Function-blocking antibodies to human vascular adhesion protein-1: A potential anti-inflammatory therapy

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Human vascular adhesion protein-1 (VAP-1) is a homodimeric 170-kDa sialoglycoprotein that is expressed on the surface of endothelial cells and functions as a semicarbazide-sensitive amine oxidase and as an adhesion molecule. Blockade of VAP-1 has been shown to reduce leukocyte adhesion and transmigration in vivo and in vitro models, suggesting that VAP-1 is a potential target for anti-inflammatory therapy. In this study we have constructed mouse-human chimeric antibodies by genetic engineering in order to circumvent the potential problems involved in using murine antibodies in man. Our chimeric anti-VAP-1 antibodies, which were designed to lack Fc-dependent effector functions, bound specifically to cell surface-expressed recombinant human VAP-1 and recognized VAP-1 in different cell types in tonsil. Furthermore, the chimeric antibodies prevented leukocyte adhesion and transmigration in vitro and in vivo. Hence, these chimeric antibodies have the potential to be used as a new anti-inflammatory therapy.

Introduction

The movement of leukocytes from the peripheral blood to inflammatory sites is a fundamental component of the immune response. Leukocyte extravasation from the vasculature is a multi-molecular process that begins with the initial capture of flowing leukocytes via selectin/carbohydrate interactions. Subsequent to this, leukocytes stably adhere to the vessel wall when their integrins bind to endothelial-expressed Ig superfamily members. Leukocytes may then migrate through the endothelial lining of the vessel and into surrounding tissues (for review see [1]).

Leukocyte accumulation and degranulation at sites of inflammation can result in extensive tissue damage if inflammation is not quickly resolved. In the lung, this may lead to adult respiratory distress syndrome [2], and in the heart to myocardial injury [3]. The blocking of leukocyte extravasation into inflamed tissues would potentially help to prevent the majority of pathology associated with harmful inflammation and can be theoretically done at any point in the multi-step
paradigm of leukocyte emigration. In this work we have investigated the feasibility of blocking the adhesive interactions of vascular adhesion protein-1 (VAP-1, AOC3) by developing antibodies as potential therapeutic agents.

VAP-1 is a homodimeric 170-kDa sialoglycoprotein which functions as a semicarbazide-sensitive amine oxidase (SSAO) and as an adhesion molecule that regulates adhesion and transmigration of leukocytes from the luminal surface of the endothelium to the basolateral surface. VAP-1 is stored in intracellular granules in an enzymatically inactive form and is also expressed on the cell surface in an active form [4–8]. The expression of VAP-1 is more restricted than that of most other adhesion molecules, being expressed only in germinal center dendritic cells, smooth muscle cells and adipocytes in addition to endothelial cells. It is also absent from all leukocytes, potentially giving additional benefits in specificity.

VAP-1 has been shown to bind lymphocytes in rotating adhesion assays using transfected rat endothelial cells [9] and in flow-based models using human hepatic sinusoidal endothelial cells [10] and rabbit endothelium [8]. In addition to this, VAP-1 knockout mouse models have shown that lack of the protein leads to increased rolling velocity and a decreased rate of leukocyte transmigration [11]. Recent work has also demonstrated that VAP-1 functions as a regulator of neutrophil transmigration [12]. These studies have shown that neutrophils bind to VAP-1 at two sites and that blockade of either site reduces neutrophil transmigration into the tissues [12]. The antibodies described in this study block one of these sites, the other is the SSAO enzyme active site of VAP-1. VAP-1 is therefore a target for immunotherapy aimed at preventing leukocyte transmigration and ultimately enhancing the resolution of inflammation.

Previously we have generated a number of murine monoclonal antibodies specific for human VAP-1 [13, 14] but there are several obstacles in use of murine antibodies in human therapy. It has been reported that the serum half-life of murine antibodies in man is significantly shorter than that of human antibodies and, in some indications, they may insufficiently activate effector functions [15]. However, the principal issue is that the administration of murine antibodies to humans may result in the development of human anti-murine antibodies (HAMA) [16], limiting the use of murine antibodies in long-term therapy, especially when repeated administrations are needed. Therefore, it is desirable to genetically engineer the murine antibodies to make them more human-like and thus render them less immunogenic. One approach is to replace the constant domains of the mouse antibody with those of a human antibody and thus generate chimeric antibodies [17]. Chimeric antibodies are likely to maintain the original antigen-binding specificity and affinity but be less immunogenic than their murine counterpart [18, 19].

We now describe the production and characterization of chimeric mouse/human anti-VAP-1 antibodies derived from three of our anti-VAP-1 antibodies for potential therapeutic use. By combining the variable domains of the parental mouse antibodies with a modified human IgG2 constant region (G2Δα; [20]), the resulting antibodies should have reduced binding to Fc receptors compared to natural IgG molecules and not activate complement or trigger antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, we have exploited the ability of the murine antibodies to block VAP-1-mediated adhesion whilst reducing their potential to cause side effects due to immunogenicity or activation of effector functions.

Results

Cloning of murine Fd and light chain cDNA

We have previously generated a number of well-characterized and cloned hybridoma cell lines producing mouse monoclonal antibodies specific for human VAP-1 which have been successfully used for in vitro and in vivo studies [13, 14]. To clone the DNA encoding the variable regions of these antibodies, total RNA was isolated from the hybridoma cell lines, and the Fd region and light-chain DNA amplified by RT-PCR. For the Fd, ten degenerate 5’ primers and one degenerate 3’ primer were used in an array of PCR [21], successful amplifications yielding DNA fragments of approximately 630 bp. Light-chain cDNA with sizes of approximately 660 bp were amplified using seven degenerate 5’ primers and one 3’ primer [21]. These cDNA were cloned and their DNA sequences determined.

