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Research paper

Molecular characterization of hybridoma subclones spontaneously switching at high frequencies *in vitro*

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ABSTRACT

The hybridoma technology allows the production of large quantities of specific antibodies of a single isotype. Since different isotypes have special effector functions and are distributed distinctively throughout the body, it is often useful to have a library of switch variants from the original monoclonal antibody. We have shown previously that forced expression of activation induced cytidine deaminase (AID) in hybridomas increased their very low frequency of class switch recombination (CSR) *in vitro* only ~7–13 fold. Since we had previously identified rare hybridoma subclones that spontaneously switched at more than 100 times higher frequencies, we have now examined those higher switching variants to search for ways to further increase the frequency of isotype switching *in vitro*. AID was not responsible for the ~100 fold increase in CSR, so we used whole-genome gene expression profiling to provide a platform for studying candidate molecular pathways underlying spontaneous CSR in hybridomas.

1. Introduction

Isotype class switching from IgM to IgG, IgA or IgE allows antibodies to retain the same antigen specificity but to carry out different effector functions and be distributed differently throughout the body (Stavnezer et al., 2008). Hybridomas produce monoclonal antibodies that express one isotype but these antibodies can be made more useful both for *in vitro* assays and for their effectiveness *in vivo* if they can be switched in culture to provide a library of monoclonal antibodies all with the same specificity but capable of carrying out different subsets of effector functions. Class switch recombination (CSR) juxtaposes the rearranged heavy chain V(D)J region that was expressed with the μ constant region to one or another of the downstream Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα mouse constant regions by intrachromosomal recombination, with the deletion of the intervening DNA. *In vivo* and in some cell lines, this process is initiated by activation induced (cytidine) deaminase (AID) which deaminates cytidine residues in the single stranded DNA at donor and recipient switch (S) regions that are just upstream from the constant region genes, converting them to uridine residues. The G–U or G-abasic mismatches created are processed by base excision and mismatch repair in an error prone manner to produce staggered single stranded DNA breaks on each strand that can be converted into double-stranded DNA breaks which are then processed by non homologous end joining pathways (NHEJ) (Stavnezer et al., 2008).

Most hybridomas switch from one isotype to another in culture at low frequencies of 10^{-5}–10^{-6} (Radbruch et al., 1980), making it labor intensive and time consuming to obtain class switched variants *in vitro*. This was true for the 36–65 hybridoma, that makes an IgG_1 monoclonal antibody that binds to the hapten p-azophenylarsonate (Ars) (Marshak-Rothstein et al., 1980; Spira and Scharff, 1992). Hybridomas are generated by fusing primary B cells to malignant plasmacytoma cell lines (Kohler and Milstein, 1975) and represent a stage of B cell differentiation that does not usually express AID. We have previously shown that, like most hybridomas, 36–65 cells do not express AID (Iglesias-Ussel et al., 2006). We showed that the forced expression of AID in 36–65 cells, and some other hybridomas, increased the frequency of CSR only 7–13 fold

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(Iglesias-Ussel et al., 2006). In contrast, using many iterative cycles of brute force sib selection we had in the past been able to isolate variants of the 36–65 hybridoma that spontaneously switched from IgG_1 to IgG_{2b} and IgG_{2a} at ~100 times higher frequencies of 10^{-3}–10^{-4} (Spira et al., 1994). We have now compared the low-switching 36–65 hybridomas to their sister hybridomas that switched at a ~100-fold higher frequency and observed that AID expression could not be detected in the higher switching variants, indicating that different factors must be responsible for the ~100-fold increase in switching. We also found that the differences in the frequency of switching between the high and low spontaneously switching variants are retained even after forced expression of AID. We have used microarray gene expression profiling to compare these hybridomas that spontaneously switch at ~100 times different frequencies *in vitro* in an attempt to identify combinations of factors that might be responsible for spontaneous switching.

2. Materials and methods

2.1. Cell lines and cell culture conditions

Low and high spontaneously switching variants from 36–65, an A/J hybridoma that produces an IgG_1 anti-p-azophenylarsonate monoclonal antibody, were grown as previously reported (Spira et al., 1994). For convenience we have shortened the original names used in reference (Lin et al., 1996) throughout the text. Thus, 36–65.L derives from the low-switching variant 36–65.12.7.30.7.2, while 36–65.H is a high-switching variant newly isolated from 36–65.12.7.23.20.10.10.11.9. Naïve spleen B cells were obtained from two 6 week old C57BL/6 mice. Splenocytes were isolated, depleted of T cells, grown in RPMI 1640 medium containing 10% FCS and stimulated with 40 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) and 25 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 4 days. These animal experiments were approved by the Albert Einstein College of Medicine Animal Use Committee.

