Research Article

Human scFv SIgA expressed on Lactococcus lactis as a vector for the treatment of mucosal disease

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The gastrointestinal tract is a complex niche and the main port of entry of many pathogens that trigger a wide range of diseases like inflammatory bowel disease (IBD) and colon cancer. Antibodies are effective for treating such diseases, but a system capable of local delivery at the site of the pathology, thus avoiding systemic side effects, is not yet available. Here we report a novel recombinant scFvSIgA1 protein produced by Lactococcus lactis, anchored to the bacterial membrane, which retains its full immuno-recognizing potential. This scFv fragment employed was specific for a colon cancer epitope, epithelial glycoprotein protein-2 (EGP-2). Accordingly L. lactis expressing this chimeric protein was capable of binding cells expressing this epitope. Expression of specific antibodies on bacteria may allow local delivery of anticancer agents produced by such bacteria in conjunction with the antibody and provides a new avenue in the quest for targeted drug delivery.

Keywords: Lactococcus lactis / Local delivery system / pIgR / Recombinant / scFv SIgA

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1 Introduction

The gastrointestinal tract is the locus for many different pathologies, including cancer and inflammatory disease, whose treatment remains problematic. Although substantial insight has been gained in the pathogenesis of these diseases [1, 2], translating this knowledge to novel therapy remains troublesome. These problems derive from the difficulty of delivering functional protein therapeutics to the mucosa of the digestive tract, mainly as a consequence of the protein-structure hostile nature of the lumen of the tract and the occurrence of unwanted side effects when such proteins are introduced in a systemic fashion. Molecules that normally reside in the tract, however, should be able to withstand protein degradation. The principal antibody on most mucosal surfaces is secretory Ig A (SIgA). It is a polypeptide complex consisting of dimeric IgA (SIgA). It is a polypeptide complex consisting of dimeric IgA (SIgA).
produced IgA are reduced, e.g., in selective IgA deficiency, and in HIV patients IgA is reduced or absent. Similar conditions appear when patients are treated with immunosuppressive drugs. Evidently, however, immunoneutralization of specific disease-related proteins is also an exiting possibility (e.g., neutralization of mucosal TNF in Crohn’s disease; [3]). The possible use of SlgA for the treatment of disease is further facilitated by the advent of DNA recombinant technology which now enables the generation of so-called chimeric minibodies incorporating selective antibody fragments (single chain variable fragments, scFv). The problem however as to how to get such molecules at their intended site of action in the digestive tract remains.

Poly Ig receptor (pIgR) is expressed on all the intestinal epithelial cells [4]. Dimeric IgA, secreted by the plasma cells, binds to the pIgR present on epithelial cells of the mucosa and is subsequently transported to the lumen of the gut. Then the IgA is released into the gut lumen together with the extracellular part of the pIgR as SlgA [5]. pIgR is composed of six domains, with the sixth domain containing the protease cleavage site. This cleavage site on the pIgR can be exploited for specific drug delivery because epithelial cells have protease activity capable of specifically hydrolyzing and cleaving this site in the complex. A part of the pIgR remains bound to the polymeric Ig to become SlgA or SlgM.

A specific receptor for the IgA Fc region (FcRRI) exists; it is a novel apoptotic unit active systemically but not in the mucosa. In addition to its anti-inflammatory and inhibitory signaling action, it seems essential for controlling tumor growth [6]. A number of reports showed that therapeutic antibodies are able to trigger immune responses via FcRI. These antibodies can either be intact IgA or bispecific antibodies (BsAb) that recognize both the ectodomain for FcRI and the tumor antigen of interest [7]. In a comparison of hapten-directed antibodies of different human isotypes, IgA2 was found to be more effective. Also, IgG isotypes recruits neutrophils and induces tumor cell death [7]. Similarly, an IgA1 antibody against EpCAM proved more effective than IgG1 recruiting neutrophils to kill EpCAM-positive tumor cells in solid tumors [8]. Ideally, such antibodies would only be delivered at the intended site of action, i.e., the digestive tract where the EpCAM-bearing colon (pre-) cancer cell resides, but strategies for gut-specific delivery have been poorly researched hitherto.

