Chapter 4. DISCUSSION

Purification of human Hp phenotype has been hampered for years due to its structural diversity in Hp 2-1 and 2-2 (61, 70-72). In the present study, plasma without any additional manipulations (e.g., ammonium-sulfate precipitation) was utilized for the isolation of each phenotype. The solution property of each specific type isolated from the mAb-affinity column was consistent with its molecular forms, of which Hp 1-1 was homogeneous in both charge and size in contrast to the other two types (Figs. 1 and 2).

Since Hp 1-1 represents the simplest unit of Hp (β-α-α-β), we speculate that the molecular hindrance for the "functional surface" of this type is minimal; the overall antigenic reactive sites might be altered for the antibody binding with polymeric Hp 2-1 or 2-2. To test this hypothesis, we initially determined the immunoreactivity of each phenotype of Hp with sequence-dependent β-chain specific mAb. The immunoreactivity of Hp 1-1 for these mAb (8B1 for example) was higher than that of Hp 2-1 and 2-2 (59). A similar result was also seen using a sequence-dependent α-chain specific mAb 3H8.

Furthermore, we demonstrated that 3B7 and 4H11 were immunoreactive to Hp 2-1 and 2-2 polymers, but not to 1-1 (Fig. 3). One of the possibilities is

that the epitope for these mAb is not fully exposed. Another potential explanation is that these mAb recognized a newly formed epitope that is oriented in a 3 D-configuration, where it only takes place in the unique polymeric forms of Hp 2-1 and 2-2. Such notion is consistent to our Western blot analysis using a PAGE gel containing a reducing reagent (Table 2), showing the conformation-dependent nature of these mAb. Unfortunately, the exact epitope could not be addressed presently. Nonetheless, each of these conformational mAb is believed to be directed against its own 3D-epitope as demonstrated by the hemoglobin inhibition experiment (Table 2 and Fig. 7). It is conceivable that a mAb specific only to Hp 1-1 might be produced when Hp 1-1 is used for immunization. For example, we have recently generated a mAb that only recognizes a thermo-denatured β-lactoglobulin, when immunizing thermo-denatured β-lactoglobulin (66, 67). MAb prepared against human hepatic lipase using its active or inactive form as an immunogen can also distinguish between active and inactive forms of lipase (64). Using mAb as a probe to study the structural and functional relationship of a given protein has been popular and reviewed (73). It provides a powerful tool in defining the functional location within the molecule. Some unique low-density-lipoprotein (LDL) mAb have even been used for discriminating between patients with and

without coronary artery disease (74, 75). To this end, the present study offers the future prospect of preparing a specific mAb for each type that can be used for less time-consuming Hp phenotyping in large populations. From a technical point of view, the type specific mAb can potentially be used for all the individuals, if predetermination of phenotype becomes as necessary as blood typing. For example, a recent study postulates that the relative benefit or harm of vitamin therapy on the progression of coronary artery stenoses in women is dependent on Hp type. The influence of Hp type seemed to be stronger in women with diabetes (58).

Since the 3D structure of each specific Hp type is lacking, the exact mechanism involved in the recognition of these 3B7 and 4H11 mAb to the Hp polymers remains elusive. Interestingly, the immunoreactivity of Hp was totally abolished in the presence of a reducing reagent (Table 2) revealing that the overall structure of Hp was crucial in maintaining the necessary conformation for these two mAb. On the other hand, the α-chain specific mAb 3H8 was “sequentially dependent” as evidenced by our antigenic mapping using the synthetic peptide containing only 15 residues (Fig. 9) and its ability to react with the recombinant α1 and α2. The conformation of the latter was essentially disordered in structure as compared to α-helical enriched Hp

molecules (Fig. 11). With respect to the epitope specificity, the antigenic site was located within the residues 31-45 as determined by the peptide array (Fig. 9). The overlapped peptide 22-36 possessed a partial immunoreactivity suggesting that the C-terminus (residue 36) beyond Tyr-37 was involved.

