The use of recombinant vaccinia virus to generate monoclonal antibodies against the cell-surface glycoprotein endoglin

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Abstract Characterization of novel cell-surface protein molecules, initially identified by cDNA cloning techniques, usually requires the generation of specific antibodies to further analyze their biochemical and/or functional properties. Here we report a simple method, using recombinant vaccinia virus, for the generation of monoclonal antibodies (mAb) to the cell-surface antigen endoglin. A recombinant vaccinia virus carrying a cDNA encoding human endoglin was inserted into the thymidine kinase locus under the control of the 7.5k vaccinia virus promoter. Infection of Balb/c mice with this recombinant virus led to the generation of specific polyclonal antibodies, as demonstrated by the antisera reactivity against human endoglin transfectants. The spleen cells of these infected animals were fused to myeloma cells, allowing efficient generation of several hybridomas which secrete mAbs to human endoglin, as evidenced by their reactivity with purified endoglin as well as with endoglin transfectants. Some of the mAbs selected seem to be specific for regions of endoglin conserved among different species as evidenced by their cross-reactivity with chicken endoglin. These results underline the utility of recombinant vaccinia virus to generate antibodies with novel properties to new cell surface proteins such as endoglin.

Key words: Monoclonal antibody; Vaccinia virus; Surface antigen; Endoglin (human)

1. Introduction

Monoclonal antibodies (mAb) specific to cell-surface antigens have systematically provided a means of identifying and characterizing the structure and function of many integral membrane glycoproteins. The fast advances in molecular biology cloning have allowed the isolation of novel genes encoding new membrane proteins. However, the initial characterization of these novel gene products is usually hampered by the lack of specific antibodies. To circumvent this problem some investigators have employed the approach of modifying the cDNA sequence by including a sequence tag within the coding region. Examples of these encoded tags include hexahistidine, influenza haemagglutinin (YPYDVPDYA), vesicular stomatitis virus glycoprotein (YTDIEMNRLGK), and c-myc (EQKLISEEDL) peptides, which can be easily recognized, within the corresponding fusion protein, by commercially available antibodies. However, the utility of this tag method is exclusively restricted to the analysis of those cells expressing the gene of interest by transfection. On the other hand, some authors have used direct immunization of the animals with cDNA [1,2], but the antibody titers of the corresponding sera are usually low.

Based on the efficient immune response elicited upon infection with the vaccinia virus, the technology of constructing recombinant vaccinia viruses has opened up a new avenue to the production of safe and efficacious vaccines [3,4]. In terms of antibody response, the most successful antigens expressed from recombinant vaccinia viruses have been surface glycoproteins such as rabies G-protein, influenza haemaglutinin, or the rinderpest virus HN and F-proteins. Here, we have constructed a recombinant vaccinia virus expressing human endoglin, a surface component of the TGF-β receptor complex [5]. Infection of Balb/c mice with this recombinant virus has allowed the efficient generation of hybridomas producing specific mAbs to endoglin.

2. Materials and methods

2.1. Recombinant vaccinia virus construction

Culture of the Western reserve strain vaccinia virus and isolation of recombinant virus was performed as described [6]. Basically, plasmid pCEXV containing the L-isoform of human endoglin [7] was digested with *Eco*RI, and the resulting fragment was incubated with DNA polymerase I (Klenow fragment) and inserted into the *Sma*I site of vaccinia virus plasmid pSC11. The resulting plasmid is a vector that coexpresses the *CD105* gene inserted downstream of the 7.5k vaccinia virus promoter and the *Escherichia coli* β-*galactosidase* gene under the control of the vaccinia virus 11k late promoter and flanked by viral thymidine kinase sequences. Thus, upon recombination in infected cells, both genes were inserted into the thymidine kinase locus of the vaccinia virus genome [8].

2.2. Generation and selection of mAbs to endoglin

Balb/c mice were injected intraperitoneally with 10^7 pfu of either VV-L-Endo or VV-TK- in PBS on days −30 and −14. At day 0, animals were bled and spleen cells were fused with SP2 mouse myeloma cells at a ratio of 4:1 according to standard techniques [9]. Hybridoma culture supernatants were sequentially screened first by ELISA against purified endoglin coated to plastic, by flow cytometry analysis against rat myoblast transfectants expressing endoglin, and by Western blot analysis with purified endoglin. Hybridomas producing mAbs specific for endoglin were subjected to two rounds of cloning by limiting dilution in flat-bottomed 96-well plates.

