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Anti-tumor activities of peptides corresponding to conserved complementary determining regions from different immunoglobulins

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ABSTRACT

Short synthetic peptides corresponding to sequences of complementarity-determining regions (CDRs) from different immunoglobulin families have been shown to induce antimicrobial, antiviral and anti-tumor activities regardless of the specificity of the original monoclonal antibody (mAb). Presently, we studied the *in vitro* and *in vivo* antitumor activity of synthetic peptides derived from conserved CDR sequences of different immunoglobulins against human tumor cell lines and murine B16F10-Nex2 melanoma aiming at the discovery of candidate molecules for cancer therapy. Four light- and heavy-chain CDR peptide sequences from different antibodies (C36-L1, HA9-H2, 1-H2 and Mg16-H2) showed cytotoxic activity against murine melanoma and a panel of human tumor cell lineages *in vitro*. Importantly, they also exerted anti-metastatic activity using a syngeneic melanoma model in mice. Other peptides (D07-H3, MN20v1, MS2-H3) were also protective against metastatic melanoma, without showing significant cytotoxicity against tumor cells *in vitro*. In this case, we suggest that these peptides may act as immune adjuvants *in vivo*. As observed, peptides induced nitric oxide production in bone-marrow macrophages showing that innate immune cells can also be modulated by these CDR peptides. The present screening supports the search in immunoglobulins of rather frequent CDR sequences that are endowed with specific antitumor properties and may be candidates to be developed as anti-cancer drugs.

Introduction

Chemotherapy is a major approach to treat various cancers. Side effects, however, are commonly reported because of the inability to deliver the appropriate amount of drug specifically to tumor cells without affecting normal cells. Drug resistance, clearance and biodistribution are also problems to be faced in the various treatment protocols [7]. Aiming at specifically targeting tumor cells and endothelial cells of neoangiogenesis, or to stimulate the immune system, proteins, monoclonal antibodies and peptides have been used [26]. Due to size restrictions, antibodies and large protein ligands may have poor delivery to tumors and exhibit toxicity to the liver and bone marrow. In contrast, peptides have the advantage of small size, good tumor penetration and biocompatibility.

Apart from the immune peptides derived from tumor cell protein antigens that are mostly known as T $CD8^+$ epitopes, anti-cancer peptides can act directly through inhibition of angiogenesis, induction of apoptosis or by inhibiting protein–protein interactions, signal transduction pathways, enzymes and gene expression [7,10,20,26,27]. Some of them penetrate tumor cells and remodel actin cytoskeleton with inhibitory effects on cell migration [8] or severely affect actin dynamics leading tumor cells to apoptosis [2].

Short-sized peptides can act directly on tumor cells without affecting normal cells, thus evolving as an alternate strategy to conventional chemotherapy [26]. Peptides can also act as carriers of cytotoxic agents and radioisotopes conveying them to the tumor microenvironment. Those derived from native proteins have been described to present antimicrobial activities similar to peptides of innate immunity (defensins, cathelicidins, histatins) [6]. More than 30 peptides and their oncolytic properties against solid tumors have been recently reviewed [13].

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In a previous work, Polonelli et al. [24] showed that synthetic CDR peptides can exhibit different antimicrobial, antiviral and antitumor activities *in vitro*, *in vivo* and/or *ex vivo*, in a way that was compared to early molecules of innate immunity [17]. This contribution extended previous results showing the remarkable therapeutic activity of an engineered synthetic peptide included in the variable region of a recombinant yeast killer toxin-like anti-idiotypic antibody against experimental mucosal and systemic candidiasis [23]. Both articles opened a new field of interest focusing on the bioactivity of internal sequences of immunoglobulins [18]. As a prototype of the CDR bioactive peptides, C7H2 derived from mAb C7 V_H CDR 2, showed significant antitumor activity *in vitro* and *in vivo* [24] notwithstanding the original specificity of the mAb, directed to a *Candida albicans* cell wall mannoprotein. C7H2 induces apoptosis in melanoma cells, by interacting with, and altering β-actin dynamics associated with abundant generation of superoxide anions [2]. Additional evidence of IgM/IgG-CDR-derived synthetic peptides with antitumor activities, including the description of microantibodies, has also been obtained [9]. The prototype, a 17-amino acid microantibody, derived from a mouse mAb was able to inactivate HIV *in vitro* [14].

Recent evidence on immunomodulatory activities of Ig-CDR peptides has also been obtained [11,19]. Cytotoxic effects have also been described for constant regions of immunoglobulins [12,22]. As a proof of concept of the bioactive potential of Ab CDR peptide sequences, we extended these studies and report on the differential antitumor activities of synthetic peptides with sequences identical to CDRs of the light (L_1 and L_3) and heavy (H_2 and H_3) chains of different immunoglobulin families. The CDRs chosen have more often been implicated in bioactivity than L_2 and H_1. We show here the biological activity of different CDR peptides as promising molecules displaying relevant *in vitro* and *in vivo* antitumor activities against malignant melanoma.