Using the sequence information, combined with the knowledge of the properties of the original antibodies, three murine antibodies, Mo2D10, MoTK8-14 and Mo1G6, were selected for constructing chimeric antibodies. Fig. 1 shows the predicted amino acid sequences of the variable regions of these three anti-VAP-1 antibodies. Comparison of these sequences with sequences collated by Kabat et al. [22] revealed that the V\text{H} genes of 2D10, TK8-14 and 1G6 belonged to mouse V\text{H} subgroups IB, IIB and IB, respectively. Correspondingly, the V\text{L} genes of 2D10 and 1G6 were members of mouse V\text{L} subgroup V whereas the V\text{L} gene of TK8-14 belonged to subgroup VI.
Construction and production of chimeric antibodies

The murine variable region cDNA were expressed with modified IgG2 and k light chain human constant regions in the rat myeloma cell line YB2/0. Stable cell lines were established which yielded 1–7 mg purified antibody per liter of culture medium. When analyzed by SDS-PAGE under reducing conditions, the purified antibodies, designated Ch2D10, ChTK8-14 and Ch1G6, each migrated as two homogeneous bands representing the heavy chain (approximately 50 kD) and light chain (approximately 22 kD; data not shown). To aid analysis of results, 2D10 antibodies were made with unmodified wild-type human IgG1 and IgG2 constant regions and a control antibody, which does not bind VAP-1, ChNPLys, was constructed with the modified IgG2Aa constant region.

VAP-1 binding properties of chimeric antibodies

The binding of the chimeric antibodies to human VAP-1 was analyzed by flow cytometry (Fig. 2). All three chimeric antibodies bound specifically to human VAP-1 expressed on the surface of a rat endothelial cell line (Axl cells) with no binding to mock-transfected cells detected. Ch2D10 showed the highest apparent avidity for VAP-1, requiring a concentration of approximately 0.1 μg/mL to achieve 50% saturation of mean fluorescence, compared to 0.2 μg/mL for Ch1G6 and 0.8 μg/mL for ChTK8-14.

The VAP-1 binding properties of the chimeric antibodies were directly compared to those of the original mouse antibodies in competition experiments (data not shown). A constant amount of biotinylated mouse antibody was mixed with increasing amounts of unlabeled competitor mouse or chimeric antibody, and the amount of labeled antibody able to bind to VAP-1-expressing Axl cells was analyzed by flow cytometry. Ch2D10 and ChTK8-14 competed to a similar extent as their mouse counterparts but Ch1G6 showed better inhibition than Mo1G6. In the preparation of the hybridoma cell line producing Mo1G6, NS-1 cells, which produce irrelevant antibody light chains, were used as the fusion partner [23]. Thus, it is very likely that a proportion of light chains within the Mo1G6 preparation are non-specific whereas Ch1G6 contains only VAP-1-specific light chains, explaining its improved VAP-1 binding.

To further test the recognition properties of the chimeric antibodies, human tonsil sections were stained with the chimeric and original monoclonal antibodies. Both types of antibodies stained high endothelial venules (HEV) and a subpopulation of flat-walled venules in the tonsil (Fig. 3). In addition, follicular dendritic cells and vascular smooth muscle cells in tonsil were positive with both antibody series. Neither

Figure 2. VAP-1 binding activity of chimeric antibodies. The binding of the chimeric antibodies and irrelevant control antibody at varying concentrations to the Axl cells expressing human VAP-1 was analyzed by flow cytometry. Mock-transfected Axl cells were used as a negative control. Results are expressed as percentage of maximal mean fluorescence.
chimeric antibodies nor the original monoclonals stained lymphocytes, epithelial cells or other structures. This suggests that the replacement of the antibodies’ Fc regions had not altered their binding specificity.

**Figure 3.** Functional characterization of the chimeric antibodies and the original mouse monoclonal antibodies in human tissue. Tonsil sections were stained with biotinylated MoTK8-14 (A), ChTK8-14 (B), Mo2D10 (C), and Ch2D10 (D) and negative control antibody (E) followed by streptavidin-phycocerythrin. Two of HEV (in A–D) are pointed out by arrows and germinal centers (GC) labeled. Original magnification ×500.

**Figure 4.** Flow cytometric analysis of antibody binding to human FcγRI. B2KA cells expressing human FcγRI on their surface were incubated with the mouse (A) and chimeric (B) anti-VAP-1 antibodies, biotinylated anti-human κ chain antibody and FITC-conjugated ExtrAvidin. The mean channel fluorescence (MCF) for 20 000 events was measured for each sample. Human 2D10 IgG1 antibody was used as a positive control.

**Effector function properties of chimeric antibodies**

Binding of the murine and chimeric antibodies to human FcγRI was tested by flow cytometry using cells transfected to express this Fc receptor in isolation. The chimeric antibodies showed no binding above background levels even at 100 μg/mL (Fig. 4B). In contrast, all three murine antibodies showed some degree of binding (Fig. 4A). MoTK8-14 and Mo1G6, both with IgG2a heavy chains, bound as strongly as the human 2D10 IgG1 control. Their binding curves had midpoints falling between 0.5 and 1 μg/mL, indicating an association constant of $2 \times 10^6$–$3 \times 10^6$ M$^{-1}$. Binding of Mo2D10 was approximately $10^3$-fold lower but still significant at concentrations above 10 μg/mL.

The level of complement-mediated cell lysis initiated by anti-VAP-1 antibodies was assessed by the release of $^{51}$Cr from VAP-1-expressing cells in the presence of antibody and guinea pig (Fig. 5A) or human (Fig. 5B) complement. In these assays, the control 2D10 IgG1 and 2D10 IgG2 antibodies were both shown to cause consistent and reproducible lysis in ECV304-V cells in the presence of guinea pig but not human complement. The antibody Ch2D10, with the modified 2D10 IgG2 constant region, did not cause lysis of ECV304 cells under any of the conditions, suggesting that the modifications inhibit its ability to induce complement-mediated cell lysis.