Despite the high production costs and the occurrence of systemic side effects, antibody-based immunotherapy has become an effective treatment for a number of different cancers. Therapeutic mAb can be used to induce tumor cell death by a variety of mechanisms. For example, by cross-linking antigens on tumor cells leading to apoptosis, cell cycle arrest or the inhibition of cell proliferation [9]. Smaller recombinant engineered antibody fragments and variants thereof are now emerging as credible conventional antibody-based immunotherapy. These fragments preserve the targeting specificity of whole mAbs and can be economical, furthermore possess a wide range of diagnostic and therapeutic efficacy. The first clinical trial using scFv in ovarian cancer patients was reported in 2000 by the group of Alvarez et al. [10]. This successful clinical trial opened a new era to many recombinant antibodies fragment variants like diabodies, triabodies, etc. These fragments are extensively studied in various human carcinomas such as colorectal, breast, and small cell lung cancer [11–13]. The possibility to engineer this treasure of immunoneutralizing DNA sequences into stable IgA molecules greatly adds to the appeal of SlgA exotherapy, despite the as yet unsolved production and delivery problems.

A possible target for SlgA exotherapy is epithelial glycoprotein-2 (EGP-2), a well-established target antigen in colorectal cancer that is overexpressed on the cell surface of various human carcinomas [11, 12]. Whereas in normal epithelia, EGP-2 expression is limited to the baso-lateral membrane and thus inaccessible for SlgA molecules present in lumen of the tracts, colorectal cancer cells lose this polarity of EGP-2 expression [14, 15]. The advantage of targeting EGP-2 is that it is not present in the circulation and has been extensively studied in antibody-mediated imaging [16–18]. The minibody MOC31 scFv-Fc recognizes human EGP-2 and harbors human constant gamma 1 chain Fc domain and is thus ideally suited for engineering into SlgA for colon cancer SlgA exotherapy. Such SlgA exotherapy still critically awaits a solution to the delivery problems.

Importantly a new mucosal delivery strategy has emerged via the execution of a first clinical trial using transgenic Lactococcus lactis producing IL-10 to treat Crohn’s disease, which was published by Braat et al. [19]. If L. lactis can be coached to produce scFv, the window to clinically useful SlgA exotherapy for colon cancer therapy and for other treatment diseases, will be opened. This consideration prompted us to construct scFvMOC31 IgA for targeting colon cancer employing L. lactis as a heterologous expression system. The fragment antigen binding (Fab) region was derived from mAb MOC31 that can recognize EGP-2 and it was extended with constant γ gene. The sixth domain (Sc) contains the cleaving sites and the remaining five domains of pIgR were cloned resulting in MOC31-SlgA1. Finally, we expressed this scFv as a part of natural membrane protein of L. lactis bacteria. Membrane expression was confirmed by Western blotting. Despite these many changes in the MOC31 protein, it could still recognize the EGP-2 antigen which was transiently expressed on the Ramos B cells. Thus, in this paper, we report an important next step in the quest for anticancer SlgA exotherapy. Furthermore, the ability of antibody-expressing bacteria to bind to specific motifs also enables specific targeting to cell expressing such motifs and should thus allow treatment of these cells with bacterially produced products co-engineered into the bacteria with the antibody.
Table 1. Primer list, which was used to amplify different gene to construct scFv SIgA

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Restriction enzyme sites</th>
<th>Gene of interest to amplify</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgAEcoRI2F</td>
<td>CTGAATTCGGAGGCAGCCCCAAGGG</td>
<td>EcoRI</td>
<td>Cα region</td>
</tr>
<tr>
<td>2</td>
<td>IgHindIII2B</td>
<td>CCAAGCTTGTAGCAGGCTGCTCCA</td>
<td>HindIII</td>
<td>Cα region</td>
</tr>
<tr>
<td>3</td>
<td>SC1bgl2kas1F</td>
<td>AGATCTAGGGGCCGACGCTGCTCTGATG</td>
<td>AflIII &amp; HindIII</td>
<td>SC1 sixth domain of PIgR</td>
</tr>
<tr>
<td>4</td>
<td>SC1pfo1af2B</td>
<td>TCCTGGACTTAAGCTGCGGAAATCCAC</td>
<td>Van91I &amp; AflIII</td>
<td>SC1 sixth domain of PIgR</td>
</tr>
<tr>
<td>5</td>
<td>SC2hind3cla1F</td>
<td>CAAAGAAGCTTATGGAATGTTGTCCTCCGCCGAGGAGTG</td>
<td>HindIII &amp; ClaI</td>
<td>SC2 five domains of PIgR</td>
</tr>
<tr>
<td>6</td>
<td>SC2van91sal1B</td>
<td>AATCCATAGGTGTTAGCTGACGCTGCCGAGCTCCCTTCCTTC</td>
<td>Van91 Sal</td>
<td>SC2 five domains of PIgR</td>
</tr>
<tr>
<td>7</td>
<td>extraSC1Afl2age1cla1F</td>
<td>CCTTAGAGCGGCTATGAGATGTCGTC</td>
<td>AflII, ClaI</td>
<td>Extra SC1 sixth domain of PIgR</td>
</tr>
<tr>
<td>8</td>
<td>ExtraSC1pfo1B</td>
<td>TCCTGGAGAATCCACAGATG</td>
<td>AflII</td>
<td>Extra SC1 sixth domain of PIgR</td>
</tr>
<tr>
<td>9</td>
<td>LL-AflIII-startsIgA-F</td>
<td>ACGCCTGACCCATGGAAGAGCA</td>
<td>AflII</td>
<td>Extra Afl III site</td>
</tr>
<tr>
<td>10</td>
<td>LL-AflIII-BasIII-sIgA-B</td>
<td>ACTTAAGGCGGCCGCTCCGGGAATCCACA</td>
<td>AflII, BasIII</td>
<td>Extra Afl III site</td>
</tr>
<tr>
<td>11</td>
<td>Van91I-AflIII-end-sIgA F</td>
<td>CCAACCTATGGACGGCTATGGAATGAGAGCAGTG</td>
<td>Van91F</td>
<td>Extra Afl III site end SIgA</td>
</tr>
<tr>
<td>12</td>
<td>Muni-end-sIgA B</td>
<td>CAATTGTTCCAGGGGAGGTT</td>
<td>Muni</td>
<td>Extra Afl III site end SIgA</td>
</tr>
</tbody>
</table>

The restriction enzymes introduced into the vector are underlined and bold.

2 Materials and methods

2.1 Construction of scFvMOC31IgA1/2

The Ca1/2 genes were amplified from normal ileum of human cDNA with EcoRI/HindIII restriction sequences (Table 1) using PCR method. Cy1 region present in MOC31IgG plasmid [13] was replaced with Cα1 and Cα2 using above mentioned restriction enzymes to get MOC31-IgA1 and MOC31-IgA2 constructs, respectively (Fig. 1B).

2.2 Construction of scFvMOC31IgA1SC1

The sixth domain of human SC was amplified from SC-his6 plasmid [20] and was designated as SC1. MOC31SC1IgA1 and MOC31SC1IgA2 were constructed by inserting SC1 using BglII/PfoI restriction enzymes into MOC31-IgA1 and MOC31-IgA2 (Fig. 1C).

2.3 SC2

The remaining five domains of human SC (referred as SC2) were amplified from SC-his plasmid. For subcloning, S’ HindIII and S’ Van91I were introduced in SC2. The entire five domains were cloned into the MOC31SC1IgA1 and MOC31SC1IgA2 to get MOC31SIgA1/SIgA2 (Figs. 1D and 2A).

