activation. The key method for obtaining atomic resolution structural information is X-ray crystallography, a technique accounting for 88% of structures deposited in the Protein Data Bank. Unfortunately, this technique requires milligram amounts of purified protein, which has to subsequently be induced to form crystals. Both protein production and crystallization can be difficult tasks particularly for membrane-bound, multidomain and multisubunit macromolecular complexes.

GPIb-IX-V demonstrates all these difficulty criteria. In such cases structural biologists use the divide-and-conquer approach, dividing the proteins into smaller, more manageable fragments, for example, deleting the membrane-spanning regions. These approaches previously led to structure determination of the extracellular domain of GPIbα and its complex with VWF-A1 domain.4,5 A strategy that can be used in even more stubborn cases is to crystallize the protein of interest in the presence of large fusion-tag, as recently exemplified for β-adrenergic and Toll-like receptors.6 This approach has been taken a step further by McEwan et al, who grafted 3 segments of the GPIX subunit onto the homologous GPIbβ subunit (see figure).7 McEwan et al now describe the structures of the extracellular domains of both GPIbβ and this GPIbβ/IX chimera.1 The observation that GPIX cannot be expressed on cell surface by itself, but only in the presence of GPIbβ, provides an assay for the effects of mutations on the interaction between these proteins through the analysis of surface expression in transiently transfected cells.

One of the key advances provided by McEwan and colleagues is the description of the quaternary association of GPIb-IX subcomplex (the GPV subunit is not essential for most functions of the receptor). Clearly, one needs to be cautious when interpreting the interfaces observed in the GPIbβ/IX chimera crystals as reflective of the functional interaction between GPIbβ and GPIX in the intact receptor complex. However, this conclusion is the simplest explanation of the available data and is supported by several pieces of evidence: (1) it was demonstrated, based on surface expression analysis and coimmunoprecipitation experiments, that the GPIX loops grafted in the GPIbβ/IX chimera are sufficient to mediate association with GPIbβ; (2) the crystal structure of the GPIbβ/IX chimera reveals interactions of GPIX loops with GPIbα sequences that are consistent with association in the native complex; (3) analogous interactions are observed in at least 2 crystal forms of the GPIbβ/IX chimeric protein; and (4) mutagenesis of residues in the observed interface results in a loss of the ability of GPIbβ to support surface expression of GPIX in transiently transfected cells. Because interactions between transmembrane helices make a major contribution to the association of receptor subunits, the self-association of the GPIbβ/IX chimera observed in the crystals is not observed in solution. The nature of self-association of the current GPIbβ/IX chimera makes it unsuitable to study its interactions with GPIbα directly, but this may be possible after grafting further GPIX segments into the GPIbβ scaffold. While the current data allow a possible arrangement for the binding of 2 GPIβ subunits to GPIX to be deduced, as presented by McEwan et al, further data are required to develop a unique model.

The paucity of structural data on the GPIb-IX complex has also meant that the molecular basis of a number of mutations leading to Bernard-Soulier syndrome has remained unclear. A combination of structural and surface expression analysis now provides an immediate explanation for 8 mutations in the GPIbβ extracellular domain and suggests different mechanisms leading to disease. While 6 of the mutations affect proper folding and secretion of GPIbβ, 2 exhibit no such effect. Instead, these 2 mutations result in a lack of GPIX cell-surface expression when GPIbβ and GPIX are coexpressed, suggesting they perturb the functional interaction between GPIbβ and GPIX. Indeed, the corresponding residues are present near the GPIbβ-GPIX interface revealed by the GPIbβ/IX chimera structure.

The structures presented by McEwan et al also have general implications in terms of protein structure.1 GPIbβ and GPIX unusually contain only 1 copy of the leucine-rich repeat (LRR) structural motif. As the stability of LRRs and more generally solenoid9 protein structures depends on stacking of several repeats, a single repeat would not be expected to adopt a stable structure. The structures reveal that a stable structure is formed by the stacking of 3 solenoid layers through the help of repeat-flanking sequences.

The new structural information not only advances the functional understanding of the GPIb-IX-V complex, but also provides new opportunities for developing antithrombotic therapeutics with a potential for reduced bleeding side effects.