2.2. Soft agar cloning

4 ml of 0.4% SeaPlaque agarose (FMC Bioproduct) in 20% FCS medium was placed in a 60-mm culture plate (Falcon-Becton Dickinson) and solidified at 4 °C for 10 min. 10^3 cells in 1 ml of medium were laid over the top of the soft agar and placed at 4 °C for 10 min. Cells were grown at 37 °C for ~7 days and clones were collected and placed into a 96-well plate, as previously described (Iglesias-Ussel et al., 2006).

2.3. Transfection conditions

5×10^6 cells from two low (L25, L27) and two high (H23, H27) switching hybridomas were transfected with 10 μg human AID expressing vector (pCEP4-hAID) or an empty vector control linearized with EcoRV and NruI using a GenePulser electroporator (Bio-Rad) at 950 μF, 450 V and 200 Ω. Cells were plated in 96-well plates at 10^4 cells/well, selected with hygromycin B (Calbiochem) and ~2 weeks later stable transfectants were picked and expanded in culture. Total RNA was isolated using Trizol (Invitrogen) and hAID mRNA expression was determined by RT-PCR, as previously described (Iglesias-Ussel et al., 2006).

2.4. ELISA spot assay (ESA)

The assay was performed as previously reported (Iglesias-Ussel et al., 2006). Plates were pre-coated with a 1/500 dilution of the anti-mouse antibody against the corresponding isotype (Southern Biotechnology) and blocked with 2% BSA-TBS. Cells were plated and grown in culture for 18 h. Spots were developed with biotinylated antibody against the corresponding isotype (Southern Biotechnology) and 5-BCIP substrate (Amresco) and counted using a dissecting microscope. Median frequencies of switching were calculated. When no spots were detected, one spot was assigned to allow determination of median frequencies.

2.5. Western blotting

5×10^6 cells were lysed on ice for 30 min with 200 μl lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA pH 8 and complete protease inhibitor cocktail from Roche). The lysates were centrifuged at full speed and supernatants removed and boiled in 2× Tris–glycine SDS sample buffer (Invitrogen). Protein samples from 5×10^5 cells were separated on a 4–20% Tris–glycine gel (Invitrogen), transferred to a PVDF membrane (Invitrogen) and blotted with anti-AID mouse monoclonal IgG_1 antibody (Cell Signaling), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Southern Biotech) or anti-beta actin mouse monoclonal IgG_{2a} antibody (Sigma, 5316) followed by HRP-conjugated goat anti-mouse IgG2a antibody (Southern Biotech). Bands were visualized with a LAS-3000 imager (FujiFilm).

2.6. Gene expression profiling analysis by microarrays

Total RNA from the L25, L27, H23 and H27 hybridoma subclones (Fig. 1) was purified from 5×10^6 cells using RNeasy

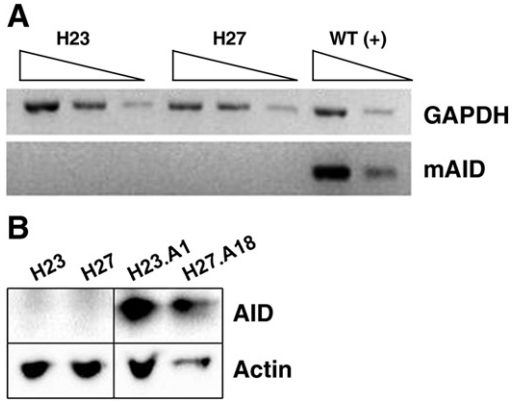

Fig. 1. Higher spontaneous frequency of switching is not due to AID expression. (A) Semiquantitative RT-PCR. Total RNA was extracted from the two high-switching hybridoma subclones (H23 and H27) as well as naïve mouse spleen cells stimulated with LPS and IL-4 for 4 days (WT+), as described in Materials and methods, and treated with DNaseI. RT-PCR one step (Invitrogen) was performed from 1 μg of total RNA and two five-fold dilutions using primers for mouse AID (mAID) (118 and 119 (Muramatsu et al. (1999)) and GAPDH (Zhang et al., 2001). (B) Western blot. AID protein levels in 36–65 hybridomas switching at high frequencies before (H23, H27) and after AID transfection (H23.A1, H27.A18).