2.3. ELISA

Endoglin was purified by an immunoaffinity chromatography technique from human placenta as previously described for other cell surface glycoproteins [9,10]. Purified endoglin was diluted in 25 mM Tris-HCl, 150 mM NaCl, pH 8.2, at a final concentration of 10 μg/ml and 50 μl of this solution were incubated overnight at 4°C in each well of plastic 96-well flat-bottomed plates. Following saturation (2 h at room temperature) of the remaining free plastic sites with 200 μl of 2% w/v bovine serum albumin in phosphate-buffered saline (PBS;

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pH 7.4), wells were washed 3 times with PBS and incubated for 30 min at room temperature with 50 μl of the corresponding hybridoma culture supernatant. After three washes with PBS, 50 μl of a 1:1000 dilution in RPMI medium of peroxidase-conjugated goat anti-mouse IgG (Sigma) were added to each well and incubated for 30 min. After three additional washes with PBS, 100 μl of the peroxidase substrate *o*-phenylenediamine dissolved (0.12% w/v) in 0.2 M Na_2HPO_4, 0.1 M citric acid buffer, pH 5, (0.12% w/v) and 0.108% v/v H_2O_2 were added to each well and the reaction allowed to proceed until detectable yellow-brown colour is developed. Absorbance of each well at 450 nm wavelength is finally measured in a SLT-Lab Instruments (Austria) ELISA reader.

2.4. Flow cytometry

L6E9 rat myoblast transfectants expressing human endoglin were generated as described [7]. For flow cytometry analyses, these cells or chicken fibroblasts (5×10^5) derived from primary cultures (a generous gift from Dr. J. Martín-Pérez, IIB-CSIC, Madrid, Spain) were incubated with the indicated antibodies for 30 min at 4°C in a flexiwell plate (Dynatech). After three washes with 200 μl of RPMI, FITC-conjugated sheep anti-mouse IgG secondary antibody (Sigma) was added and incubation proceeded for an additional period of 30 min at 4°C. Finally, cells were washed twice with PBS and their fluorescence was estimated with a FACScan (Becton Dickinson), using logarithmic amplifiers. Antibodies used were from the indicated culture supernatants of hybridomas (anti-endoglin), or from sera of vaccinia infected mice at a 1:1000 dilution. Control mAbs used in this study were TS2/16 (anti-human CD29), X63 (negative control) and TEA 1/62.2 (anti-human endoglin) [11].

2.5. Western blot analysis

Purified endoglin from human placenta ($\approx$1 μg/lane) was subjected to SDS-PAGE on 7% polyacrylamide gels under non-reducing conditions. Protein was electrophoretically transferred to nitrocellulose membranes using a semi-dry transfer cell (Bio-Rad). Filters were blocked with binding buffer (10 mM Tris-HCl, 100 mM NaCl, 0.2% Tween-20, pH 7.5), containing 5% non-fat dry milk for 2 h at room temperature. Specific immunodetection was carried out by incubation with the corresponding mAb in binding buffer 5% non-fat dry milk for 1 h at room temperature. After extensive washing in binding buffer, antibody binding to the membrane was detected with peroxidase-conjugated goat anti-mouse antibody (Sigma). The presence of endoglin was revealed using a chemiluminescence assay (ECL detection kit, Amersham Ibérica, Madrid, Spain).

2.6. Immunoprecipitation analysis

Human promonocytic U-937 cells were treated with 10 ng/ml of phorbol esters to overexpress endoglin as described [12]. Treated cells were iodinated with 0.5 mCi of ^{125}I-labeled sodium iodine (ICN Biomedicals, Irvine, CA) using 1,3,4,6-tetrachloro-3α,6a-diphenylglycoluril (Iodogen, Sigma, St. Louis, MO) and lysed in PBS containing 1% Triton X-100, 1% BSA, 1 mM PMSF, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4, for 15 min. Lysates were immediately clarified by centrifugation at $700\times g$ for 30 min and precleared with 30 μl of protein A–Sepharose (Sigma). For immunoprecipitations, equal amounts of radioactive cell lysates were incubated at 4°C with 100 μl of corresponding hybridoma culture supernatants or 5 mg of purified mAb. After 2 h, 100 μl of culture supernatant containing rat anti-mouse IgG mAb 187.1 were added and incubation proceeded for an additional period of 2 h at 4°C. Finally, immunoprecipitates were removed by additions of 30 μl of protein A–Sepharose, incubation for 1 h with continuous stirring and centrifugation at $200\times g$. Samples were electrophoresed on SDS–7% polyacrylamide gels followed by exposure and analysis in a Molecular Imager System (GS-525, Bio-Rad).

