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Selection and Evaluation of Specific Single Chain Antibodies against CD90, a Marker for Mesenchymal and Cancer Stem Cells

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Abstract

Background: CD90, a membrane-associated glycoprotein is a marker used to identify mesenchymal stem cells (MSCs). Recent studies have introduced CD90, which induces tumorigenic activity, as a cancer stem cell (CSC) marker in various malignancies. Blocking CD90 activity with anti-CD90 monoclonal antibodies enhanced anti-tumor effects. To date, highly specific antibody single-chain variable fragments (scFvs) have been isolated against various targets and showed promising results in cancer immunotherapy.

Methods: A phage antibody was produced from a scFv library using M13KO7 helper phage. The phage library was panned against a CD90 epitope. To select specific clones, PCR and DNA fingerprinting were performed and common patterns were identified. The panning results were confirmed by phage ELISA.

Results: Of 20 clones selected after panning, 16 shared identical fingerprints. One clone from this group reacted specifically with the epitope in phage ELISA. The average absorbance of wells coated with the CD90 peptide was significantly greater than that of wells containing no peptide (p=0.03).

Conclusions: Currently, recombinant antibodies are used not only as highly specific detection tools, but due to their specific characteristics, are applied in targeted cancer therapies. The anti-CD90 scFv selected in this study has the potential to be used to detect MSCs and target CSCs and offers promising strategies for treatment of various cancers.

Keywords: Cancer stem cell, CD90, Mesenchymal stem cell, Single chain antibody

Introduction

Thy-1 (CD90), a 25-37 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein on the external leaflet of the lipid bilayer, localizes to lipid rafts (1-3). It is expressed on many cell types including neuronal cells, thymocytes, lymphocytes, fibroblasts, activated endothelial cells, keratinocytic stem cells, hematopoietic and mesenchymal stem cells (MSCs), ovarian follicular cells, and some cancer cells (4-6). Recent studies have introduced CD90 as a marker for cancer stem cells (CSCs) in various malignancies (2, 7). It has been reported that Thy-1 is involved in oncogenesis, T cell activation, apoptotic signaling, inhibition of neurite outgrowth, leukocyte and melanoma cell adhesion and migration, fibroblast proliferation, tumor suppression, and cell death (8-10). A number of studies show the participation of CD90 in MSC self-renewal and differentiation (11). Study results indicate that CD90 controls MSC differentiation by acting as an obstacle in the pathway of differentiation commitment (12). These functions indicate the importance of Thy-1 as a regulator of cell-cell and cell-matrix interactions (3).

Two independent adult stem cell populations are found in bone marrow; these are hematopoietic stem cells (HSCs) and MSCs (13). Mesenchymal stem cells undergo mesodermal lineage-specific differentiation to osteocytes, adipocytes, and chondrocytes. The potential of these cells to

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differentiate to connective tissue has been utilized for tissue engineering (14, 15). Mesenchymal stem cells are ideal candidates for adult stem cell-based therapy and as gene carriers due to their hypoimmunogenic and immunomodulatory properties. These primitive cells migrate to injury sites and regulate immune responses by inhibiting cytokine release and enhancing tissue repair (15). It has been shown that some functions of bone marrow MSCs, including the formation of glandular structures, expression of keratinocyte-specific protein, re-epithelialization, and angiogenesis aid in wound healing and limit scar size. They also play a crucial role in the repair of ischemia-damaged cardiac tissue by differentiating into cardiomyocytes and vasculature cells and regenerating blood vessels (16).

The International Society for Cellular Therapy has defined some criteria to identify MSCs. These include clonogenic activity, extended proliferation, adherence to plastic, and differentiation into mesenchymal lineages, chondrocytes, adipocytes, osteoblasts, and possibly pericytes, myocytes, and neurons (17). Mesenchymal stem cells are positive for CD105, CD73, and CD90, negative for CD34, CD19, CD45, CD11a, and HLA DR (13, 17), and should undergo mesenchymal differentiation in vitro (13). Mesenchymal stem cells are immunoprivileged and thus escape immune surveillance due to the lack of HLA-DR expression and associated co-stimulatory molecules.

