Cancer Letters 312 (2011) 168–177

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Molecular targeting of malignant glioma cells with an EphA2-specific immunotoxin delivered by human bone marrow-derived mesenchymal stem cells

Xin-lin Sun [a,b], Zhi-min Xu [a,b,c,1], Yi-quan Ke [a,b,*], Chang-chen Hu [d], Shi-yong Wang [a,b], Geng-qiang Ling [a,b], Zhong-jie Yan [a,b,c], Yi-jing Liu [a,b], Zhen-hua Song [a,b], Xiao-dan Jiang [a,b], Ru-xiang Xu [b,c]

[a] Department of Neurosurgery, Zhujiang Hospital, Southern Medical University, Guangzhou, China
[b] Institute of Neurosurgery, Key Laboratory on Brain Function Repair and Regeneration of Guangdong, Southern Medical University, Guangzhou, China
[c] Department of Neurosurgery, The Military General Hospital of Beijing PLA, Beijing, China
[d] Department of Neurosurgery, Shanxi Provincial People's Hospital, Taiyuan, China

ARTICLE INFO

Article history:
Received 13 May 2011
Received in revised form 28 July 2011
Accepted 30 July 2011

Keywords:
Immunotoxin
EphA2
Mesenchymal stem cells
Malignant glioma
Gene therapy

ABSTRACT

Immunotoxins have shown great promise as an alternative treatment for brain malignancies such as gliomas, but their failure to penetrate into the tumor mass remains a major problem. Mesenchymal stem cells exhibit tropism to tumor tissue and may serve as a cellular vehicle for the delivery and local production of antitumor agents. In this study, we used human bone marrow-derived mesenchymal stem cells (hMSCs) as a vehicle for the targeted delivery of EphrinA1-PE38, a very specific immunotoxin against the EphA2 receptor that is overexpressed in gliomas. hMSCs were transduced with adenovirus to express secretable EphrinA1-PE38. Our *in vitro* assays confirmed the expression, release and selective killing effect of the immunotoxin produced by hMSCs. Furthermore, the intratumoral injection of engineered hMSCs was effective at inhibiting tumor growth in a malignant glioma tumor model. These results indicate that gene therapy utilizing EphrinA1-PE38-secreting hMSCs may provide a novel approach for the local treatment of malignant gliomas.

1. Introduction

Glioblastoma multiforme (GBM) is the most common adult malignant brain tumor with poor prognosis. Despite advances in the current treatment of GBM with surgery, radiotherapy, and chemotherapy, the average survival of patients with GBM remains only 14 months [1]. Novel strategies with increased specificity and efficacy are in great demand.

Among the novel approaches for GBM therapy, immunotoxins have emerged as potent tools for killing tumor cells. Immunotoxins are fusion proteins that contain a targeting moiety that is chemically or recombinantly linked to a cytotoxic moiety such as Diphtheria toxin (DT) and Pseudomonas exotoxin (PE) [2]. Monoclonal antibodies and growth factors are used as the targeting moieties in the construction of recombinant immunotoxins because of their specificity and high binding affinity to the target cells. Nearly all protein toxins work by enzymatically inhibiting protein synthesis [3]. Although immunotoxins represent a novel and potent treatment for malignant gliomas, their clinical application has been hampered by the inability to locate at the tumor mass and non-specific associated neural toxicity [4]. Thus, a new approach for immunotoxin delivery to overcome these obstacles is vital.

* Corresponding author. Address: Department of Neurosurgery, Zhujiang Hospital, Southern Medical University, Gongye Road 253, 510282, Guangzhou, China. Tel.: +86 020 61643266; fax: +86 020 84311562.
E-mail address: zjkeyiquan@yahoo.com.cn (Y.-q. Ke).
[1] Contributed equally with the first author.

0304-3835/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2011.07.035

Recently, human mesenchymal stem cells (hMSCs) have begun to show promise as potential vehicles for delivering therapeutic genes for the treatment of brain tumors [5,6]. hMSCs possess tropism for experimental tumors, including gliomas, following intraarterial or intracranial injections [7]. Moreover, these cells can be obtained from patients without ethical concerns, easily expanded *in vitro*, and genetically modified with viral vectors for the delivery of antitumor substances *in vivo* [8,9]. Several preclinical trials of genetically modified hMSCs expressing IFN-β, IL-2 and TRAIL have exhibited significant antitumor effect in glioma models [8,10]. With these studies in mind, we used hMSCs in our study as a carrier for the delivery of an immunotoxin against malignant glioma.