In order to examine ADCC, we labeled ECV304 target cells with a red fluorescent marker (PKH26) and a green fluorescent cytoplasmic marker (CFSE), in an adaptation of the fluorometric assessment of T lymphocyte antigen-specific lysis (FATAL) assay of Sheehy et al. [24]. PKH26 staining was used to identify target cells during flow cytometry and lysis of target cells was indicated by loss of cytoplasmic CFSE. Incubation of target cells with lymphocyte effector cells in the presence of 2D10 IgG1
or 2D10 IgG2 antibodies caused a consistently reproducible level of ADCC when compared to a control without antibody (Fig. 6). This lysis was dependent on the presence of the VAP-1 antigen. However the Ch2D10 antibody consistently failed to induce ADCC with CFSE loss being close to control levels in all cases. This suggests that the modifications in Ch2D10 constant region have significantly reduced its ability to induce ADCC.

Adhesion assays

Rotatory adhesion assay

An in vitro frozen section assay was used to test the capacity of the chimeric and mouse monoclonal antibodies to inhibit lymphocyte binding to HEV, which are specialized vessels through which lymphocyte extravasation takes place in lymphatic tissues. For this experiment, only Mo2D10 and MoTK8-14 antibodies and their chimeric counterparts were chosen because Mo1G6 contains a heterogeneous mixture of light chains as discussed above. All of the mouse anti-VAP-1 antibodies were able to prevent lymphocyte adherence to vascular endothelium. Treatment of the tonsil sections with the chimeric and parental antibodies resulted in comparable inhibition of lymphocyte binding to HEV (Fig. 7), indicating that the chimeric antibodies have the same function-blocking properties as their murine counterparts.

Flow-based adhesion assay

VAP-1 is expressed on the luminal surface of endothelial cells and therefore any antibody that binds to it has the potential to present Fc regions to circulating leukocytes. We investigated how immune complexes formed between rVAP-1 and either Ch2D10 or the control 2D10 IgG1 and 2D10 IgG2 antibodies would affect flowing neutrophils. Immune complexes formed between 2D10 IgG1 and rVAP-1 were able to capture flowing neutrophils (Fig. 8A) and promote their conversion to stationary adhesion (Fig. 8B). However, immune complexes formed with either 2D10 IgG2 or
Ch2D10 were less able to initiate the formation of neutrophil adhesive interactions and subsequent conversion to stationary adhesion, with the Ch2D10 antibody being comparable to the PBS control. These data suggest that VAP-1-binding antibodies based on Ch2D10 and, perhaps, native 2D10 IgG2 would not activate circulating neutrophils.

To investigate whether the tendency of 2D10 IgG2 to capture or activate flowing neutrophils above the level seen with Ch2D10 or PBS control might be significant, we mimicked the in vivo situation where immune complexes may be formed of a combination of IgG subclasses. We investigated the effect of forming immune complexes against VAP-1 using a combination of 2D10 IgG1 and 2D10 IgG2 or 2D10 IgG1 and Ch2D10 on the capture and activation of flowing neutrophils. Immune complexes formed by a mixture of 2D10 IgG1 and 2D10 IgG2 were much more able to capture and activate (Fig. 9A, C) flowing neutrophils than were immune complexes formed by a combination of 2D10 IgG1 and Ch2D10 (Fig. 9B, D). This suggests that in mixed immune complexes the presence of 2D10 IgG2 may supplement the activation signals derived from Fc receptor binding to 2D10 IgG1 whereas the modifica-
tions made to Ch2D10 appear to have reduced this supplementary activation.

Transmigration assays

Transmigration of lymphocytes can be induced in vitro by an N-formyl-Met-Leu-Phe (fMLP) gradient [25]. Since VAP-1 functions also as a regulator of transmigration [12], we investigated the effect of Ch2D10 in an in vitro assay of transmigration. We examined the ability of lymphocytes to transmigrate through a monolayer of mock-transfected or VAP-1-expressing Ax cells grown on a co-culture insert, towards fMLP (Fig. 10). The number of cells transmigrating through a monolayer of VAP-1-expressing Ax cells was greater than through a monolayer of mock-transfected Ax cells, which did not express VAP-1. This may be explained by the fact that VAP-1 contributes to the regulation of lymphocyte transmigration. As expected, when mock-transfected Ax cells were treated with Ch2D10 there was no effect on the number of cells transmigrating. Whilst ChNPllys, a non-antigen-binding version of Ch2D10, had no effect on the rate of transmigration through the VAP-1-expressing Ax cell monolayer, treatment with Ch2D10 reduced the rate to below that of mock-transfected Ax cells, suggesting active inhibition of lymphocyte transmigration by Ch2D10.

To test in vivo efficacy of Ch2D10, we used genetically modified mice that express human VAP-1 as a transgene on endothelial cells but lack murine VAP-1, and investigated leukocyte extravasation in a model of peritoneal inflammation. Compared to mice that were treated with ChNPlys control antibody, mice treated with Ch2D10 showed a 40% reduction ($p=0.008$) in the number of total leukocytes migrating into the peritoneal cavity. When analyzing the effect on granulocyte migration in particular, the reduction was even greater (55%, $p<0.01$). These results clearly show that the antibodies used in this study not only recognize human VAP-1 in an in vitro model but also show good efficacy in the reduction of both lymphocyte and granulocyte transmigration.

