行政院國家科學委員會補助專題研究計畫成果報告

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以噬菌體展出系統來篩選C型肝炎病毒特異性的重組人類抗體的研究（II）

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Generation and characterization of recombinant human antibodies specific for hepatitis C virus by phage displaying system **(II)**

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計畫主持人：楊沂淵

共同主持人：

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中文摘要

C 型肝炎病毒是主要造成人類輸血後非 A 非 B 型肝炎的病毒，它不只會造成暫時性感染的急性肝炎，亦會造成慢性肝炎、肝硬化、肝細胞癌。C 型肝炎的非結構蛋白 5 具有 RNA 聚合酵素之活性，且在病毒複製過程中扮演重要角色。在本實驗中，我們利用兩個噬菌體抗體基因庫篩選與分析對 NS5A 有專一性的抗體。包含重鏈與 kappa 或 lambda 輕鏈的基因庫大小分別為：1.3×10^{7} 和 2.1×10^{6} pfu。隨機挑選二十個菌株進行 DNA 序列分析，結果指出：這些抗體片段有四種組合，分別為 NS5L6、NS5L13、NS5L14、與 NS5K8。在這四種組合當中，其中六個屬於 NS5L6 這群且含有相同重鏈與輕鏈基因。甚至 NS5L6 的輕鏈與 NS5L13 的輕鏈幾乎相同，這可能暗示輕鏈是決定抗體具有對 NS5A 抗原專一性之關鍵。任意選擇的菌株表現蛋白後，用西方墨點法、抗人類κ或λ輕鏈抗體分析後，證實 50 kDa 的 Fab 之蛋白存在。酵素連結免疫分析法結果顯示，NS5L6、13、14 與 C 型肝炎病人血清相較下，皆具有 NS5A 結合之專一性。綜合以上所述，我們的結果說明：噬菌體表現抗體的技術，也許可取代傳統製造人類專一性單株抗體方法。在未來，這些抗體將可被用來發展成抗感染性疾病的藥物。

Abstract

Hepatitis C virus (HCV) is the major source of non-A, non-B hepatitis, which causes not only acute hepatitis by transient infection, but also chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in human. Nonstructural protein 5 (NS5) of HCV possesses RNA polymerase activity and plays a key role in viral replication. In this study, we utilized two phage display antibody libraries to generate and analyze antibodies specific for NS5A. The sizes of antibody libraries

containing kappa and lambda light chain are 1.3×10^7 and 2.1×10^6 pfu (plaque forming unit), respectively. Sequence analysis of 20 randomly selected clones indicated that these Fab fragments consisted of four groups, represented by NS5L6, NS5L13, NS5L14, and NS5K8. Of which, six NS5L6 clones contain identical heavy and light chain genes. Moreover, the light chain gene used by NS5L13 is almost identical to that of NS5L6 clones, suggesting that this light chain might be crucial for the NS5A-binding activity. The Fab expression of these chosen clones was verified as a 50 kDa protein on western blot using anti-human κ or λ light chain antibodies. ELISA results revealed that NS5L6, 13, and 14 all have NS5A-binding specificity comparable to that of sera from HCV-infected subjects. Viewed as a whole, our results suggested that the phage display antibody technology might provide an alternative way for the generation of human specific monoclonal antibodies. These generated antibodies could be useful for the development of therapeutic agents against infectious diseases in the future.

Introduction

HCV is a small positive enveloped RNA virus about 30-38 nm in diameter (Yuasa et al., 1991). The virus itself is an RNA virus of about 9,400 nucleotide bases (Choo et al., 1991). After first isolation of an antibody serologic marker by Choo (1991) and coworkers, multiple strains of HCV were recognized. The HCV is now considered as characteristic typical of the family *Flaviviridae* (Okamoto et al., 1991; Takamizawa et al., 1991). HCV possesses a 5'-untranslated region that is highly conserved among strains (Okamoto et al., 1991), which is followed by an open reading frame encoding a polypeptide with 3,010 to 3,011 amino acids in length. This polypeptide is subdivided into nine proteins by the following orders: core (C), envelop (E) E1, E2, nonstructural (NS) proteins NS1, NS2, NS3, NS4 (A/B), and NS5 (A/B) (Takamizawa et al., 1991). These proteins are formed by the cleavage of the original polyprotein by both host and viral proteases (Okamoto et al., 1991; Hijikata et al., 1993). The C region encodes the core nucleocapsid protein (Takeuchi et al., 1990). E1 has been shown to be a glycosylated envelop protein. Whether E2/NS1 represents another envelop protein or a nonstructural one remains controversial (Hahm et al., 1995). NS2 protein is a transmembrane protein, the carboxyl terminal inserts into lumen of endoplasmic reticulum; whereas the amino terminal is localized in the cytosol. The function of NS2 protein is not clear at this time, but data demonstrated that its carboxyl terminus have proteolytic function (Santolini et al., 1995). NS3 is a 70 kDa multifunctional protein. The NS3 encodes viral protease that cleaves at least four sites downstream of the viral proteins. Moreover, based on the structural analysis, the NS3 contains RNA helicase activity, NTPase and RNA binding domains (Yao et al., 1997). Recent work suggested that NS3/4A is a serine protease similar to chymotrypsin and that it helps cleave

the original polyprotein (Tanji et al., 1995). NS5 encodes two proteins NS5A and NS5B, and has been demonstrated to exist as a protein comprising two polypeptides acting as a RNA-dependent RNA polymerase (Major and Friestone 1997). NS5A contains many potential sites for phosphorylation particularly on serine at amino acid position 2197, 2204 and 2210. In HCV-infected cells, NS5A was localized on the nuclear membrane, suggesting it may be involved in the HCV replication. The life cycle of HCV is not clearly understood.

In this present study, we propose to apply phage display antibody technology to construct and characterize a panel of antibodies against HCV NS5A antigen. As mentioned above, since NS5A is considered to important for viral replication, it is a good therapeutic target molecule for use in developing anti-HCV drugs. The antibodies against HCV with high specificity generated in our proposed studies represents a promising antiviral approach to interfere with the life cycle of HCV.

Materials and methods

PCR amplification of HC and LC variable region

Total RNA was extracted from 2 x 10^6 peripheral mononuclear cells by commercial method. Briefly, Trizol buffer (1 ml) and chloroform were added to the tube to lyse the cells. After centrifugation, total RNAs were collected at the aqueous phase, precipitated by isopropyl alcohol, and dissolved in DEPC-treated water. The concentration of RNA samples was measured at OD 260. 1-10 ug of RNAs were reverse-transcribed with an oligo-dT primer to synthesize the first strand cDNA using SuperScript reverse transcriptase.

The λ L chains were amplified by PCR with the CL2 3' primer and 9 different 5' primers. All primers were synthesized as previously described. Similarly, the γ1 H chain Fd regions were amplified with the CG1Z 3' primer and 8 different 5' VH primers . The PCR were performed for 35 cycles, each consisting of a 15-second denaturation at 94°C, a 50-second priming at 52°C, and a 90-second elongation at 72°C (except for a 10-minute during the last cycle). Amplified DNA was analyzed on a 1% agarose gel.

Phage display antibody library construction

The library was constructed according to the published protocols with minor modifications. The amplified L chain DNA were pooled, digested with SacI and XbaI, and cloned into the SacI/XbaI linearized pComb3 vector. The recombinant DNA were electroporated into XL1-Blue strain E. coli. The size and the insert frequency of the resultant L chain library were determined; then phagemid DNA from the total library were prepared. Thereafter, the amplified H chain Fd region DNA were digested with SpeI and XhoI, and ligated into the SpeI/XhoI linearized phagemid DNA that already contained L chain DNA. The resultant phagemid DNA were electroporated into XL-1 Blue cells as previously. Again, the size and the insert frequency of the resultant L chain-H chain Fd library were determined. Phage production

were initiated by the addition of helper phage VCS-M13, and the recombinant phage were precipitated with 4% polyethylglycerol and 3% NaCl (W/V), resuspended in 1x PBS containing 2% BSA and stored at 4°C until used.

Competitive ELISA

The relative affinity of each HCV NS5A binding clone was determined by an indirect competitive ELISA assay using the method of Friguet et al. (1985). A fixed amount of Fab was mixed with increasing amounts of free HCV NS5A molecule (competitive, 1-400 ug/ml). After 18 hr incubation at room temperature, the mixture was transferred to ELISA plates precoated with HCV NS5A molecule. Their binding to solid-phase HCV NS5A was determined as previously (Casali et al., 1990). The dissociation constant (Kd) was calculated according to the Klotz method (Friguet et al., 1985).