Cancer stem cells (CSCs) are found in both tumors and hematological cancers and can give rise to all cell types in a given cancer sample. They are highly resistant to conventional chemo- and radiotherapies (18). CD90 expression has been reported in some CSCs. Variable expression of CD90 in high- and low-grade glioblastoma (GBM) vs. normal brain tissue has been shown. It was demonstrated that CD90 not only has an important role in CSC formation, but its expression is increased in high grade glioma and it is important for GBM CSC development (19). The presence and role of CD90 as an important marker has been also shown in hepatic, breast, pancreatic, and esophageal CSCs (20-22).

Single chain fragment variable (scFv) antibodies consist of heavy (VH) and light (VL) chain variable regions joined together by a flexible peptide linker. These antibodies are small and effective and can be easily expressed in functional form in *Escherichia coli* (*E. coli*), providing protein engineering to improve their properties (23, 24). To date, several highly specific scFvs against various targets have been isolated (25, 26) and shown promising results in cancer immunotherapy. The small size of these antibodies allows for more rapid and even penetration to and into tumors and other tissues than whole antibodies (27). These antibodies have also been used for diagnoses of infections and as biomarkers (28-30).

In this study specific scFvs were selected against CD90 using phage display technology. The reactivity and specificity of the selected scFvs were evaluated by phage ELISA.

Materials and methods

Phage Rescue

A phage antibody display library of scFv was produced as described previously (31). *Escherichia coli* containing phagemid was cultured on 2TYG agar/ampicillin (tryptone, yeast extract, glucose, agar, and ampicillin) (Merck, Germany) plates overnight at 30 ºC. The bacteria were scraped and incubated in 2TYG broth at 37 ºC for one hr. M13KO7 helper phage was added and cultures were incubated at 37 ºC for 30 min. After shaking for 30 min, the culture was centrifuged at 1.6 *xg* for 20 min and the pellet transferred to 2TY broth containing 100 µg/ml ampicillin and 50 µ/ml kanamycin, and incubated with shaking at 30 ºC overnight. Cultures were centrifuged at 2.5 *xg*. The supernatant was passed through a 0.2 µm filter, and stored at 4 ºC.

Panning

The CD90 epitope peptide (PEHTYRSRTNFTSKY), at a concentration of 10 µg/ml diluted in PBS, was coated on 4 ml polystyrene immunotubes (Nunc, Denmark) at 4 ºC overnight. After washing with PBS, the tubes were blocked with 2% skimmed milk and incubated at 37 ºC for two hr. The tubes were washed four times with PBS/Tween 20 and four times with PBS. Phage supernatant was diluted 1:1 in the blocking solution, added to the 4 ml immunotubes, and incubated at room temperature for one hour with occasional inversions. After washing, logarithmic phase TG1

E. coli was added, incubated at 37 ºC for one hour, and centrifuged at 1.6 *mg*. The bacterial pellet was re-suspended in 2TY broth media containing 10 g Bacto-tryptone (Merk, Germany) 5 g Yeast extract (Merk, Germany) 10 g NaCl (Sigma, UK) in a final volume of 1 L using double-distilled H2O, plated on 2TY agarose/ampicillin plates, and incubated at 30 ºC overnight. Four rounds of panning were performed using phage rescue supernatant from the previous round to select the epitope-specific scFv antibodies.

PCR and Mva I digestion of the Selected Clones

The VH-linker-VL inserts from the selected pre- and post-panned clones, *E. Coli* bacteria containing phagemid vector which carrying scFv genes, were amplified by PCR (94 °C for one min, 55 °C for one min, and 72 °C for two min, x 30 cycles) using R1: CCATGATTACGCCAAGCTTTGGAGCC and R2: CGATCTAAAGTTTTGTCGTCTTTCC vector primers. The PCR template was the bacterial DNA which obtained by heating a bacterial solution at 94 °C for 10 min prior to PCR cycles. To determine the fingerprinting patterns, 17 µl of the PCR product was mixed with 1 µl of *Mva* I restriction enzyme (Roche Applied Science, Germany) and 2 µl of restriction enzyme buffer, heated at 37 ºC for two hours in a dry block heater, and electrophoresed on a 2% agarose gel.

