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New peptide MY1340 revert the inhibition effect of VEGF on dendritic cells differentiation and maturation via blocking VEGF-NRP-1 axis and inhibit tumor growth in vivo

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ABSTRACT

The development and clinical application of immunostimulatory therapy provides us a new and exciting strategy in cancer treatment of which the agents act on crucial receptors. Given the fact that Neuropilin-1(NRP-1) is essential for vascular endothelial growth factor (VEGF) to inhibit LPS-dependent maturation of dendritic cells (DCs), it may present a potentially meaningful target in cancer immunotherapy. To explore this hypothesis, we synthesized a novel polypeptide called MY1340 consist of 32 amino acids with the aim of targeting VEGF–NRP-1 axis. Pull-down assay coupled with liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) was firstly conducted to identify NRP-1 as a potential MY1340 interacting protein, and the interaction between them was further confirmed by western blot. The competitive enzyme-linked immunosorbent assay (ELISA) results revealed that MY1340 was able to inhibit the binding between NRP-1 and VEGF with IC_{50} 7.42 ng/ml, better than that of Tuftsin, although a natural ligand reportedly specific for the NRP-1 receptor. The presence of VEGF significantly reduced the expression of human leukocyte antigen-DR (HLA-DR), CD86 and CD11C on DCs, and this effect was reverted by MY1340-augment p65 NF-κB and ERK1/2 phosphorylation. We also present evidence that MY1340 is remarkably efficacious in the treatment of mice bearing subcutaneous liver cancer and induced DC maturation in the tumor environment in vivo. Taken together, these results indicate that MY1340 may represent a potential efficient immune therapeutic compound within disease that are rich in VEGF.

1. Introduction

Neuropilin family (NRP-1, and NRP-2) are 120–140 kDa type I transmembrane proteins that were first identified their key function in mediating axonal guidance in the neural crest migration and is highly conserved throughout the vertebrate kingdom [1]. Despite the role in nervous system development, NRP-1 has also been described in many other biological processes including cell migration, neoangiogenesis and immune response [2–4]. The extracellular domain of NRP-1 contains distinct regions that enable numerous extracellular interactions and signaling pathways [5]. Known ligands include: class III and class IV semaphorins (SEMA3A/SEMA4A, respectively), VEGF and Tuftsin or Tuftsin-like peptide [6,7]. NRP-1 lacks inherent catalytic activity, therefore cannot signal on its own but via co-receptor complexes [8].

DCs, a class of professional antigen-presenting cells discovered by Ralph Steinman in 1973 which initiate innate and adaptive immune responses to maintain the homeostasis of immunity [9]. There are three generally accepted stages of DC differentiation: DC precursors, immature DC and mature DC [10]. Immature DCs located in the periphery screen foreign antigens including vi-ruses and microbial pathogens [11]. Immature DCs express characteristic inflammatory chemokine receptors (CCR1, CCR2, CCR5, CCR6 and CXCR1) and thus specialize in antigen capture [12]. They have only low levels of MHC class II expression at the cell surface and little or no expression of co-stimulatory molecules—such as CD40, CD80 and CD86 which is required to support T-cell proliferation [12]. Encounter with pathogens such as LPS leads to immature DC activation and migration to secondary lymphoid organs, and during the migration, they undergo maturation [13]. Maturation status is linked with up-regulation of MHC class II, co-stimulatory (CD40, CD80, and CD86) and adhesion molecules needed for the stimulation of T cells, and down-regulation of receptors and molecules used by immature DCs for the uptake of antigens [14].

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VEGF was originally thought to have a role in carcinogenesis mostly through their pro-angiogenic effect [15]. Recently, there has been a great deal of interest in VEGF as a strong negative modulator of DC differentiation/maturation, which ultimately favor tumor immune escape and outgrowth. In mouse xenograft models, melanoma was found to release VEGF that causes defective maturation of differentiated tissue-resident dendritic cells [16]. Neutralizing VEGF-specific antibody abrogated the negative effect and increased number of spleen and lymph node DCs [17]. In humans, increased VEGF levels was closely associated with reduced number of DC in tumor tissue and in peripheral blood of cancer patients [18,19]. VEGF are also responsible for maintaining a suppressive phenotype on DC in the circulation of cancer patients and consequently induce a state of immunological tolerance to tumor antigens [20]. Moreover, anti-VEGF antibodies have been used in cancer patients, demonstrating improvement in allo-stimulatory capacity of DC in these patients [7]. Interestingly, a recent study reported that $VEGF_{165}$ (VEGF-A isoform) requires the co-receptor NRP-1 to inhibit the LPS-dependent DC maturation [21]. This finding has led to speculate that blockade of $VEGF_{165}$ binding to NRP-1 may revert the negative regulation of $VEGF_{165}$ on DCs maturation and restore DCs function to inhibit tumor growth.

The extracellular domain of Nrp1 is divided into distinct subdomains (a1, a2, b1, b2, and c) that enable ligand binding [22]. The VEGF-A_{165} binding site is situated in the b1 domain of which the crystal structure has been reported previously [23]. The b1 domain of NRP-1 interacts with high affinity with the c-terminal exon 8-encoded VEGF-A_{165}, which forms a “C-wall” at one side of the binding pocket [24]. This structural basis triggered the interest of medicine researchers and different strategies have been recently employed to inhibit these interactions [25,26]. One approach developed a family of peptides (e.g. tuftsin [27], ATWLPPR [28], EG3287 [29], and CendR peptides [30], mimic of the sequence encoded by VEGF-A_{165} exon 8), that simulate the VEGF-A_{165} C-terminal sequence, bind into the specific pocket of the b1 domain of NRP-1 and can function as competitive inhibitors. We utilized the peptide TKPR, corresponding to the C-terminal amino acids of VEGF-A_{165} as a starting point for design. Thereafter, we synthesized a novel series of peptides which engages the ligand-binding pocket in the NRP-1 b1 domain. Previous trials in our laboratory using competitive ELISA test has identified peptide MY1340 the best candidate that binds NRP-1 with the most favorable binding energy. In this study, we explore what consequences does the presence of MY1340 have on VEGF-suppressed DC maturation status. The possible mechanism of immunomodulatory activity on DCs has also been investigated in vitro. Furthermore, the potential antitumor activities were also evaluated in vivo using tumor-bearing mice.