3. Results and discussion

Endoglin is a surface component of the TGF-β receptor system expressed at high levels in endothelial cells [5,13,14]. The importance of endoglin in the biology of endothelial cells is demonstrated by the fact that mutations in the coding region of the *endoglin* gene are responsible for the disease known as hereditary haemorrhagic telangiectasia type 1 [15]. Expression of human endoglin can be achieved in vitro using recombinant vaccinia virus [8]. In order to facilitate functional studies on human endoglin we were interested in generating novel mAbs to human endoglin. To attain this, mice were infected with endoglin recombinant vaccinia virus and, to assess the in vivo expression of human endoglin, the antibody specificity of their sera was analyzed by flow cytometry (Fig. 1). VV-L-Endo-infected animals displayed a high positive titer of antibodies specific for endoglin compared with the negative response of VV-TK-infected or naive animals, demonstrating the in vivo expression of endoglin upon infection with the recombinant virus. Next, spleen cells from these animals were fused to myeloma cells and the hybridoma culture supernatants were screened by ELISA against endoglin-coated wells. We found that 18 out of 496 supernatants clearly recognized purified endoglin. Of these, 12 representative mAbs are shown in Table 1. Most of these mAbs also recognized endoglin expressed on the cell surface of mouse transfectants, as determined by flow cytometry (Table 1). Moreover, some of these mAbs were also able to recognize endoglin as determined by Western blot (Fig. 2A) and immunoprecipitation under non-reducing conditions (Fig. 2B) analyses, being the molecular mass of the protein recognized 180 kDa which is consistent with the published data. The specificity of these antibodies for endoglin was confirmed by their lack of reactivity in Western blots against other purified proteins such as bovine serum albumin (BSA) or human purified β1 integrin (not shown). Interestingly, some of these mAbs did show a specific cross-reactivity against chick (and rat) endoglin (Table 2) which is an extremely useful feature that will permit the use of these antibodies to probe the functional relevance in vivo of endoglin in different animal models, such as the induction of angiogenesis in chick chorioallantoic membranes.

Taken together, these results demonstrate that recombinant vaccinia virus can be used to generate mAbs to cell surface

Table 1
Screening of hybridoma supernatants specific for endoglin

mAb	ELISA[a,c]	Flow cytometry[b,c]
X63	0.17	10.3
TS2/16	0.19	13.6
TEA1/62.2	0.72	191.9
1B4	0.33	104.1
1B5	0.62	78.0
2E5	0.37	48.2
2H6	0.61	182.6
3A8	0.36	91.5
4B2	0.49	82.6
4D3	0.89	241.0
4F9	0.37	65.1
5A10	0.30	30.2
5E5	0.41	30.1
5H1	0.68	79.5
5H11	0.41	156.2

[a]Endoglin-coated 96-well plates were incubated with the indicated hybridoma supernatants in duplicate. The bound mAb was estimated by using a goat anti-mouse IgG coupled to peroxidase and estimating the absorbance at 450 nm. Values correspond to one representative experiment out of three different ones. SD was always $<10\%$.

[b]Myoblast transfectants expressing human endoglin were stained with the indicated hybridoma supernatants and analysed by flow cytometry. Values represent the mean channel fluorescence intensity obtained.

[c]Culture supernatants containing mAb TEA1/62.2 (anti-endoglin), TS2/16 (anti-β1 integrin) or X63 (myeloma) were used as a positive and negative controls, respectively.

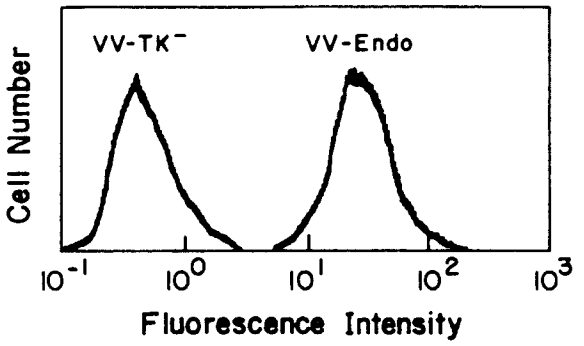

Fig. 1. Expression of recombinant endoglin in mice infected with recombinant vaccinia virus. Mice were immunized with recombinant vaccinia virus VV-Endo or with control VV-TK⁻ and sera was collected and assayed by flow cytometry against rat myoblast transfectants expressing L-endoglin. Sera from 12 different animals were found to specifically recognize human endoglin on the cell transfectants. A representative experiment is shown. The reactivity of the sera from VV-TK⁻ infected animals, used as a negative control, was similar to that obtained with sera from naive animals.