Discussion

In this work we have set out to create a function-blocking antibody against the adhesive/transmigration ability of VAP-1. Whilst we had available an array of murine antibodies known to block human VAP-1 function, there were two main obstacles to overcome in order to maximize their potential. Mouse antibodies are prone to trigger HAMA responses in man; therefore the murine anti-VAP-1 antibodies were re-engineered as mouse-human chimeric antibodies in order to render them less immunogenic [16, 19]. Secondly, all natural IgG subclasses have the capacity to recruit at least some of the human effector systems, a property detrimental to an antibody targeted to the vascular endothelial cells at sites of inflammation. For the chimeric antibodies, we chose a modified human 2D10 IgG2 constant region that has minimal interactions with effector molecules (G2Dα, [26]).

It might be argued on theoretical grounds that full humanization of antibodies, using just the complementarity-determining regions (CDR) of the rodent antibody, is desirable [27]. Whilst it is true that approximately 30% of a chimeric antibody is derived directly from the rodent antibody, compared to an estimated 5% for a humanized antibody, this 30% representing the variable regions may be highly homologous to human variable regions [19]. In fact, alignment of $V_{H}$ and $V_{k}$ sequences of the mouse anti-VAP-1 antibodies to human germ-line V gene sequences (V BASE Sequence Directory; Tomlinson et al., MRC Centre for Protein Engineering, Cambridge, UK) demonstrated that the mouse variable region sequences used for constructing the chimeric anti-VAP-1 antibodies had a high sequence similarity to identified human sequences (data not shown). Few changes would be needed to convert the chimeric antibodies to fully humanized molecules using the closest match human variable region strategy [28].

An advantage of using chimeric antibodies, rather than humanized antibodies, is that the specificity and affinity of the antigen-binding site can be more easily maintained. All three chimeric antibodies were shown to bind specifically to the surface-expressed human VAP-1. Their VAP-1 binding properties were comparable to those of their respective mouse monoclonal antibodies or, in the case of Ch1G6, improved due to the
heterogeneous nature of the murine antibody preparation. Moreover, both the mouse and chimeric antibodies stained different cell types and structural compartments of tonsils in the same manner. Ch2D10 showed the highest apparent avidity for VAP-1 and some of the later experiments concentrated on this antibody.

In the proposed therapeutic applications of VAP-1 antibodies to block the binding of leukocytes to vascular endothelium, harmful side effects could be caused by the complement and Fc receptor binding activities of the Fc region [28, 29]. For this reason, the chimeric antibodies were constructed using a modified human 2D10 IgG2 constant region, called G2Δα, which has been engineered to lack natural effector functions [20, 26]. A series of human constant regions were designed by interchanging motifs critical for FcγRI–III and complement C1q binding [30] between IgG1, IgG2 or IgG4 subclasses, an approach adopted to minimize the potential immunogenicity of the mutations. The constant regions were characterized in conjunction with anti-RhD and CD52 specificities.

This work demonstrated that the G2Δα constant region, which incorporates IgG4 residues at positions 330 and 331 within the CH2 domain, showed no detectable binding to FcγRI and FcγRIII and reduced binding to FcγRII [20, 26, 31]. G2Δα antibodies did not activate human monocytes or trigger CD16+ NK cell-mediated cytotoxicity (ADCC). In addition, anti-RhD antibody with the G2Δα constant region could specifically inhibit the triggering of human monocytes and ADCC by RhD+ red blood cells in the presence of human alloimmune anti-RhD sera.

To ensure that the property of reduced FcγR binding was maintained for the G2Δα constant region with anti-VAP-1 specificity, the binding of Ch2D10 was compared to that of 2D10 IgG1 and 2D10 IgG2 with all three classes of receptor (data not shown). For FcγRI and FcγRIII, binding could not be detected. Binding to FcγRIIa of the 131R allotype and the inhibitory receptor FcγRIIb was similar to that of 2D10 IgG2 and approximately fivefold lower than the binding of 2D10 IgG1. 2D10 IgG2 binds more highly to FcγRIIa 131H than 2D10 IgG1 but this was reduced approximately tenfold in Ch2D10, showing that the modified constant region was better suited for human therapeutic use than natural human subclasses. We compared the FcγRI binding activities of the mouse and chimeric anti-VAP-1 antibodies in order to demonstrate another advantage of the chimeric antibodies over their murine counterparts. Unlike all three murine antibodies, the chimeric molecules did not show binding to the Fc receptors even at the highest concentration tested.

The real test of any antibody claiming to have reduced ability to direct an immune response must come from assays of its effector function, principally those measuring lytic activity caused by ADCC and complement. The reduction in Fc receptor binding of Ch2D10 should reduce the binding of NK cells to any cell presenting Ch2D10 at its surface with clear implications for ADCC. Indeed, this is reflected in our in vitro ADCC assay in which lysis caused by Ch2D10 was not significantly different from control levels whereas both 2D10 IgG1 and 2D10 IgG2 promoted greater levels of lysis. In addition Ch2D10 caused no complement-mediated cell lysis in assays using either guinea pig or human complement. These data in combination with the reduced binding to Fc receptors suggest that Ch2D10 will have reduced effector function in vivo.

Assays for effector function in vitro provide an indication as to the antibodies’ characteristics in vivo; however, the cellular location of the epitope must also be taken into consideration. In this case VAP-1 is presented at the endothelial cell surface. This suggests that any antibody bound to VAP-1 has the potential to present its Fc regions to flowing neutrophils that are marginalized to the sides of the blood vessel by the ‘plug flow’ effect of weakly interacting red blood cells flowing in the center of the vessel. To examine this we used an in vitro flow-based model to examine the interaction of flowing neutrophils with immune complexes formed by IgG and rVAP-1. VAP-1 may form multimers which in turn will favor the formation of the immune complexes required for Fc receptor cross-linking and cellular activation [32, 33]. Microslides coated with 2D10 IgG1 alone, rVAP-1 alone or PBS (control) did not form any adhesive interactions with flowing neutrophils; however, those coated with rVAP-1 and then 2D10 IgG1 formed numerous adhesive interactions with flowing neutrophils, due to the formation of immune complexes on the microslide surface and the interaction of these with neutrophil CD16 and CD32 (Kirton and Clark, unpublished observations).