2.4 pNZmlePSIgA1

pNZmleP produce a membrane protein that is a malate transporter protein in *L. lactis*. The pNZmleP was constructed as described in ref. [21]. To ligate the entire MOC31SIgA1 into the pNZmleP, AflIII restriction site was used. An additional AflIII restriction site was added at the 3’ end of the MOC31SIgA1 by PCR. MOC31SIgA1 was ligated at the C-terminal of the *mlep* gene at the AflIII restriction site (Table 1). Only the cells with the right orientation plasmid can grow. Because the AflIII restriction enzymes digest ACRYGT and we used different nucleotides at the beginning and at the end of the MOC31SIgA1 sequence. Ligation mixtures were transformed to *L. lactis* NZ9000 using an electroporation protocol described by Holo and Nes [22] (Fig. 2b).

2.5 COS-7 cells transfection

Two six-well plates of COS-7 cells were transiently transfected with supercoiled plasmid DNA using the GeneJammer transfection reagent (Stratagene) according to the manufacturer’s recommendations. Transfection efficiency was normally in the range of 5–10%. After 2 days, one transfected plate was fixed with acetic acid and ethanol. These cells were used for immunofluorescent staining. The second plate was incubated for 3 days and scFvSIgA1 protein conditioned medium was aspirated and stored at −20°C until analysis. The rest of the COS-7 cells were washed twice with PBS, pelleted, and stored at −20°C until analysis. Later, the proteins from these cells were extracted and stored at −80°C.

2.6 Bacterial strains, growth condition, and induction of proteins

In our experiments *Escherichia coli* K-12 strain was grown on LB agar and broth. The cells were induced by IPTG to produce the proteins. *L. lactis* strain NZ9000 was cultivated semianerobically at 30°C in M17 broth at pH 6.5, supplemented with 1.0% (w/v) glucose and 5 μg/mL chloramphenicol when carrying pNZmleP or derivatives. For isolation of membrane vesicles, cells were grown to an OD600 of 1, after which transcription from the *nisA* promoter was switched on by the addition of 0.2% v/v culture supernatant of the nisin A producing strain NZ9700 [23]. Cells were harvested 1 h after the induction of nisin.
2.7 Membrane vesicle preparation of MOC31pNZmlepSIgA1

Cell were washed with 100 mM KPO4, pH 7.0 and then resuspended in 100 mM KPO4, pH 7.0 with 20% glycerol. Cells were recaptured in a bead beater at force 6.5 for 30 s for three times. Whole cells were removed by centrifugation at 16000 \(\times g\) for 15 min and membrane fractions were isolated by centrifugations at 267000 \(\times g\) for 30 min. Vesicles were resuspended in 100 mM KPO4, pH 7.0 with 20% glycerol and stored in \(-80^\circ\)C until used.

2.8 Immunofluorescent staining

Cover slips containing transfected cells were fixed with ethanol and acetic acid. The cells transfected with MOC31-IgA variants were stained with antihuman IgA-FITC (Dako). MOC31-IgG transfected cells were stained with Alexa fluor 488 goat antihuman IgG (H + L) (molecular probes). DAPI was used to counter stain the nuclei.

2.9 Enzyme-linked immunosorbent-assay (ELISA)

scFv MOC variant levels in cultured supernatant and in protein extract were measured with an IgA- and IgG-specific ELISA. Duplicate wells of serially diluted supernatants were analyzed as described previously [24].

2.10 Jurkat and Ramos cells FACS

Human cell lines Jurkat and Ramos were stably transfected with EGP-2 as described previously [14] and cultured in RPMI (invitrogen) media supplemented with 15% fetal calf serum. These cells were incubated for 30 min with transfected COS-7 supernatant and protein extract of different MOC variants. After a brief wash the cells were incubated with goat antihuman IgA-PE. The FACS analysis was performed on Coulter EpicTM Elite flow cytometer. Data were analyzed by using Flowjo software version 5.4.5 according to the manufactures protocol (Tree Star).
2.11 Western blotting

Expression of all MOC31 scFv variants were confirmed by Western blotting. The expected size of proteins and percentage of the gels are described in the Table 2 and gels were stained with comassie blue. For immunodetection, samples were separated by reduced SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBS-T buffer (PBS containing 0.05% Tween 20), and then incubated with different (Table 2) antibodies separately for 2 h. The membrane was washed three times with PBS-T buffer and then incubated in HRP conjugated secondary antibody for 1 h. The membrane was washed three times with PBS-T buffer and the expressed proteins were visualized using ECL plus Western blotting detection system (Amersham).