SDS-PAGE and western blot

Purified Fab antibodies with the HCV NS5A binding activity was separated by 12% SDS-PAGE under described condition (Laemmli, 1970). After separation, human IgG Fab fragment on the gel was transferred to the nitrocellulose papers by a sandwich-diffusion method. Two identical blots were obtained after transfer overnight at room temperature. For immunodetection, the membranes was blocked in 1% BSA dissolved in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 8.0) for 1 hr (10 minutes each) at room temperature and then incubated with properly diluted goat anti-human λ and γ specific antibodies conjugated with alkaline phosphotase for one additional hr. The blot was developed in substrate solution prepared by mixing reagents A and B in buffer as recommended by the manufacturer (Bio-Rad). Color development was stopped simply by rinsing the blots in distilled water.

Results and Discussion

In the present study, we have applied phage display antibody library to generate and characterize 9 monoclonal anti-HCV NS5A antibodies. The combinatorial antibody libraries were a kind gift from Dr. Maruyama Toshiyaki. The heavy and light chain gene inserts were identified as 680 bp bands on agarose gel electrophoresis. The expression of anti-HCV NS5A Fab fragment detected by western blotting as a 50 kd protein. The binding activity of these clones for NS5A protein was determined by ELISA. Moreover, their NS5A specificity was confirmed by competitive ELISA. When 100 μg/ml of free NS5A was for competition assay, the binding activity of 3 anti-NS5A antibodies was inhibited by 30%, 40% and 45%, respectively. Among these binders, NS5L6 clone showed a positive reactivity to NS5A protein blotted onto a nitrocellulose paper.

Sequence analysis of these NS5A binders showed that 6 (66%) clones had identical heavy (H) and (L) light chains V regions, represented by NS5L6.h and NS5L6.l, respectively. Of the remaining three clones, one used homologous light chain derived from the germline Vλ DPL8, which also encoded

the NS5L6.1 light chain. All nine clones showed a high number of somatic mutations that occurred in the context of antigen driven. These findings demonstrated that the gene usage of VH and VL of anti-HCV NS5A antibodies derived from HCV infected patients is most likely restricted to certain germline configuration. It will be important to study the neutralizing activity of these isolated anti-HCV IgG antibodies both in vitro and in vivo in the future.

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Reference

1. Casali P, Nakamura M, Ginsberg-Fellner F. and Notkins AL. 1990. Frequency of B cells committed to the production of antibodies to insulin in newly diagnosed patients with insulin-dependent diabetes mellitus and generation of high affinity human monoclonal IgG to insulin. *J Immunol.* 144:3741-3750.
2. Choo QI, Richman KH, and Han JH. (1991) Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci* 88,2451-2455.
3. Friguet B, Chaffotte AF, Djavadi-Ohaniance L. and Goldberg ME. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J Immunol Methods* 77:305-319.
4. Hahm B, Han DS. Back SH, Song OK, Cho MJ, Kim CJ, Shimotohno K, and Jang SK. (1995) NS3-4A of hepatitis C virus is a chymotrypsin-like protease. *J Virol* 69,2534-2539.
5. Hijikata M. Mizushima H. Tanji Y. Komoda Y. Hirowatari Y. Akagi T. Kato N. Kimura K. and Shimotohno K. (1993) Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C. *Proc Natl Acad Sci* 90, 10773-10777.
6. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
7. Major ME, and Feinstone SM. (1997) Molecular biology of hepatitis *C. J Hepatology* 25,1527-1537.
8. Okamoto H, Okada S, Sugiyama Y, Kurai K, Lizuka H, Machida A, Miyakawa Y, and Mayumi M. (1991) Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier comparison with reported isolates for conserved and divergent regions. *J Gen Virol* 72,2697-2704.
9. Santolini E, Pacini L, Fipaldini C, Migliaccio G, and La Monica N. (1995) The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J Virol* 69:7461-7471.
10. Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yashida I, and Okayama H. (1991) Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol* 65:1105-1113.
11. Tanji Y, Hijikata M, Satoh S, Kaneko T, and Shimotohno K. (1995) Hepatitis C virus-encoded nonstructural protein

NS4A has versatile functions in viral protein processing. *J Virol* 69, 1575-1581.

12. Yao N, Hesson T, Cable M, Hong Z, Kworcg AD, Le HV, and Weber PC. (1997) Structure of the hepatitis C virus RNA helicase domain. *Nature Structural Biology* 4:463-467.

13. Yuasa T, Ishikawa G, Manabe S, Sekiguchi S, Takeuchi K, and Miyamura T. (1991) The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. *J Gen Virol* 72:2021-2024.