Immune complexes formed using 2D10 IgG2 or Ch2D10 captured neutrophils at a similar level to that of control (PBS). In particular we noted that there was no significant difference in the ability of 2D10 IgG2 or Ch2D10 complexes to capture and activate neutrophils, despite their differences in previous functional assays.

In order to further differentiate the neutrophil-activating characteristics of 2D10 IgG2 from those of Ch2D10, we then went on to form immune complexes composed of either a mixture of 2D10 IgG1 and 2D10 IgG2 or a mixture of 2D10 IgG1 and Ch2D10 with rVAP-1. We saw that whilst 2D10 IgG2 was able to support capture and activation of neutrophils even in the presence of low proportions of IgG1, Ch2D10 was not able to support capture unless IgG1 was present in high proportions. This suggests that 2D10 IgG1 and 2D10 IgG2 can cooperate to capture and activate flowing neutrophils but Ch2D10 cannot cooperate with 2D10.
IgG1 in this way. The difference seen in this assay is likely to reflect the reduced binding of Ch2D10 to FcγRIII and/or FcγRIIa relative to 2D10 IgG2. A similar phenomenon was noted when looking at the activation of monocytes in response to red cells coated in mixtures of IgG1 and other antibodies [20]. Despite not activating monocytes when used alone, 2D10 IgG2 was fivefold less efficient than G2Aβ in inhibiting activation due to 2D10 IgG1.

In our in vitro and in vivo models of transmigration we saw that Ch2D10 reduced lymphocyte transmigration. This clearly demonstrates that these antibodies block sites on VAP-1 that are used in leukocyte transmigration and suggest their use in immunotherapy. This therapeutic potential was demonstrated when Ch2D10 treatment of genetically modified mice, expressing human VAP-1 instead of mouse VAP-1 from endothelial cells, significantly reduced the inflammatory response to an acute model of peritonitis. In our in vitro transmigration assay we also noted that treatment of Ax cell monolayers with Ch2D10 reduced transmigration to below that of control levels. This suggests that Ch2D10 may inhibit transmigration by signaling through the inhibitory receptor, FcγRIIb, in addition to inhibition by blocking the VAP-1 antigen.

We have shown that conversion of the murine anti-VAP-1 antibodies into mouse-human chimeric molecules has not altered their properties of epitope recognition. In addition, extensive testing of the chimeric antibody Ch2D10 has shown reduced binding to Fc receptors and reduced effector function relative to the murine progenitors and to wild-type human versions of the antibody. Thus, Ch2D10 retains ability to block lymphocyte adhesion and transmigration but should not trigger either HAMA responses or immune effector responses in vivo. Ch2D10 may be suitable for development of a therapy for chronic inflammatory disease in man.

Materials and Methods

Cloning of murine cDNA

Total RNA were isolated from hybridoma cells secreting anti-human VAP-1 antibodies [13, 14] using an Ultraspec RNA Isolation kit (Biotex). First-strand cDNA were synthesized using oligo(dT) primer and murine Moloney leukemia virus reverse transcriptase (Gibco BRL). The cDNA encoding the mouse Fd fragments and the entire light chains were specifically amplified by PCR using 200 pmol of degenerate 5’ and 3’ primers as described by Kettleborough et al. [21]. The products were cloned into pUC18 for nucleotide sequencing.

Production of chimeric antibodies

Leader and 3’-region DNA segments, containing sequences for the mouse Ig heavy chain promoter and a signal peptide as well as the appropriate splicing sites were amplified by PCR from M13VHPCR1 [34]. Overlap-extension PCR was used to join these to the 5’ and 3’ ends of the Vβ and Vκ cDNA of anti-VAP-1 antibodies 2D10, TK8-14 and 1G6. HindIII-BamHI fragments comprising leader-VH-3’ region or leader-Vκ-3’ region DNA were cloned into a pSVhgy expression vector containing a modified human IgG2 constant region gene (G2Aα; [20]) or a pSVhgy expression vector containing a human κ constant region gene [34], as appropriate. DNA sequences were confirmed by sequencing. Vectors for the control 2D10 IgG1 and 2D10 IgG2 antibodies were constructed in the same way using wild-type constant region genes [20]. For the control G2Aα antibody, ChNPLys, the heavy and light chain variable region DNA were provided from M13VHPCR1 (anti-NIP) and the original pSVhgy κ expression vector (anti-lysozyme; [34]).

For each antibody, the heavy and light chain expression vectors were co-transfected into non-secreting rat myeloma cells (YB2/0; [35]) as previously described [20]. Stable transfectants secreting the highest levels of antibody, as determined by a sandwich ELISA employing human IgG Fc- and κ chain-specific antibodies, were expanded into 2-L roller bottles with 400 mL Iscove’s modified Dulbecco’s medium, 2% FCS. The chimeric antibodies were purified from the culture supernatant by protein A-agarose chromatography. Eluted antibodies were dialyzed into PBS and filter-sterilized. Antibody concentrations were determined by measuring A280 (ε=0.714) and by sandwich ELISA and their purities were judged on Coomassie-stained 12.5% SDS-polyacrylamide gels [36].

Immunohistochemistry and flow cytometry

Antibodies to be biotinylated were dialyzed against 200 mM NaHCO3, 81 mM Na2CO3, incubated with biotinamidocaproate H-n-hydroxysuccinimide ester (Sigma, 120 μg/mg antibody) at room temperature for 2 h and then dialyzed into PBS. For immunohistochemistry, acetone-fixed frozen sections of different human tissues were first overlaid with different biotinylated murine anti-VAP-1 antibodies, their chimeric derivatives or class-matched negative control antibodies, followed by incubation with streptavidin-phycocerythrin (Becton-Dickinson). After washing, the coverslips were attached with Fluoromount (Southern Biotechnology Associates, Inc.). Sections were observed via fluorescence microscopy.