Phage ELISA

Wells of a 96-well polystyrene plate were coated with 150 µl of peptide at 100 µg/mL, and incubated at 4 °C overnight. The plate was washed with PBS, wells were blocked with 2% skimmed milk, and the plate was incubated at 37 ºC for two hr. After washing the wells with PBS/Tween and PBS, phage supernatant containing the selected scFv diluted 1:1 with blocking solution was added to the wells and incubated at room temperature for two hr. To remove the unbound phages, wells were washed three times with PBS/Tween and three times with PBS. Rabbit anti-fd bacteriophage antibody (Sigma, Germany) (1:100) was added to each well and plates were incubated at room temperature for 1.5 hr. After washing, HRP-conjugated anti-rabbit antibody (1:1000) (Sigma, Germany) was added and the plate was incubated at room temperature for one hr. The wells were washed and 150 µl of Amino-bis-3-ethylbenzothiazoline-6-sulfonic (ABTS) acid (Sigma-Aldrich, Germany) solution (10 mg ABTS, 20 ml citrate buffer pH=4, and 6 mL H2O2) was added and the absorbance was read after 30 min at 405 nm using an ELISA reader. Negative controls included wells without peptide, with an unrelated prostate stem cell antigen peptide, with an unrelated scFv to HER2, and with M13KO7. All assays were performed in triplicate and the average optical densities (ODs) were calculated.

Statistical analysis

The Mann-Whitney U test was used to compare the average absorbance of the sample wells with the average of the no peptide controls.

Results

PCR and Mva I restriction digests

Seventeen pre- and 20 post-panned clones were amplified by PCR and the PCR products were digested with *Mva* I. A 950 bp product representing the VH-linker-VL fragments was amplified from all 20 post-panned clones (Fig. 1). Digestion of the 20 PCR products with *Mva* I resulted in one dominant restriction digest pattern, shared by 16 of the clones (Fig. 2). One clone with the common pattern was selected for further evaluation. The fingerprint patterns of 17 clones from the library before panning are shown in Figure 3. The variety of the fingerprints before panning shows the diversity and heterogeneity of the library, while the similarity of the fingerprints after panning demonstrates the effectiveness of the panning process.

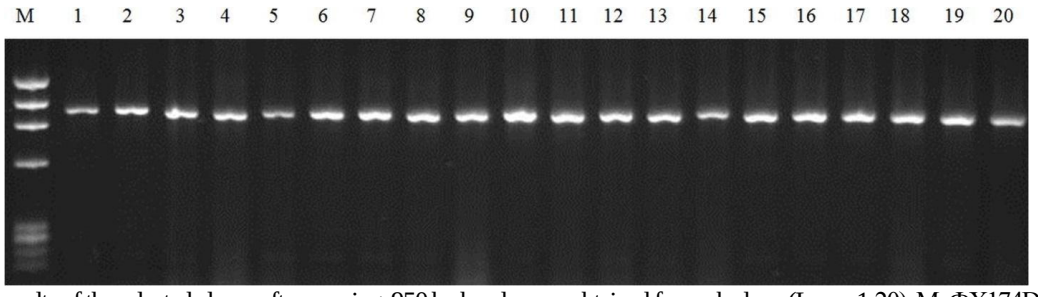

Fig. 1. PCR results of the selected clones after panning. 950 bp bands were obtained for each clone (Lanes 1-20). M: ΦX174DNA marker.