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**CLINICAL TRIALS**

Comment on Wierda et al, page 5126

**Anti-CD20: tales of identical twins?**

Anna Schuh Oxford Biomedical Research Centre

In this issue of Blood, Wierda et al show in a retrospective analysis of 206 patients that prior rituximab exposure does not affect the efficacy of subsequent ofatumumab treatment.1
Although these results will have to be confirmed prospectively, they begin to unravel clinically significant differences between these anti-CD20 antibodies. This is important for 2 reasons:

1. The addition of the monoclonal anti-CD20 antibody rituximab to the fludarabine and cyclophosphamide chemotherapy backbone in first line therapy is the first ever drug combination to have altered the natural history of chronic lymphocytic leukemia (CLL). Besides, the same combination has shown superior progression-free survival (PFS) in second-line treatment. A significant number of CLL patients are therefore treated with rituximab combinations. However, 20% and 40% of patients treated with rituximab, fludarabine, and cyclophosphamide (FCR) in first and second line, respectively, relapsed within 2 years of finishing treatment and are unlikely to benefit from further rounds of FCR. There is, therefore, an urgent need to identify effective modalities for this poor prognosis group of patients.

2. The precise mode of action of ofatumumab and indeed rituximab remain poorly understood. The clinical observations presented by Wierda et al provide clinical evidence that although both are Type I antibodies directed against the same cell-surface molecule, they are likely to act through very different mechanisms. There are striking differences between rituximab and ofatumumab. Whereas rituximab is a chimeric antibody, ofatumumab (HuMax-CD20) is a fully human anti-CD20 monoclonal antibody. Ofatumumab binds to a different epitope than rituximab and is thought to confer greater complement-mediated cellular cytotoxicity with some additional antibody-dependent cellular cytotoxicity. Ofatumumab is also the only anti-CD20 antibody not to have direct contact with the 3 critical amino acids (170-172) responsible for classic type I and II action. It shows enhanced activity against CLL cells expressing low levels of CD20. Besides, contrary to ofatumumab, rituximab monotherapy is of limited efficacy. Although comparison between trials should be made with caution, the recently published phase 2 data on FC-O combination therapy in first line shows inferior overall response rate (ORR) and PFS compared with FCR.

In the current study, the authors used follow-up data of the single-arm phase 2 Hx CD20 406 study on safety and efficacy of single-agent ofatumumab in bulky refractory (BF ref) or fludarabine and alemtuzumab refractory (FA ref) disease. Results of the interim analysis of the first 138 patients led to accelerated FDA approval of ofatumumab for double refractory patients. The ORR in this study was 58% in the FA ref group and 47% in the BF ref group. Perhaps the most significant improvement was in overall survival (OS), which was 13.7 and 15.4 months in the FA ref and BF ref responder groups, respectively, compared with 9.8 and 10.2 months in nonresponders.

Now, Wierda et al proceed with a retrospective analysis of 206 patients from this study: 117 were previously treated with rituximab (98 rituximab-refractory), and 89 were rituximab-naive. For rituximab-treated, rituximab-refractory, and rituximab-naive patients, ORR was 43%, 44%, and 53%; PFS was 5.3, 5.5, and 5.6 months; and OS was 15.5, 15.5, and 20.2 months. There were no significant differences in ofatumumab-related infusion reactions or hematologic or infectious adverse events between subgroups.

What do these findings mean for our patients? They are the first attempt to evaluate the impact of prior rituximab exposure on efficacy of a next-generation anti-CD20 antibody. It is important to highlight the weaknesses of the study: this is a retrospective ad-hoc analysis of a study never designed to answer this question. Besides, patient numbers in some of the subgroups are small. These limitations do not allow any definite clinical conclusions to be drawn and ideally, results should be validated in a prospective manner. However, the data clearly tell us that patients with rituximab-treated or rituximab-refractory disease should not be excluded from clinical studies investigating other anti-CD20 antibodies. Importantly, mechanisms of resistance to anti-CD20 antibodies seem to vary between different antibodies. While the German CLL8 data clearly demonstrate that patients with TP53 abnormalities did not benefit from FCR treatment, there is some evidence from the Hx CD20 406 study that ofatumumab might be effective in at least some of these patients. Furthermore, the United Kingdom analysis of compassionate use ofatumumab included patients with TP53 abnormalities who benefitted from ofatumumab. However, the available data on single-agent ofatumumab in FCR-refractory patients with CLL also demonstrate that responses are generally of short duration and no complete responses were achieved. Therefore, future efforts need to focus on evaluating the role of maintenance and combination therapy with novel targeted agents such as BCR inhibitors.

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Anna Schuh