Adherent Ax cells expressing human VAP-1 or Ax cells transfected with human VAP-1 cDNA in an inverse orientation in the vector (mock control; [9]) were grown in RPMI 1640, 20% FCS, 2 mM L-glutamine, 1 mM Na-pyruvate, 10 μM β-ME, 1% non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.75 mg/mL geneticin. To measure binding to VAP-1 by flow cytometry, the cells were treated with Cell Dissociation Buffer (Gibco BRL) to obtain single-cell suspensions, pelleted and resuspended in wash buffer (PBS, 0.1% w/v; BSA, 0.1% v/v). The cells were pelleted at 106 cells/well in 96-well plates and resuspended in 100 μL/well wash buffer containing chimeric antibody. After 30-min incubation.
on ice, the cells were washed three times with 150 μL wash buffer/well and then incubated for 30 min on ice with 100 μL of 39 μg/mL FITC-conjugated anti-human IgG (Fc-specific, Sigma). The cells were washed as previously and fixed by adding 100 μL wash buffer containing 1% v/v formaldehyde and kept at 4°C until analysis by flow cytometry (FACScan, Becton Dickinson). A minimum of 10 000 gated events were collected for each well and the results analyzed using Lysis II software.

The binding of mouse and chimeric antibodies to FcγRI was similarly measured by flow cytometry using transfected cells expressing FcγRI cDNA (B2Ka; S. Gorman and G. Hale, unpublished). Following incubation with dilutions of the anti-VAP-1 antibodies or control human 2D10 IgG1 antibody, the cells were incubated with 20 μg/mL biotin-conjugated goat anti-human κ chain antibody (Sigma) or biotin-conjugated goat anti-mouse IgG (Fab-specific, Sigma) as appropriate, followed by 20 μg/mL ExtrAvidin-FITC (Sigma). Surface expression of FcγRI was confirmed by staining with a CD64 monoclonal antibody (Serotec, Oxford, UK) and FITC-conjugated goat anti-mouse IgG antibodies (Sigma).

Complement-mediated cell lysis

ECV304 cells were transfected with VAP-1 (ECV304-V; [9]) or a mock control (ECV304-M), harvested, adjusted to 1×10^5/mL in serum-free media containing 200 μCi/mL of ^51^Cr and incubated for 45 min at 37°C. RPMI containing 0.5% serum was then added, the cells spun and the supernatant poured off. ECV304-V cells were transferred to a 96-well plate, with human or guinea pig complement (10%) and with antibody or medium only (control) and the plate incubated at 37°C for 45 min. The plate was spun at 250 × g for 5 min, and 50 μL of each well’s supernatant transferred to a plate containing 150 μL/well of scintillation fluid that was then read using a scintillation counter.

Antibody-dependent cell-mediated cytotoxicity

The lysis of ECV304-V (target) cells by lymphocytes (effectors) was measured using the FATAL assay developed by Sheehy et al. [24] Lymphocytes were isolated from whole blood by layering onto Histopaque followed by centrifugation at 450 × g for 20 min. Lymphocytes were harvested, washed and adjusted to 5×10^5/mL ECV304 media. ECV304-V or ECV304-M cells were grown to confluence in 25-cm² tissue culture flasks, removed via trypsin and then labeled with the red fluorescent membrane label PKH-26 using a linker kit (Sigma), and the green fluorescent cytoplasmic label CFSE (1×10^6 M). ECV304 cells were washed to remove excess dye and adjusted to 1×10^6/mL in ECV304 media. Antibody (10 μg/mL) or PBS control was added to the ECV304 cells and incubated for 1 h at 37°C. The cell pellet was disrupted and resuspended in 1 mL of the lymphocyte suspension to give an effector-to-target ratio of 50:1. The mixed cell suspension was incubated for 4 h at 37°C, fixed in 1% paraformaldehyde and then analyzed by flow cytometry. Lysis of target cells by chimeric antibodies was expressed as a percentage of lysis achieved by wild-type 2D10 IgG1 antibody.

**Rotary adhesion assay**

The capacity of different antibodies to block lymphocyte binding to endothelial cells was studied using an in vitro frozen section assay. Human tonsils were obtained from elective tonsillectomies. Freshly cut 8-μm sections of frozen tonsils were first incubated for 30 min with 100 μL of RPMI 1640 medium supplemented with 5% AB-serum (Finnish Red Cross) and 10 mM HEPES, pH 7.2, containing 100 μg/mL of different anti-VAP-1 antibodies or class-matched negative control antibodies. Thereafter, the sections were overlaid with 3×10^6 peripheral blood lymphocytes. Under constant rotation, lymphocytes were allowed to bind to vascular endothelium in the sections for 30 min at 7°C. Following this, the non-adherent cells were gently washed off and the adherent cells were fixed to the sections overnight in ice-cold PBS containing 1% v/v glutaraldehyde. The lymphocytes adhered to at least 100 HEV per sample were counted under dark-field illumination and the results expressed as percentage of negative control binding.

**Flow-based adhesion assay**

The flow assay was set up essentially as previously described by Sheikh and Nash [37]. Briefly, microslides were connected at one end to a Harvard syringe pump via a length of tubing, and neutrophils or PBS were drawn through the microslide via an electronic switching valve (Lee Products, Gerards Cross, UK). A constant wall shear stress of 0.1 Pa was created within the microslide by the selection of an appropriate withdrawal rate for the syringe pump. A CCD video camera module (XT-ST50CE; Sony, Thatcham, UK) was connected to an inverted light microscope (Axiovert 25; Zeiss, Göttingen, Germany) so that movie records of flowing neutrophils could be made. Data were written directly to a PC using StreamPix image capture software (Norpix, Montreal, Canada).