glycoproteins like endoglin. This system takes advantage of the DNA recombinant techniques, allowing efficient immunization with the vaccinia virus containing the cDNA coding for the protein of interest. Other methods use naked DNA vaccines to generate specific antibody responses [1,2]. These DNA vaccines consist of plasmid DNA expression vectors that, when administered to an animal, result in the induction of antigen-specific immunity. Thus, the elicited immune response depends on the efficiency of the gene transfer process which is usually very low. This is in contrast with the broad infection of a natural mammalian host with vaccinia virus, whose efficiency is only limited by lethality at very high doses. In our hands, relatively low doses of vaccinia were sufficient to immunize animals against the recombinant protein. Under these conditions, the vaccinia virus is cleared up from the circulation in approximately 6–8 days upon infection [3] leaving the animal completely uninfected by the time of the cell fusion. This represents an advantage over the retroviral expression systems of surface antigens [16] as the retrovirus is stably integrated into the cells of the infected animal. Finally, recombinant vaccinia virus can efficiently express endoglin in vitro at the surface of infected cells [8], allowing not only the immunization of the animals, but also the screening of specific hybridoma, in the absence of purified antigen. In summary, this report indicates that successful immunization with a recombinant vaccinia virus can be combined with the hybridoma technology to yield specific mAbs with novel properties (cross-species reactivity) to a human cell surface antigen like endoglin.

Table 2
Reactivity of hybridoma supernatants with endoglin expressed on chicken fibroblasts

mAb	Mean fluorescence intensity[a,b]
X63	6.0
TS2/16	6.8
TEA1/62.2	10.2
1B4	132.8
2E5	246.5
2H6	12.0
3A8	53.6
4B2	8.4
4D3	7.6

[a]Chicken fibroblasts were stained with the indicated hybridoma supernatants and analysed by flow cytometry. Values represent the mean channel fluorescence intensity obtained. Culture supernatant containing mAb X63 (myeloma) was used as a negative control.

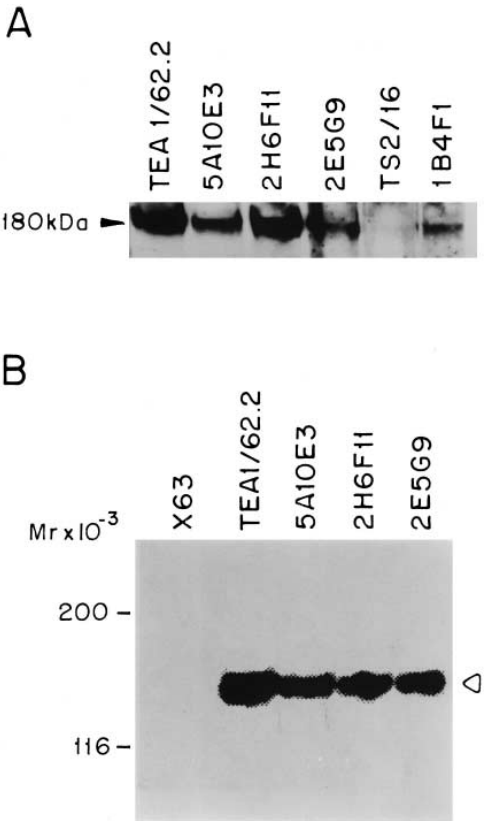

Fig. 2. Characterization of mAb to endoglin. A: Western blot analysis. Purified endoglin from human placenta was subjected to SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Filters were incubated with the indicated mAb and the presence of endoglin was revealed using a chemiluminescence assay as a 180 kDa band. B: Immunoprecipitation analysis. PMA-treated, U-937 cells were ^{125}I-labeled, lysed and immunoprecipitated with the indicated mAb. Samples were subjected to SDS-PAGE under non-reducing conditions and specific bands detected with a Molecular Imager System. mAb TEA1/62.2 (anti-endoglin) and X63 were used as a positive and negative controls, respectively. The position of endoglin is indicated by an arrow head.

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