Several lines of evidence indicate that the anticipated antigenic site was closely associated with the putative residues 31-39 (or RYQCKNYYK). First, the size of an epitope is relatively small usually containing 6-9 amino-acid residues (22, 63, 76, 77), then it is possible to narrow down the reactive site from the immunoreactivity in overlapped peptides (67). This region consisting of charged residues (Arg-31, Lys-35, and Lys-39) is consistent to the notion that charged amino acids are usually involved in a given antigenic site playing a key role in determining the epitope specificity (67). Second, within the proposed residues 31-39 (nine residues), there are five residues that differ from the sequence of mouse (see Fig. 9 for more details). Third, single mutation at Tyr-32 of recombinant α1 chain resulted in a total loss in immunoreactivity (Fig. 11). Fourth, the mapped epitope sequence is exactly repeated twice in the α2 subunit, consistent with the doubled immunoreactivity found in recombinant α2 as compared to α1 (Fig. 11).

However, the immunoreactivity of this defined epitope in whole Hp 2-1 or

2-2 molecule was attenuated and almost identical to 1-1. One of the potential explanations is that only one defined copy of the two epitopes in the α2 (for example, the one at NH_2-terminus) of 2-1 or 2-2 is available for the 3H8 mAb. This copy is evenly expressed on the Hp surface (Fig. 9). Another possibility is that one randomly expressed copy or less of the two epitopes in α2 is reactive for the mAb. By chance, this mAb reacted equally with three Hp types and therefore was suitable for the immunoassay for Hp determination (Fig. 8). Nevertheless, the immunoreactivity of α2 subunit was markedly decreased in Hp 2-1 and 2-2 confirming our hypothesis that the surface availability was limited for the binding of sequence-dependent mAb in Hp polymers. It is worth mentioning another α-chain specific mAb (2A3, Table 2) that also possessed identical immunoreactivity against the three Hp types. But due to its being inhibited by the hemoglobin binding (Fig. 7); it lacks utility in Hp determination, and thus was presently not studied for antigenic mapping. The mapped antigenic sequence for mAb 3H8 (residues 31-39) provides us with a valuable reference and an opportunity in the future for the preparation of a polyclonal antibody against this specific region (78). Such a regional-specific polyclonal antibody could be potentially used for standardizing the method in Hp determination of all types.

The Hp concentrations of 1-1 subjects we determined were differentially higher than that of 2-1 and 2-2. These data are consistent to some studies (30), but not to others (38, 79). Such discrepancy may due to the diverse specificity of the polyclonal antibodies used in the immunoassays.

Taken together, we have identified the unique conformational difference among the antigenic structure of three Hp phenotypes using mAb. Such structural diversity may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders (79, 80). These effects are correlated with a phenotype-dependent modulation of oxidative stress (39). For example, Hp 2-2 and 2-1 are associated with an increased risk for the development of nephropathy in patients with diabetes mellitus. Nakhoul et al (81) have postulated that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved. Melamed-Frank et al (40) have recently demonstrated that the binding of Hp 1-1 to hemoglobin is much superior to that of Hp 2-2. The mechanism involved, however, is not fully understood. The fashion of hemoglobin binding seems to be similar to our α-chain mAb 3H8, which binds better to Hp 1-1 than to 2-1 and 2-2. This phenomenon may affect the clearance rate of hemoglobin containing heme, so is

responsible for the development of oxidative stress induced diseases in patients with Hp 2-2 phenotype (82). Another essential feature is that all the non-human species possess only the Hp 1-1 type. The biological activities of Hp, such as antioxidant (39, 83), angiogenesis (84), hemoglobin-binding (85), and receptor-meditated Hp-hemoglobin complex clearance (86), are originally "designed" for Hp 1-1. The evolved Hp 2-1 and 2-2 due to the insertion of a partially redundant sequence of α chain resulted in not only large size of the particles, but also significant alternation of the surface epitopes as demonstrated in the present study. The rearranged epitopes produced their own conformation-dependent mAb, and attenuated their binding to Hp 1-1. Conceivably, the newly oriented surface may also attenuate its biological activities as mentioned above. Finally, we speculate that a mAb specific to Hp 1-1 can be produced when mice are immunized with Hp 1-1. Originally, we thought this could be achieved by given Hp 2-1 which also contains 1-1 molecules. The experiment to address this possibility is currently in progress using Hp 1-1 as an immunogen.

In conclusion, the present study provides new insight in understanding the structural diversity of Hp phenotypes. The antigenic mapping further demonstrates the "masked" region involved in the molecules of Hp 2-1 and 2-2

and provides a valuable reference and strategy in producing a useful immunochemical reagent.