To investigate the role of IgG subclass in neutrophil capture and activation, immune complexes were formed in situ by coating the microslide with purified rVAP-1 at 10 μg/mL for 60 min and then incubating with either 2D10 IgG1, 2D10 IgG2, Ch2D10 or a mixture of antibodies (10 μg/mL antibody total). Neutrophils at a density of 1×10^6/mL were perfused through the microslide for 3 min, with the final 2 min being recorded as a movie file. The neutrophil bolus was then followed by a 1-min washout with PBS. Within the 2-min recording of the neutrophil perfusion, three separate CCD camera fields (280 μm²/field) were recorded, each for 30 s. Neutrophils in free flow appeared as blurred streaks; those interacting with the surface rapidly transformed to stable adhesion. The number of neutrophils interacting with the surface in each of the three fields observed was quantified to give adhesive interactions/mm²/min and used to calculate a mean for each individual experiment. At the end of the washout period, the number of neutrophils in three microscope fields (630 μm²/field) was counted.

**In vitro transmigration assay**

VAP-1 expressing or mock-transfected Ax cells were grown to confluence on a co-culture insert in a 24-well plate well
Culture medium both above and below the insert was replaced with medium containing TNF-α (100 ng/mL) and the cells incubated at 37°C for 2 h. All culture medium was exchanged for media containing antibody (2 or 50 μg/mL) and the cells incubated for 2 h at 37°C. Antibody-containing medium was removed and then replaced in the insert with 300 μL of medium containing PBMC (1 × 10⁶/mL) and below the insert with 900 μL of medium containing FMLP (1 × 10⁻⁷ M). After 2 h of incubation at 37°C, the inserts were removed and the number of transmigrated cells counted by light microscopy.

In vivo peritonitis model for transmigration

To generate mTIEhVAP-1 transgenic/VAP-1 knockout (VAP-KO+TG) mice, mTIEhVAP-1 line E35 mice expressing human VAP-1 on the vasculature [38] were crossed to VAP-1 knockout mice that were previously created by using conventional gene targeting techniques to replace the mouse VAP-1 gene with a nonfunctional mutant allele [11]. The mTIEhVAP-1 transgenic, mouse VAP-1 mutant allele and endogenous mouse VAP-1 allele were all identified by PCR screening of purified genomic DNA with specific primers and verified immunohistochemically with human and mouse VAP-1 antibodies [38].

Inflammation was induced in peritoneal cavities of VAP-KO+TG mice by i.p. injection of 1 mL PBS containing 4% thioglycollate medium (Sigma-Aldrich Chemie, Germany). The mice were treated with i.v. injection of Ch2D10 or ChNPlys control antibody (nine and six mice, respectively; 200 μg antibody/mouse). At 4 h after the induction of inflammation, cells were collected from the peritoneal cavity by washing it with 10 mL RPMI containing 5 U/mL Heparin Leo (Løvens Kemiske Fabrik, Denmark) and counted. Leukocyte subtypes were analyzed from lavage fluid smears after Reastain Quick-Diff (Reagena, Finland) staining.