Experimental

CDR peptide design and synthesis

The CDR amino acid sequences were obtained according to the rules of Kabat [15] and Chothia [1,4] beyond the AbM method, or "definition of AbM" created from Accelrys software (Oxford Molecular's AbM or antibody modeling software), as described. The protein BLAST program of NCBI-NLM was used for choosing the CDR sequences with more than 80% homology within a family of immunoglobulins. Priority was given to CDRs derived from variable light chains L_1 and L_3 and heavy chains H_2 and H_3. Table 1 shows the different sequences of peptides sorted out for the present investigation.

Table 1
Amino acid sequences of 17 CDR peptides with their respective access codes from NCBI BLAST.

Peptide	Amino acid sequence	Access codes
LA3-L1	RASQGISSWLA	BAC80166.1
B9-L1	RASQSISSYLN	AEA48887.1
C36-L1	KSSQSVFYSSNNKNYLA	CAB44483.1
MS1-L1	TLSSGHSSYAIA	AAS21063.1
MN20-L3	QQYSGYPY	2BRR_L
V330-H2	VISYDGSYKYYADSVKG	BAI51287.1
CM04A05-H2	VISGSSGRTHYADSVKG	CAL06666.1
D2-H2	GIIPIFGAANYAQ	ABV70971.1
CE48-H2	INSGGGGTYYADSVKG	CAL04002.1
Mg16-H2	EIYYSGSTNYNPSLKS	ABI35660.1
1-H2	SLYYSGTTFYNPSLKS	CAD44679.1
HA9-H2	YIYYSGSTNYNPSLKS	AAO86898.1
D07-H3	DLRNHVFWSGYSTSFDY	ABE97346.1
A7-H3	DRGRGLISYYYYGMDV	AAW29179.1
F1-H3	ASYGSRGWYFDV	CAA80000.1
F6-H3	DQGGDDYGDYYYYYGMDV	ABP98185.1
MS2-H3	DTVMATPYYFDY	AAM88150.1

Tumor cell lines and cell culture

The murine B16F10 cell line of melanoma cells was subcloned at the Experimental Oncology Unit, Federal University of São Paulo, UNIFESP and the cell line B16F10-Nex2 was obtained and deposited at Banco de Células Rio de Janeiro (BCRJ 0342). Cell lineages of human melanoma (A2058), human colon carcinoma (HCT), human breast cancer (MCF-7 and SKBR-3), mouse fibroblasts (3T3), murine melanocytes (melan-A) and human foreskin fibroblast (HF) were provided by Ludwig Institute for Cancer Research, São Paulo, Brazil and Dr. Luiz F. Lima Reis (Hospital Sirio-Libanez, São Paulo). Cells were cultured at 37 °C, under humid atmosphere and 5% CO_2, in RPMI-1640 medium with 10 mM *N*-2-hydroxyethylpiperazine-*N*2-ethanesulphonic acid (HEPES), 24 mM sodium bicarbonate, 40 mg/L gentamicin, pH 7.2 and 10% fetal calf serum (FCS). U87-MG cell line (glioblastoma) was provided by Dr. Osvaldo K. Okamoto, University of São Paulo and was maintained in DMEM medium supplemented as for the RPMI-1640 medium.

Cell viability assay

Tumor cells (10^4) were seeded in 96-well plates and incubated with all peptides at different concentrations ranging from 0 to 1 mM. Viable cells were quantified using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich, St. Louis, MO) assay. After 24 h of incubation with peptides, 10 μl of MTT solution (5 mg/ml) in 16 phosphate-buffered saline (PBS) was directly added to the cells, followed by incubation for 3 h at 37 °C. Absorbance was measured at 570 nm with an automated spectrophotometric plate reader (SpectraMax-M2, Molecular Devices Software Pro 5.4, Sunnyvale, CA). Cell viability was expressed as percent values in comparison with untreated control cells. The experiments were performed in triplicate. Alternatively, viable cells were quantified using the Trypan Blue exclusion assay.

Murine bone marrow cells and macrophage differentiation

Fresh bone marrow cells were used to generate macrophages using L929-cell supernatant conditioned medium (LCCM) as a source of granulocyte/macrophage-colony stimulating factor (GM-CSF). Cells were resuspended in 10 ml bone marrow differentiation medium, which consists of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY), 30% LCCM, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded in non-tissue culture treated Optilux Petri dishes (BD Biosciences, Franklin Lakes, NJ) and incubated at 37 °C in 5% CO_2 atmosphere. After 4 days, 10 ml of fresh medium was added per plate and incubated for additional 3 days. To obtain macrophages, the supernatants were discarded and the adherent cells were washed with 15 ml of sterile PBS. Macrophages were detached gently using a cell scraper and PBS. The cells were centrifuged at 200 × *g* for 5 min and resuspended in 10 ml of RPMI 1640 (Gibco, Grand Island, NY). Cells were counted and cultured in tissue culture plates for 12 h.

Macrophage NO production

Nitric oxide (NO) production was assessed by measuring the accumulation of nitrite in the culture medium using the Griess reaction. 5×10^5 Bone marrow macrophages were seeded in 24-well