The specificity of immunotoxin therapy relies on the presence of a molecular marker that is highly overexpressed on tumor cells and nearly absent on normal cells [2,7]. Recent evidence has shown that the EphA2 receptor is overexpressed in GBM compared to normal brain tissues [11,12]. EphA2 is a member of the Eph receptor tyrosine kinase family, whose 16 members can be further divided into "A" and "B" classes, based on sequence homology and binding affinity to their ligand, the Ephrin [13]. Accumulating evidence has indicated that overexpression of EphA2 is associated with the development and progression of a number of human malignancies, including those of breast [14], prostate [15], melanoma [16] and ovary [17] as well as in GBM. Furthermore, recent data have shown that EphA2 is overexpressed not only on the plasma membrane of tumor cells but also in the tumor-associated vasculature [18,19]. Thus, the EphA2 receptor represents a very attractive target for immunotoxin therapy.

In this report, the extracellular domain of EphrinA1, the specific ligand of EphA2, was fused to PE38, the truncated form of PE, to engineer EphrinA1-PE38-secreting cells. hMSCs were genetically manipulated with an adenoviral vector encoding EphrinA1-PE38. Our study show that EphrinA1-PE38 can be released from hMSCs and that these cells selectively kill U251 cells *in vitro* and in a glioma model.

2. Materials and methods

2.1. Cell culture

The human glioma cell lines U251, A172, SHG-44, U87 and human breast cancer cell line T47D were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection. The human glioma cell lines U118 and U373 were purchased from the American Type Culture Collection. Human umbilical vascular endothelial cells (HUVEC) were a generous gift from Dr. Zhengping Xu (Zhejiang University School of Medicine, China). U-251[EphrinA1](+) cells were generated in our laboratory by transfection with pIRES2-EphrinA1-EGFP as previously described [20]. Details of the U-251[EphrinA1](+) cell generation are provided in Supplementary data. Human mesenchymal stem cells (hMSCs) were isolated and cultured as previously described [21]. These hMSCs have a fibroblast-like morphology and express CD105 (>90%), CD29 (>90%), and CD44 (>90%) but lack expression of CD14, CD34, and CD45. All of the cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).The cells were grown at 37 °C in 95% air, 5% CO2. At confluency, the cells were trypsinized (0.25% trypsin with 0.1% EDTA), Cultures were passaged at a ratio of approximately 1:3.

2.2. Western blot analysis

Western blot analysis was performed as previously described [11]. Protein lysates isolated from tumor cells were separated by SDS–PAGE and transferred to nitrocellulose membranes. Blots were incubated with anti-EphA2 (clone D7, Millipore, Billerica, MA, USA), anti-EphA3(clone C-19, Santa Cruz, CA, USA), anti-EphA4 (clone S-20, Santa Cruz) and anti-EphA5 (clone C-16, Santa Cruz,) followed by species-specific secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG, Sigma, Munich, Germany). The antibody specific for β-actin (Clone AC-74, Sigma) were used to confirm equal sample loading.

2.3. Adenoviral vectors and ex vivo gene transduction

The PE38 gene was ligated into the pAdTrack-CMV vector by HindIII and EcoRV sites from the pRB391 plasmid. The extracellular domain of EphrinA1 lacking GPI-anchorage (aa:1–182) was amplified by PCR using the pCMV-Sport6-EphrinA1 plasmid (GeneCopoeia, Rockville, MD, USA) as a template. The following primers were used: 5′GTATAGATCTATGGAGTTCCTCTGGGCCCCTCTCTTG3′ (forward, BglII site underlined) and 5′GTCTAAGCTTTGTGA CCGATGCTATGTAGAACCCGCACCTC3' (reverse, HindIII site underlined). The resulting fragment was fused upstream of the PE38 fragment by BglII and HindIII sites to construct the recombinant plasmid pAdTrack-EphrinA1-PE38. The new plasmids were identified by restriction enzyme digestion and DNA sequencing.

The adenovirus was packaged in 293 cells using the AdEasy Adenoviral Vector System following the manufacturer's protocol (Agilent, Santa Clara, CA, USA). The virus titer was determined by infection of 293 cells with serially diluted vector stocks, followed by observation of GFP-positive cells. After three turns of amplification and purification by density gradient centrifugation, high titer recombined Ad-EphrinA1-PE38 adenovirus was harvested and stored in −80 °C until use.

For *ex vivo* gene transduction, 2×10^5 hMSCs were placed in a 24-well plate 1 day before adenovirus infection. The cells were infected with Ad-EphrinA1-PE38 at 800 pfu/cell (unless otherwise stated) for 2 h. Subsequently, the viral supernatants were replaced with fresh medium. The transduction efficiency was confirmed by fluorescence microscopy and viable cells were counted by the trypan blue exclusion assay for 1 week.

2.4. Detection of transgene expression in hMSCs

EphrinA1-PE38 transgene expression in transduced hMSCs cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA