and human IGF receptor is 85% at the C-terminal and 98% at the tyrosine kinase domain [30]. In addition, heterodimeric hybridization of receptors have been described regarding IGFR and Insulin Receptor (IR) [31,32] which are of the same tyrosine kinase family and have a high degree of sequence similarity [33]. This phenomenon may also explain the binding and internalization of UMAP3 with UM cells.

In summary, at least three UMAPs could be identified using phage peptide libraries and in vitro panning. In addition, synthetic constructed peptides that corresponded to these UMAPs were shown to successfully internalize targeted UM cells. Whether these UMAPs can be used clinically for in vivo imaging (radiolabelled) or targeted therapy remains to be investigated.

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