Design, synthesis and study of multimeric peptidic conjugates for a new approach of anti-tumoral immunotherapy

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Abstract

Recruitment of natural human antibodies for targeted destruction of tumoral cells has emerged as a very promising immunotherapy strategy. Although several compounds have been proposed in this area, efforts have to be made to increase the specificity towards cancer cells and to optimize the response of immune system. Here we reported a new bimodal compound named ARM, combining four cRGD-based peptidomimetics anchored on a cyclodecapeptidic platform to target cells displaying the $\alpha_v\beta_3$ integrin overexpressed on a majority of tumor cells, with another peptidic scaffold displaying a tetravalent α -L-Rhamnose, for the recruitment of endogenous anti-Rhamnose antibody present in human serum.

Introduction

Although significant progress has been made these last years in treatment modalities to combat cancer, current therapeutic regimens are still deficient due to severe side effects for the patient [1][2]. Recently, an original antitumoral approach-based immunotherapy has emerged, consisting in the recruitment of pre-existing natural antibodies present in human bloodstream and their redirection against tumor cells thereby activating antibody-mediated immune responses such as complement-mediated cytotoxicity (CDC) or antibody-dependent responses, i.e. antibody-dependent cellular phagocytosis (ADCP) or antibody-dependent cellular cytotoxicity (ADCC)[3][4] (Fig. 1). While several antibody recruiting molecules (ARMs) have been reported today, the immune response activated by such a compound has to be improved. Here we evaluated a novel bifunctional conjugate comprising a cluster of α -L-rhamnose (Rhm) as antibody binding module (ABM) to recruit high titers of natural antibody and a cluster of cRGD as tumor binding module (TBMs) displayed on a molecular scaffold to simultaneously target multiple tumoral membrane proteins (Fig. 2). The cRGD peptidomimetic has been used to target tumor cells displaying the $\alpha_v\beta_3$ integrin while the expression is restricted in normal tissues but highly expressed in tumor tissues [5]. The rhamnose is a monosaccharide not expressed in human cells but prevalent in microbes. Studies have shown that anti-Rhm antibodies are widely present in human serum and are able to form relatively stables complexes with rhamnose indicating that this carbohydrate might be very attractive for activation of endogenous immune response for targeted anti-tumoral immunotherapy [6]. A cyclodecapeptide scaffold, largely described by our group as well as chemoselective ligations (copper-catalyzed alkyne-azide cycloaddition) were used to elaborate modules decorated with four cRGD ((cRGD)₄-TBM) or tetravalent rhamnose units ((Rhm)₄-ABM). Both modules were first tested independently of each other with human serum containing anti-Rhm antibodies for ABM and with U-87 MG glioma cells expressing $\alpha_v\beta_3$ integrins or MCF-10a deficient in integrin for TBM. (cRGD)₄ TBM and (Rhm)₄ ABM were then assembled and the cytotoxic property of the bimodal compound was evaluated.

Figure 1: Antibody recruiting molecule (ARM) forming a ternary complex between a tumoral cell and endogenous antibodies leading to the immune-mediated clearance of the targeted cells.

Figure 2: Chemical structure of ARM with tetraivalent cRGD as TBM and tetraivalent rhamnose as ABM

Integrin-binding and antibody-binding assays

To assess the ability of our tetraivalent cRGD module to bind specifically cancer cells, we first quantified by flow cytometry the $\alpha_v\beta_3$ integrin expression at the surface of U-87 MG cells and MCF-10a using a commercial monoclonal anti- $\alpha_v\beta_3$ integrin antibody conjugated to the R-Phycoerythrin fluorochrome (23C6 mAb-R-PE). Figure 3a shows the level of $\alpha_v\beta_3$ integrin expression in the two cell lines after immunostaining with the labeled antibody. The binding property of our tetraivalent (cRGD)₄-TBM towards $\alpha_v\beta_3$ integrins was then evaluated by measuring the fluorescence of the U-87 MG cells and MCF-10a used as control, when incubated with the cyanine 5-(cRGD)₄-TBM labeled module. Cells were first incubated 1 hour at 37°C in presence of 5 μ M of the fluorescent compound. At this temperature, a 15-fold binding improvement for the U-87MG cells was measured, while binding for MCF-10a was only enhanced by a factor 5 (Fig. 3b). In order to minimize the internalization of the (cRGD)₄-TBM, incubation with cells was investigated at 4°C and the cells fluorescence analyzed. At this lower temperature, the binding of (cRGD)₄-TBM for the U-87MG cells was only 7-fold increase, while a 3-fold binding improvement was measured for the MCF-10a cells (Fig. 3c). In regard to these experiments, we decided next to evaluate the cytotoxicity of the (cRGD)₄-TBM-(Rhm)₄-ABM conjugate at 37°C.

Figure 3: Fluorescence histogram counts of cell suspensions. a. 23C6 anti-integrin Ab-R-PE fluorescence (black: MCF-10a and U-87 MG autofluorescence; blue: MCF-10a with antibody; red: U-87 MG with antibody) b. Cy5 fluorescence after incubation with (cRGD)₄-TBM at 37°C and c. at 4°C (cyan: MCF-10a autofluorescence; yellow: U-87 MG autofluorescence; blue: MCF-10a with compound; red: U-87 MG with compound)

The ability of (Rhm)₄-ABM to recruit endogenous antibodies present in serum was then evaluated in an indirect ELISA-type competition assay. In this test, a polymer functionalized with rhamnose (PAA-Rhm) was immobilized in a multi-well plate. A binding competition of the natural anti-Rhm antibodies present in human serum with the PAA-Rhm or (Rhm)₄-ABM present at different concentrations was performed. Binding of the anti-Rhm antibody to the PAA-Rhm was revealed using a secondary antibody conjugated to the Horse Radish Peroxydase which in presence of its substrate led to an absorbance signal at 490 nm. An IC₅₀ of 122 μ M, measuring the ability of the compound to inhibit 50% of antibody binding to the PAA-Rhm was determined for (Rhm)₄-ABM which correspond to a 30 times increase in the ability of the compound to recruit antibodies in serum compared to the rhamnose monomer, or an 8-fold binding improvement per rhamnose unit.