3 Results

3.1 Construction of scFvMOC31: SIgA1

DNA encoding the Ca1 and Ca2 of IgA1/IgA2 was generated by PCR. The resulting 1081 and 1053 bp PCR product encoded amino acids 330 and 317 of Ca1 and Ca2, respectively. These DNA fragments were inserted separately in the EcoRI and HindIII of eukaryotic expression vector pMOC31IgG1, yielding plasmid pMOC31IgA1/IgA2. Sequence analysis confirmed the correct and in-frame of the pMOC31IgA1/IgA2 (data not shown).

3.2 SC1

SC1 is the sixth domain of the SC that contains the proteolytic cleavage site. SC1 DNA fragment was amplified from the entire SC vector. BglII and PfoI that are present within the framework one region were used for inserting the SC1. Using these restriction sites, 58 nucleotides from the end of FR1 and beginning of CDR1 region were deleted. This resulted in a new plasmid pMOC31IgA1SC1.

3.3 SC2

SC2 contained the two domains of the SC was amplified with HindIII and Van9JI site. A stop was inserted at the beginning of the second domain, because the domains 3 and 4 are used by the Streptococcus pneumoniae to invade the epithelial cells [25]. The resulting 700 bp PCR product was successfully cloned into the MOC31IgA1SC1 plasmid to get MOC31SIgA1.

3.4 pNZmlePSIgA1

The entire MOC31SIgA1 was ligated to the plasmid pNZmleP at the AflIII restriction site. AflIII has a special feature since it recognize ACRYGT (where R stands for A or G and Y for C or T). In plasmid pMOC31SIgA this rec-
3.5 COS-7 cells transfection

COS-7 cells were transiently transfected with the entire MOC31 variant. Right orientation of the constructs was validated by this method as well as by sequencing. The transfection efficiency of eukaryotic expression vector MOC31SIgA1 was 10% as shown by immunopositive for antihuman IgA-FITC antibodies (Fig. 3). The presence of secreted scFv MOC31 variants in the transfected COS-7 cells supernatant were analyzed by ELISA (data not shown). The positive control MOC31IgG1 was produced in higher quantity than the other scFv variants.

3.6 Western blotting

Western blotting was performed on all the MOC variants. MOC31 transfected COS-7 supernatant and COS-7 cell lysate were used for Western blotting. All the products gave a expected band with a correct size (Fig. 4). Membrane vesicles were prepared from MOC31pNZmlePSIgA1 followed by Western blotting for pNZmleP and pNZmlep-SIgA1. Total protein precipitation of L. lactis with TCA was performed and used for Western blotting. From the above set of experiments we concluded that all the MOC variants were produced, but with a low yield. In order to test the functional activity of these proteins, the following experiments where performed.

3.7 Jurkat and Ramos cells FACS

Flow cytometric analysis of the EGP-2 retrovirally transduced cell lines Jurkat and, Ramos cells confirmed the cell surface expression of EGP-2. MOC31-IgA containing supernatant were able to bind to these EGP-2 on these cell surfaces and gave strong increase in percentage of staining by functionally active scFv. The binding of MOC31-IgA containing supernatant to the EGP-2 expressed Ramos cells proves that IgA is indeed functional (Fig. 5).

4 Discussion

The clinical trial employing IL-10 expressing L. lactis for the treatment of Crohn's disease has demonstrated that such bacteria constitute a viable strategy for the mucosal delivery of therapeutically interesting proteins [19]. This consideration prompted us to explore the potential usefulness for such bacteria to deliver the gut-stable IgA molecules. This would be important because already a large variety of antibody fragments is available and immunoneutralizing a large spectrum of clinically interesting molecules and their local production in a gut-stable fashion could be exceedingly useful. Here we report a novel recombinant scFvSIgA1 protein

Figure 3. COS-7 cells were transfected with MOC31 vectors, followed by staining with antihuman IgA FITC and DAPI.