2. Materials and methods

2.1. Chemicals

The peptide MY1340 ([(TKPRKHG)2-K]2-K-G), the control peptide G4 ([(GGGGGGG)2-K]2-K-G) and Tuftsin were newly synthesized via a practical approach of Fmoc solid-phase peptide synthesis by Scilight-Peptide Inc., Beijing, China. The structure of MY1340 was shown in Supplementary Fig. 1. 5-Fluorouracil (5-FU) was purchased from Kingyork Group CO., Ltd. (Tianjin, China).

2.2. DC generation and maturation

DCs were generated from filter buffy coats (FBC)-derived monocytes donated by healthy human donors who gave informed consent (23). Human Monocyte Isolation Kit II (Miltenyi Biotec) was used to select and purify $CD14^+$ cells according to the instructions. Then these monocytes (5×10^5 cells/ml) were cultured in a 12-well plate (Becton Dickinson Biosciences) with 1000 U/ml of GM-CSF (Leukine, Berlex) and 500 U/ml of IL-4 (R&D Systems) and in 2 ml X-VIVO (Life Technologies), half of the media was changed every two days. On day 6, cells were stimulated for 48 h with 25 ng/ml LPS (Solarbio). Recombinant human $VEGF_{165}$ (R&D Systems) was used single or in combination with MY1340 during DC differentiation. After a total of 8 days, mature DCs were harvested.

2.3. Cell lines and mice

Hepatoma carcinoma cells (H22) was established in our laboratory and cultivated in RPMI-1640 (Gibco) containing 10% FBS (Gibco), 1% penicillin and streptomycin (Hyclone) at 37 °C in an atmosphere at 5% CO2. Male C57BL/6 J mice (6 weeks old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were fed in a temperature-controlled room on a 12 h:12 h light-dark schedule with food and water ad libitum.

2.4. Pull down assay, LC-MS/MS and western blot

To identify the MY1340-interacting proteins on DCs, pull-down assay followed by LC-MS/MS and western blot was performed described previously [31,32]. Briefly, MY1340 was linked with biotin (synthesized by Scilight-Peptide Inc., Beijing, China) were synthesized and added in the DCs (2×10^7) culture system at 10uM for combination. G4 and beads were tested parallelly under same conditions. G4, synthesized with glycine at the same length with H4 and also labeled with biotin was used as a negative control. Then cells were washed and centrifuged at 300*g* for 10 min. MY1340 binding cells were harvested and lysed in RIPA buffer with protease inhibitor cocktail (Merck) and PMSF (Solarbio) on ice for 10 min. Cell lysate was centrifuged at 24,000*g* for 5 min at 4 °C and the supernatant was collected as whole cell extracts (WCE). 50 μl streptavidin nanobeads (Biolegend) were added in the WCE. After 30 min incubation on ice, the MY1340-interacting protein complex-coated magnetic nanobeads were placed on the magnet for 3 min, washed with PBS three times. Then the magnetic nanobeads-biotin labeled MY1340-interacting proteins complex were centrifuged at 14,000*g* for 5 min and the supernatant was put on SDS-PAGE for separation. Protein concentration was determined by BCA assay. The control was G4 instead of MY1340 to interact with DCs. The target protein bands were cut down. In-gel trypsin digestion was performed. The digested products were extracted with acetonitrile (ACN) and dried in vacuum. LC-MS/MS was performed on Q-Exactive and analyzed with Proteome Discoverer based on human NCBI Ref Seq protein database. Western blot was further used to verify the interacting protein with human NRP-1 antibody using the WCE (R&D Systems).

To address whether biotin-linked MY1340 binds NRP-1 directly, we run another pull down assay to answer this question. Briefly, biotin labeled-MY1340 (500 ng/ml) incubated with purified NRP-1 (500 ng/ml), which was expressed in E.coli, in Tris buffer with protease inhibitor cocktail in 1.5 ml EP tube. 50 μl streptavidin nano-beads were added in the system. G4 and beads were tested in parallel under same conditions. After 30 min incubation on ice with constant rotation, the tubes were placed on the magnet for 3 min, washed with PBS three times followed by centrifugation at 14,000*g* for 5 min and the supernatant was used for Western blot to verify the interacting protein with human NRP-1 antibody.

2.5. Competitive enzyme-linked immunosorbent assay (ELISA)

In the competitive ELISA assay, 500 ng/ml NRP-1 in 0.1 M $NaHCO_3$ (PH = 8.6) were used to coat the microtiter plates (MaxisorpTM PS, NUNC) incubating overnight at 4 °C. The plate was blocked for 2 h with 1% BSA (Sigma) in PBS at 37 °C. After the washing step, MY1340 and Tuftsin at different concentrations (0.457 ng/ml to 1000 ng/ml) were added in the system together with $VEGF_{165}$ at 50 ng/ml and incubated at room temperature in dark for 2 h. Then the plate was washed followed by adding with 500 ng/ml Anti-VEGF (R&D Systems) and