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Tailor-made fibroblast-specific and antibiotic-free interleukin 12 plasmid for gene electrotransfer-mediated cancer immunotherapy

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ABSTRACT

Electrotransfer mediated delivery of interleukin-12 (IL-12) gene, encoded on a plasmid vector, has already been demonstrated to have a potent antitumor efficacy and great potential for clinical application. In the present study, our aim was to construct an optimized IL-12-encoding plasmid that is safe from the regulatory point of view. In light of previous studies demonstrating that IL-12 should be released in a tumor localized manner for optimal efficacy, the strong ubiquitous promoter was replaced with a weak endogenous promoter of the collagen 2 gene, which is specific for fibroblasts. Next, to comply with increasing regulatory demands for clinically used plasmids, the expression cassette was cloned in a plasmid lacking the antibiotic resistance gene. The constructed fibroblast-specific and antibiotic-free IL-12 plasmid was demonstrated to support low IL-12 expression after gene electrotransfer in selected cell lines. Furthermore, the removal of antibiotic resistance did not affect the plasmid expression profile and lowered its cytotoxicity. With optimal IL-12 expression and minimal transgene non-specific effects, i.e., low cytotoxicity, the constructed plasmid could be especially valuable for different modern immunological approaches to achieve localized boosting of the host's immune system.

1. Introduction

Since its discovery in 1989, interleukin 12 (IL-12) has been demonstrated to be a promising anticancer agent that is capable of mediating an endogenous antitumor immune response against a variety of malignancies (Lasek et al., 2014). Although the first clinical studies, using systemic administration of recombinant IL-12 protein resulted in dose limiting toxicities (Cohen, 1995), the development of different gene therapy strategies could harness the powerful action of this pro-inflammatory cytokine by localizing it to the tumor site, lowering the systemic toxicity. Gene therapy clinical studies with IL-12 were conducted either by peritumoral injection of ex vivo transduced autologous fibroblasts or dendritic cells or by direct in situ delivery of the IL-12 gene in viral and non-viral vectors (Lotze et al., 1996; Kang et al., 2001; Hernandez-Alcoceba et al., 2016; Mahvi et al., 2007). Particularly attractive for clinical application is the gene electrotransfer (GET)-mediated delivery of IL-12 (7), primarily due to its ability to deliver genes in a localized manner (Young and Dean, 2015; Yarmush et al., 2014). IL-12 GET was made possible by the progression of the biomedical applications of electrotransfer (Yarmush et al., 2014; Bimonte et al., 2016) and cloning the active IL-12 fusion gene, which consists of p35 and p70 subunits, in plasmid expression vectors (Lee et al., 1998).

The active component of plasmid expression vectors is the expression cassette that carries a transgene and a promoter, driving its transcription (Tolmachov, 2009). Traditionally, this promoter has been the strong constitutive human cytomegalovirus (CMV) promotor that is also used in most IL-12 expressing plasmids. Now, it is widely accepted that the CMV promoter does not provide sustained expression and is susceptible to inactivation in eukaryotic cells due to its viral origin (Sum et al., 2014; Kamensek et al., 2010). Therefore, in newer expression vectors, viral promoters are often replaced with endogenous promoters (Sum et al., 2014).

Among the endogenous cellular promoters, tissue specific promoters have added value because they target specific cell types, where particular transcription factors are expressed (Gill et al., 2009). Skin-specific promoters have already shown promise for DNA vaccination by GET into the skin (Vandermeulen et al., 2009). One promoter candidate suitable for targeting the skin and the connective tissue around the tumor is the promoter of collagen gene. Collagen is the main component of the extracellular matrix (ECM) and is manly produced by fibroblasts,

Abbreviations: IL-12, interleukin 12; GET, gene electrotransfer; CMV, cytomegalovirus; EMA, European medicines agency; ORT, operator-repressor titration; SVEC4–10, murine endothelial cells; HUVEC, human umbilical vein endothelial cells; L929, murine fibroblasts; LPB, murine fibrosarcoma cells; WI-38, human fibroblasts; GFP, green fluorescent protein; EF-1α/HTLV, hybrid elongation factor-1α promoter; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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which surround and are contained within the tumor (Kharaishvili et al., 2014). It consists of 2 a1 chains that are encoded by COL1A1 and COL1A2 genes (National Center for Biotechnology Information, 2016). The promoter of the human COL1A2 gene was tested in our previous study, where we showed that it was able to drive low, localized expression from the transfected skin (Kos et al., 2015).

When designing potential clinically applicable plasmid vectors, the biosafety issues need to be considered. Although plasmid vectors are relatively safe compared to viral vectors, most plasmids carry an antibiotic resistance gene that is needed for plasmid maintenance and production in host bacteria (Gill et al., 2009). This antibiotic resistance gene in the plasmid backbone represents a safety concern due to the risk of horizontal gene transfer of antibiotic resistance traits to environmental and commensal microbes and hypersensitive reactions in the patient to the antibiotic, which can be found in the final product (Mignon et al., 2015). Therefore, the European Medicines Agency (EMA) recommends avoidance of antibiotics when producing plasmids that are intended for human use (The European Agency for the Evaluation of Medicinal Products, 2016). In addition to regulatory safety concerns, antibiotic resistance genes are responsible for other drawbacks in plasmid performance, including lower transfection efficiency, transcriptional inactivation and inflammatory reactions caused by the presence of large prokaryotic sequences (Sum et al., 2014). Different antibiotic-free selection strategies have been developed in recent decades (Vandermeulen et al., 2011). One such strategy is called operator-repressor titration (ORT) and is based on the plasmid mediated repressor titration that activates a bacterial host selectable marker (Williams et al., 1998). ORT technology was previously utilized for preparing plasmids for DNA vaccination and preparing the plasmid antiangiogenic metargidin peptide (AMEP) that was used in a clinical trial, confirming the GET safety (Spanggaard et al., 2013).