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References

1. Kubes, P. The complexities of leukocyte recruitment. Semin. Immunol. 2002. 14: 65–72.
2. Rivkind, A. I., Siegel, J. H., Guadalupi, P. and Littleton, M., Sequential patterns of eicosanoid, platelet, and neutrophil interactions in the evolution of the fulminating post-traumatic adult respiratory distress syndrome. Ann. Surg. 1989. 210: 355–373.
3. Siminak, T., Flores, N. A. and Sheridan, D. J., Neutrophil interactions with endothelium and platelets: possible role in the development of cardiovascular injury. Eur. Heart J. 1995. 16: 160–170.
4. Salmi, M. and Jalkanen, S., Different forms of human vascular adhesion protein-1 (VAP-1) in blood vessels in vivo and in cultured endothelial cells: implications for lymphocyte-endothelial cell adhesion models. Eur. J. Immunol. 1995. 25: 2803–2812.
5. Salmi, M. and Jalkanen, S., Human vascular adhesion protein 1 (VAP-1) is a unique sialoglycoprotein that mediates carbohydrate-dependent binding of lymphocytes to endothelial cells. J. Exp. Med. 1996. 183: 569–579.
6. Arviliommi, A. M., Salmi, M., Kalimo, K. and Jalkanen, S., Lymphocyte binding to vascular endothelium in inflamed skin revisited. A central role for vascular adhesion protein-1 (VAP-1). Eur. J. Immunol. 1996. 26: 825–833.
7. Yong, K. F., Williams, A., Hubscher, S. G., Salmi, M., Jalkanen, S. and Adams, D. H., Vascular adhesion protein-1 and intercellular adhesion molecule-1 mediate T-cell binding to human hepatocellular carcinoma. Biochem. Soc. Trans. 1997. 25: 2575.
8. Yegutkin, G. G., Salminen, T., Koskinen, K., Kurits, C., McPherson, M. J., Jalkanen, S. and Salmi, M., A peptide inhibitor of vascular adhesion protein-1 (VAP-1) blocks leukocyte-endothelium interactions under shear stress. Eur. J. Immunol. 2004. 34: 2276–2285.
9. Smith, D. J., Salmi, M., Bono, P., Hellman, J., Leu, T. and Jalkanen, S., Cloning of vascular adhesion protein 1 reveals a novel multifunctional adhesion molecule. J. Exp. Med. 1998. 188: 17–27.
10. Lalar, P. F., Edwards, S., McNab, G., Salmi, M., Jalkanen, S. and Adams, D. H., Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. J. Immunol. 2002. 169: 983–992.
11. Stolen, C. M., Marttila-Leihiala, F., Koskinen, K., Yegutkin, G. G., Turja, R., Bono, P., Skurulk, M., et al., Absence of the endothelial oxidase AOC3 leads to abnormal leukocyte traffic in vivo. Immunity 2005. 22: 105–115.
12. Koskinen, K., Vainio, P. J., Smith, D. J., Pihlavisto, M., Ya-Herttuala, S., Jalkanen, S. and Salmi, M., Granuloctye transmigration through the endothelium is regulated by the oxidase activity of vascular adhesion protein-1 (VAP-1). Blood 2004. 103: 3388–3395.
13. Salmi, M. and Jalkanen, S., A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. Science 1992. 257: 1407–1409.
14. Kurkijarvi, R., Adams, D. H., Leino, R., Mottonen, T., Jalkanen, S. and Salmi, M., Circulating form of human vascular adhesion protein-1 (VAP-1): Increased serum levels in inflammatory liver diseases. J. Immunol. 1998. 161: 1549–1557.
15. Piroflosky, L., Casdeavall, A., Rodriguez, L., Zuckier, L. S. and Scharff, M. D., Current state of the hybridoma technology. J. Clin. Immunol. 1990. 10 Suppl. 6: 55–125.
16. Isaacs, J. D., The antiglobulin response to therapeutic antibodies. Semin. Immunol. 1990. 2: 449–456.
17. Presta, L. G., Antibody engineering. Curr. Opin. Biotechnol. 1992. 3: 394–398.
18. Bruggemann, M., Winter, G., Waldmann, H. and Neuberger, M. S., The immunogenicity of chimeric antibodies. J. Exp. Med. 1989. 170: 2153–2157.
19. Clark, M., Antibody humanisation: A case of the Emperor’s new clothes? Immunol. Today 2000. 21: 355–412.
20. Armour, K. L., Clark, M. R., Hadley, A. G. and Williamson, L. M., Recombinant human IgG molecules lacking Fcgamma receptor I binding and monocyte triggering activities. Eur. J. Immunol. 1999. 29: 2613–2624.
21. Kettleborough, C. A., Saldanha, J., Ansell, K. H. and Bendig, M. M., Optimization of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain reaction. Eur. J. Immunol. 1993. 23: 206–211.
22. Kabat, E., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foller, C., Sequences of proteins of immunological interest, 5th Edn. National Institutes of Health, Bethesda 1991.
23. Salmi, M., Smith, D. J., Bono, P., Leu, T., Hellman, J., Matikainen, M. T. and Jalkanen, S., A mouse molecular mimic of human vascular adhesion protein 1. Mol. Immunol. 1997. 34: 1227–1236.
24. Sheehy, M. E., McDermott, A. B., Furlan, S. N., Klenerman, P. and Nixon, D. F., A novel technique for the fluorometric assessment of T lymphocyte antigen specific lysis. J. Immunol. Methods. 2001. 249: 99–110.
25. Abda, R., Chevaleyre, C. and Salmon, H., Effect of cryopreservation on chemotaxis of lymphocytes. Cytobiology 1998. 36: 184–193.
26. Armour, K. L., Atherton, A., Williamson, L. M. and Clark, M. R., The contrasting IgG-binding interactions of human and herpes simplex virus Fc receptors. Biochem. Soc. Trans. 2002. 30: 495–500.
27 Riechmann., L., Clark., M. R., Waldmann, H. and Winter, G., Reshaping human antibodies for therapy. Nature 1988. 332: 323–327.

28 Gorman, S. D., Clark, M. R., Routledge, E. G., Cobbold, S. P. and Waldmann, H., Reshaping a therapeutic CD4 antibody. Proc. Natl. Acad. Sci. USA 1991. 88: 4181–4185.

29 Bruggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jefferis, R., Waldmann, H. and Neuberger, M. S., Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J. Exp. Med. 1987. 166: 1351–1361.

30 Clark, M. R., IgG effector mechanisms. Chem. Immunol. 1997. 65: 88–110.

31 Armour, K. L., van de Winkel, J. G., Williamson, L. M. and Clark, M. R., Differential binding to human FcgammaRIIa and FcgammaRIIb receptors by human IgG wildtype and mutant antibodies. Mol. Immunol. 2003. 40: 585–593.

32 Moser, R., Etter, H., Oligati, L. and Fehr, J., Neutrophil activation in response to immune complex-bearing endothelial cells depends on the functional cooperation of Fc gamma RII (CD32) and Fc gamma RIIB (CD16). J. Lab. Clin. Med. 1995. 126: 588–596.

33 Frohlich, D., Sperti, O. and Moser, R., The Fcgamma receptor-mediated respiratory burst of rolling neutrophils to cytokine-activated, immune complex-bearing endothelial cells depends on L-selectin but not on E-selectin. Blood 1998. 91: 2558–2564.

34 Orlandi, R., Gussow, D. H., Jones, P. T. and Winter, G., Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 1989. 86: 3833–3837.

35 Kilmartin, J. V., Wright, B. and Milstein, C., Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. J. Cell Biol. 1982. 93: 576–582.

36 Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970. 227: 680–685.

37 Sheikh, S. and Nash, G. B., Continuous activation and deactivation of integrin CD11b/CD18 during de novo expression enables rolling neutrophils to immobilize on platelets. Blood 1996. 87: 5040–5050.

38 Stolen, C. M., Madanat, R., Marti, L., Kari, S., Yegutkin, G. G., Sariola, H., Zorzano, A. and Jalkanen, S., Semicarbazide sensitive amine oxidase overexpression has dual consequences: insulin mimicry and diabetes-like complications. FASEB J. 2004. 18: 702–704.