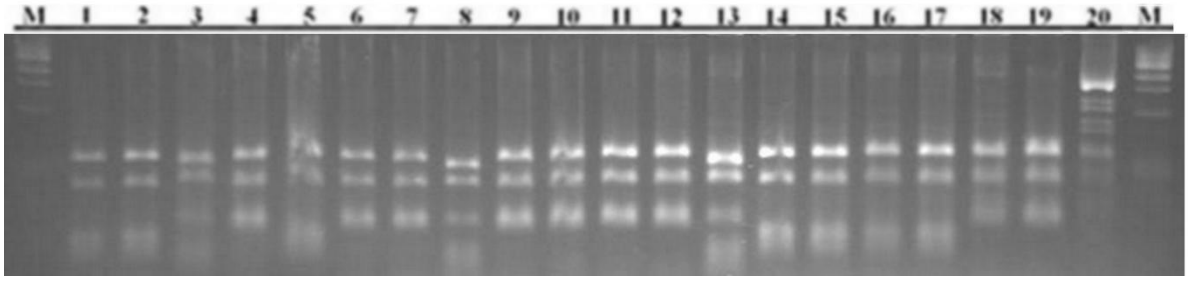

Fig. 2. *Mva* I restriction digests of the clones selected after panning. A common pattern was obtained in lanes 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 16, 17, 18, and 19. Lanes M: molecular weight marker.

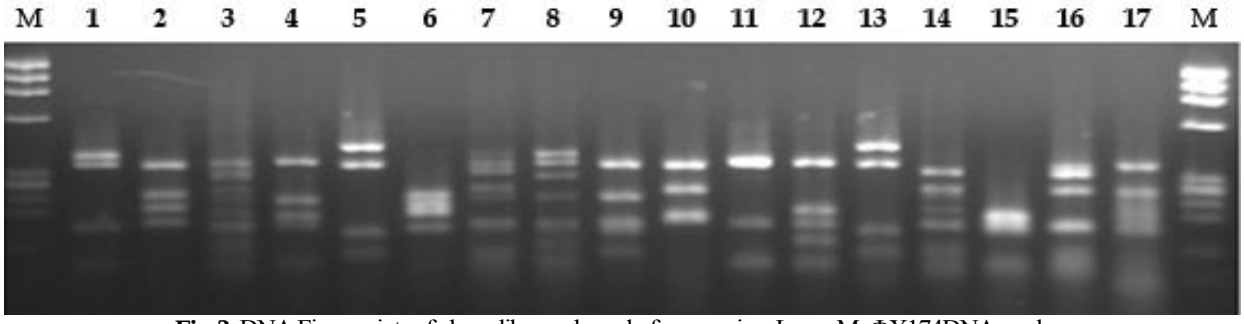

Fig. 3. DNA Fingerprints of phage library clones before panning. Lanes M: ΦX174DNA marker.

Phage Enzyme Linked Immunosorbent Assay

Specific binding of the selected scFv antibodies to the CD90 peptide was analyzed by phage ELISA. The absorbance of wells coated with the corresponding peptide was significantly greater than that of the wells containing no peptide (p=0.03), with average ODs of 0.71 and 0.17, respectively. No significant differences were found between the no peptide wells an unrelated peptide, unrelated scFv, or M13KO7. (P>0.05) (Fig. 4).

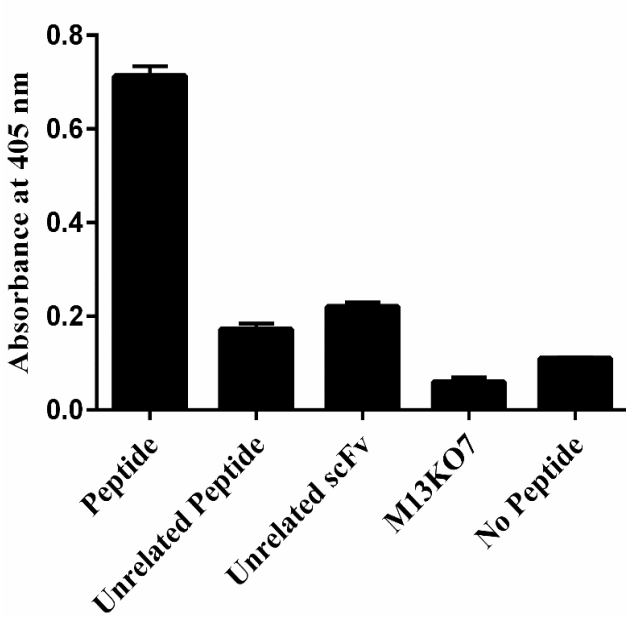

Fig. 4. ELISA results of anti CD90-scFv against the CD90 peptide. Unrelated peptide, unrelated scFv, M13KO7, and no peptide were used as negative controls.