Cytotoxicity of the (cRGD)₄ TBM - (Rhm)₄ ABM bimodal compound

The cytotoxicity of the bimodal construct (ARM) was evaluated using the commercial kit CellTiter-Glo® (Promega). This test determine the number of viable cells in culture in multi-well plates based on the quan-

tification of the ATP present, an indicator of metabolically active cells. The assay consists in measuring a bioluminescent signal proportional to the amount of the intracellular ATP. U-87MG cells displaying the $\alpha_v\beta_3$ integrins have been incubated with or without human serum (HS) and in presence of ARM (5 or 20 μM). The higher bioluminescence signal was measured with cells not incubated with ARM and serum, while the bioluminescence signal was almost undetectable for the dead cells obtained by treatment with hydrogen peroxide. For the cells treated with the lowest concentration of ARM (5 μM) and in presence of human serum, a decrease of the bioluminescent signal of 28% was observed indicating a cytotoxicity induced by HS and the ARM (Fig. 4a). An increase by four of the ARM concentration induced a rise in the cytotoxicity by the same factor showing a dose dependent effect (Fig. 4b). We clearly showed that our ARM conjugate is able to recruit antibodies in human serum and activate an immune leading to the tumor cell destruction.

Figure 4: U-87 MG cell cytotoxicity induced by the bimodal ARM in presence (+HS) or in absence (-HS) of human serum, calculated using the bioluminescence titration of the intracellular ATP. a. 5 μM b. 20 μM

In conclusion, we elaborated a bimodal molecule displaying four cRGD motifs for binding to $\alpha_v\beta_3$ integrins expressed on tumor cells and a tetravalent rhamnose units for recruitment of anti-Rhm antibodies naturally present in human serum. The tetravalent cRGD displaying module showed strong binding for tumor cells expressing the $\alpha_v\beta_3$ integrins. Similarly, the tetravalent rhamnose scaffold showed the higher ability to recruit anti-rhamnose antibodies than the rhamnose monomer. Finally, the bifunctional molecule resulting from the assembly of the two modules showed cytotoxicity when tested with U-87 MG tumor cells. These results shows that increasing the valency of the two binding modules is an efficient way to increase the destruction of cancer cells by bifunctional antibody recruiting molecules.

Materials and Methods

Cell lines and cultivation

Cell lines U-87 MG was purchased from the American Type Culture Collection (Manassas, VA, USA). Cell line MCF-10a was provided by Dr. Y Chebloune from the Grenoble Alpes University, France. U-87 MG was cultured in MEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. MCF-10a was cultures in MEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 100 ng/ml chelora toxin B subunit, 10 $\mu\text{g}/\text{ml}$ insulin and 20 ng/ml EGF. Cells were cultured adherently in complete medium at 37°C in an humidified atmosphere with 5% CO₂. Cells harvest and passaging were done after reaching 80% confluency by washing cells with PBS and detaching them from tissue culture flasks after incubation for 6 minutes at 37°C with trypsin/EDTA. The cells are then counted and diluted in complete medium or experiment buffer.

Flow cytometry

Evaluation of the $\alpha_v\beta_3$ integrins expression was assessed by using a monoclonal antibody 23C6 conjugated to R-Phycoerythrin. Evaluation of the TBM was assessed by determining the amount of tetravalent cRGD conjugated with Cy5 binding to the cells. Each cell line were harvested and 1.0×10^6 cells were resuspended in 1 ml of ice-cold binding buffer HBSS during 30 minutes. After a wash with HBSS, the cells were incubated at 37°C and 4°C with 5 μM of fluorescent tetravalent cRGD-ABM for 1 h in the dark, or with 23C6 mAb PE conjugated (1:10) at 37°C during 30 minutes. Subsequently, the cells were centrifuged, wash once in PBS and resuspended in 1 ml of PBS and finally kept on ice until being analyzed. Flow cytometry was performed on a BD FACSCalibur platform (Becton Dickinson, Heidelberg, Germany).

Cell Titer Glo assay

A cytotoxicity test was performed in a white bottom 96-well-plate. The cells were harvested by trypsin/EDTA treatment, counted and suspended in complete medium to a density of 5.0×10^5 cells/ml. Subsequently, the cells were seeded at $100 \mu\text{l}$ /well and incubated for 16 h at 37°C . Glycopeptide ARM was diluted from stock solution (1 mM) into MEM at 5 and $20 \mu\text{M}$ concentrations. After incubation of the peptide for 1 h at 37°C and 1 wash with MEM, $100 \mu\text{l}$ of MEM with 20% v/v of Human Serum was added to each well, following by a final incubation for 2 h at 37°C . The amount of live cells in each well was determined by luminescence using the CellTiterGlo viability assay per the manufacturer's instruction (Promega). The maximal killing values were untreated. Dead cells obtained by treatment with MEM with 5% v/v hydrogen peroxide were used as control.

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