In this study, we aimed to prepare an optimized expression plasmid encoding IL-12 under the transcriptional control of a fibroblast specific promoter and principally devoid of an antibiotic resistance gene. The applicability of the new plasmid was verified by determining its production yields after amplification in *Escherichia coli* (*E. coli*) as well as its expression profile and cytotoxicity after GET into different cell lines.

2. Materials and methods

2.1. Plasmid construction

Two commercially available plasmids were used in the study, a plasmid encoding the green fluorescent protein (GFP) that is under the control of the human CMV immediate early enhancer and promoter (pEGFP-N1, Clontech, Basingstoke, UK) and a plasmid encoding mouse IL-12 (mIL-12) that is under the control of a constitutive hybrid elongation factor- 1α promoter EF-1α/HTLV (pORF-mIL-12, Invivogen, Toulouse, France). The source plasmid for a fibroblast specific collagen promoter (Col) sequence was the pDD424 plasmid, which was obtained from prof. David A. Dean (University of Rochester, NY, USA) (Dean, 2013). The pCRBluntPsiCat X-mark plasmid, which was obtained from CobraBio (Keele, UK), was used to prepare the antibiotic-free plasmid. Plasmid were prepared by restriction endonuclease molecular cloning, which was followed by ligation and transformation into competent *E. coli* cells (Tolmachov, 2009; Vosberg, 1977) using reagents (Miniprep kit, TransformAid Bacterial Transformation kit, *E. coli* strain JM107, restriction enzymes, ligation kit, and gel extraction kit) from Thermo Scientific (Waltham, MA, USA). A tissue-specific reporter plasmid (pCol-EGFP) was prepared by cloning the Col promoter sequence from pDD424 into the *SacI* and *PstI* restriction site of plasmid pEGFP-N1. Using *SalI* and *NheI* restriction enzymes, an mIL-12 encoding sequence was excised from pORF-mIL-12 and a therapeutic plasmid expressing mIL-12 under the control of Col promoter (pCol-mIL-12) was constructed by cutting the plasmid pCol-EGFP with *SalI* and *XbaI* restriction enzymes to replaced GFP encoding sequence with the mIL-12 encoding sequence. The mIL-12 expression cassette with the Col promoter was then cloned into the pCRBluntpsiCat plasmid using *XhoI* and *MunI* restriction enzymes, and the therapeutic plasmid expressing mIL-12 under the Col promoter and lacking the antibiotic resistance gene (pCol-mIL-12-ORT) was produced using the antibiotic-free maintenance system ORT® and X-mark™ (CobraBio). DH1-PEPA and DH1-ORT *E.coli* strains, which are needed for preparing antibiotic-free plasmids, were obtained from CobraBio. The newly constructed plasmids were sequenced using MacroGen services (Amsterdam, NL).

2.2. Plasmid yields

For the transfection experiments, plasmids were isolated and purified using an EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany) according to the instructions provided with the kit. The plasmid DNA was eluted in endotoxin-free water (Qiagen) to concentration of 1 mg/ml. The purity and yields were spectrophotometrically determined (Epoch Microplate Spectrophotometer, Take3™ Micro-Volume Plate, BioTek, Bad Friedrichshall, Germany). Additionally, the concentration and identity were confirmed by restriction analysis on an electrophoretic gel.

2.3. Cells

Murine SVEC4–10 endothelial cells, human umbilical vein endothelial cells (HUVEC), murine L929 fibroblasts, murine LPB fibrosarcoma cells, and human WI-38 fibroblasts were used in the experiments. SVEC-10 and HUVEC cell were cultured in advanced Dulbecco modified Eagle medium (ADMEM), and L929, LPB and WI-38 cells were cultured in advanced Eagle minimum essential medium (AMEM) supplemented with 5% fetal bovine serum (FBS, Gibco, Paisley, UK), 10 mM L-glutamine (Gibco) and 50 μg/ml gentamicin (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). For experiments, cells were maintained in monolayers until they reached 80–90% confluence.

2.4. Gene electrotransfer

Cells in the exponential growth phase were trypsinized, washed in appropriate media and centrifuged for 5 min at 1500 rpm. After centrifugation, cells were resuspended in ice cold electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, and 2 mM $MgCl_2$) and prepared at a final concentration of 2.5×10^7 cells/ml. The cell suspension (40 μl) was mixed with 10 μl (4 pmol) of each plasmid and pipetted between stainless steel parallel plate electrodes and square-wave electric pulses (8 × 100 V, 5 ms, 1 Hz) were delivered by a generator of electric pulses (Faculty of Electric Engineering, University of Ljubljana, Ljubljana, SI). Afterwards, GET cells were plated on Petri dishes (Greiner Bio-One, Frichenhausen, Germany) for further experiments.

2.5. Expression of GFP and mIL-12

The expression profiles of plasmids were measured two days after GET. The expression of the GFP reporter gene was measured by flow cytometry, and the expression of mIL-12 therapeutic gene was determined on the mRNA level by the quantitative reverse transcription polymerase chain reaction (qRT-PCR), as well as on the protein level with enzyme-linked immunosorbent assay (ELISA). The expression results after GET of tissue specific plasmids (pCol-EGFP, pCol-mIL-12, and pCol-mIL-12-ORT) were presented as fold values compared to the values obtained after GET of the constitutive plasmid (pEGFP-N1, pORF-mIL-12).

2.5.1. Flow cytometry

48 h after transfection, cells were collected and resuspended in phosphate-buffered saline (PBS) and analyzed with a FASCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of