Discussion

Mesenchymal stem cells are broadly used in clinical settings to treat tissue injuries. They also have important applications in the treatment of autoimmune disorders due to their immunomodulation characteristics and secretion of bioactive molecules (15, 32).

Several monoclonal antibodies have been used to characterize MSCs and determine the presence or absence of specific markers (33). CD90 is an MSC marker (17) and anti-CD90 monoclonal antibodies have been shown to induce apoptosis in CSCs (34). Due to several desirable advantages of single chain antibodies, these recombinant antibodies have been used in a number of detections and clinical applications (35).

In this study a phage antibody display library of scFv was used to select specific scFvs against an immunodominant CD90 peptide. This peptide has been applied as an immunogen for production of an anti-CD90 monoclonal antibody that has been effective in targeting of CD90 in other studies (36, 37). Of 20 clones selected after panning, 16 (80%) were identical. One of these clones was selected for phage ELISA.

Panning has been widely used to select specific scFvs against various antigens. Wang et al. (38) selected specific scFvs against VEGFR1 using panning. It was shown that antibodies selected

through panning had sufficient affinities for molecular imaging, and also targeted drug delivery, without need for further affinity maturation. Specific antibodies against CD133, a CSC marker, were selected by panning a phage antibody against CD133-purified proteins (39). Selection of specific scFvs against IL-25 and RTF by panning demonstrated high anti-proliferative and apoptotic effects of these scFvs against breast and prostate cancer cells, respectively (40, 41).

The anti-CD90 scFv bound the corresponding epitope specifically in the phage ELISA assay while no significant binding was observed in the no- or unrelated peptide wells. The average absorbance of wells coated with the peptide of interest for the selected scFv was 6.5-fold greater than that of the wells containing no peptide. Similarly, Thathaisong et al. (42) showed that the OD of specific scFvs against Influenza-A virus H5N1 subtype at 405 nm was two-fold greater than that of negative controls in a positive phage ELISA. In our study, the lack of scFv binding to the negative controls indicated the specificity of the selected scFvs with the CD90 epitope. The specificity of panned scFvs against fumonisin was demonstrated by Hu et al. in a phage ELISA assay (43). To determine the reactivity of selected scFvs after panning a library of scFvs against CTLA4 and EGFR antigens, phage ELISA revealed that the selected clones were specific for the corresponding epitopes (44, 45).

CD90 not only is expressed on MSCs and used as a marker for their identification, but is also found on a number of CSCs and influences oncogenesis in various types of malignancies (16, 18). CD90 has been a candidate marker for CSCs of high-grade gliomas, and its role in the generation of tumor vasculature has been reported (19); therefore, blocking this marker could inhibit glioma tumor growth. It has been demonstrated that high CD90 expression in hepatocellular carcinoma (HCC) tissues correlates with venous filtration in HCC patients, and CD90+ cells obtained from HCC cell lines increased tumorigenicity, tumor invasion, chemoresistance, and metastasis. CD90 is a potential biomarker for HCC-CSCs. It was also shown that CSC activity was elevated through increased Notch pathway activity in CD90+ CSCs (46). In pancreatic adenocarcinoma and gastric cancer (47, 48) it has been shown that CD90 promotes tumorigenesis. Therefore, CSC targeting offers a promising strategy for eradication of various cancer types. The specific anti-CD90 scFv selected in this study can be used not only to detect MSCs, but due to its small size, deep penetration, human origin, and high affinity properties, is an ideal agent for CSC-targeted therapy to block CD90-mediated tumorigenesis. The scFv gene could also be cloned in an expression vector along with enzyme or toxin genes to produce fusion peptides to enhance the antibody's inhibitory activity. Mesenchymal stem cell targeting using scFvs might be an effective strategy for future cancer therapy.

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