Figure 4. Analysis of recombinant scFv polypeptide, proteins expression and their identity confirmed by immunodetection using specific antibodies. The polyacrylamide with different percentage of SDS-containing gels was prepared. The M of the scFv and Ab indicated was calculated from protein markers run in parallel.
produced by *L. lactis* anchored to their membrane which retains its full immuno-recognizing potential. As a model of an example intestinal disease for which such SIgA exotheraphy might be useful, we targeted our scFv to colon cancer. With the recent advances in molecular biology, it has become possible to translate improved characterization of tumor antigens to tailored design of recombinant antibodies specific for these antigens. ScFv molecules on their own generally do not have any built-in effector capacity nor are they able to engage natural effector molecules. We generated an eukaryotic expression plasmid, scFvSIgA1, that specifically could recognize antigen. To demonstrate the usefulness of our vector system we analyzed it specificity by binding to EGP-2 antigen, but it is obvious that the same strategy can be employed with any scFv fragment.

Upon exposure to nontolerated antigens, the human humoral immune response generated a remarkable range of antibodies. In this study we attempt to design tumor targeting human recombinant scFvSIgA1 with binding specificity for the antigen EGP-2. This molecule contains an antigen binding site (Fab) which specifically binds to the EGP-2 antigen, constant a gene as their Fc region and the SC domains. The sixth domain of SC that contains the cleavage site was designated as SC1 and was inserted at the beginning of the Fab region and other domains of SC were included after the Fc region (Fig.2). EGP-2 is abundantly present in the cells of human carcinomas of different origins and more importantly such carcinoma cells express this protein at apical side of the cell (in contrast to untransformed cells) making this epitope accessible for SIgA from the lumen. Various mAbs directed against EGP-2 have been extensively studied in diagnostic and therapeutic approaches in cancer [14, 15]. As colon cancer which also express high amount of EGP-2, this disease is a candidate for experimental treatment with the scFvSIgA producing organism created in the present study. In this context it is especially interesting that the antibody can be used to target the bacterium to the cancer cell, whereas a secondary produced bacterial product can then be employed to kill the cancer cell. In this context it is important to mention that whereas the SC2 part makes the scFvSIgA more stable [26] in the intestinal niche, the SC1 contains a peptidase cleavage site. This site can be cleaved by the protease enzymes produced by the epithelial cells. By fusing small molecules like cytokines or signaling molecules between the SC1 could be delivered locally to the affected cells.

The IgA Fc region was used to construct scFvSIgA because IgA is a mucosal Ig and contains intrinsic properties generally considered to confer protection to the Ig in the hostile gut environment. Many monoclonal antibodies and scFv are specifically targeted against EGP-2 in tumor related to systemic immunity [27]. These antibodies or the scFv contains the IgG Fc region. Upon binding to the Fc receptor on effector cells they elicit effector function like antigen - dependant cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) response and apoptosis [28]. It is well documented that FcRII elicit immune responses more efficient than IgG Fc receptors [29]. Overall it seems that the use of IgA Fc region may be superior both in terms of stability of the chimeric molecule and in terms of bioeffectivity, and thus offers advantages for the development of antibodies to treat intestinal carcinoma and other mucosal disease.

Delivery of therapeutical proteins locally have many advantages over the administrating systemically [30]. This recombinant human scFvSIgA is successfully expressed on the surface of the bacteria like *L. lactis* which is generally regard as safe (GRAS). Hence, delivery via oral administration of the organism for treating gastrointestinal cancer cells should be possible. Interestingly, the specificity of the antibody can be altered by changing the Fab region. By selecting appropriate *V* _H_ genes one can generate natural antibodies or pathogen specific antibodies, of obvious interest for treatment of IgA deficiency patients or other intestinal diseases.

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5 References