these patients, however, real efficacy of these therapeutic approaches have remained questionable due to lack of randomized controlled trials (immune interventional strategies against chronic HBV infection).[11] In addition, the doses of these immune modulatory agents, duration of therapy, therapeutic protocols have not been optimized for different chronic viral infections.

When clinicians were trying to develop immune therapy against chronic viral infections and cancers by polyclonal immune modulators, it became evident that administration of polyclonal immune modulators may be detrimental for patients with chronic virus infection. Studies have revealed that nonantigen-specific immune responses are related to tissue damages, whereas, antigen-specific immunity is needed for control of viral replication and also reduction of tissue damages in many chronic viral infections.[12,13] This opened a new field of clinical applications of immune therapy for patients with viral infections and cancers. In order to induce antigen-specific immunity in chronic viral carriers and cancer patients, viral-related antigens or tumor-associated antigens have been used.[14-16] This therapeutic approach has been regarded as vaccine therapy. In addition to antigen-based vaccines, epitope-based vaccines and DNA vaccines have also been used to induce viral or cancer-specific immunity.[17,18] In general, these therapeutic approaches are safe, but mixed signals have been found regarding their efficacies. In some studies, vaccine therapy has shown antiviral as well as immune modulatory potentials. On the other hand, the efficacy of vaccine therapy is not so promising in other clinical trials.[19,20]

In the mean time, the concept of cell-based immune therapy originated. Initially, T-cell-based immune therapies have been applied in patients with cancers.[21-23] Due to better understandings about cellular and molecular events regarding induction and maintenance of antigen-specific immunity, it became evident that antigen-presenting dendritic cells (DCs) are critical regulators of immunity.[24,25] Different preclinical trials also revealed that antigen-specific immunity can be induced and maintained in chronic viral carriers and cancer patients by administrating antigen-pulsed DCs.[26-28] The first report about safety and efficacy of antigen-pulsed DC vaccine in patients with cancer has been published by Hsu et al in 1996.[29]

Limitations of Immune Therapy against Chronic Infections and Cancers

Different types of immune therapies have been applied in patients with chronic viral infections and cancers during last three decades (Fig. 2). Thousands of publications are available about immune responses of these patients, however, few immune therapeutic approaches have received a general acceptance as therapeutic approaches in clinics. Immune therapeutic approaches against chronic viral infections and cancers are facing several challenges (Fig. 3) and these will be described first to provide a road map for solution.

Fundamental Differences between Animal Models of Human Diseases and Patients with Chronic Viral Infections and Cancer

Due to ethical and scientific limitations, the concept of immune therapy usually originates from animal studies and the therapeutic regimen is first optimized in animal models of human diseases. After assessing safety and efficacy of these maneuvers in normal volunteers, clinical trials are conducted in patients. Animal models of human diseases provide important information when critical cellular and molecular mechanisms about host/virus or host/cancer cell interactions cannot be studied in details in human. However, there are fundamental differences regarding pathological processes in human diseases and animal models of human diseases. This has become an important issue in the context immune therapy against chronic viral infections and cancers. Due to tremendous development of molecular and cellular biology, it is now possible to produce transgenic mice that represent an animal model of chronic viral infections. Also, availability of tumor cell lines allows production of animal models of cancers. Various immune therapies, such as polyclonal immune modulators, vaccine therapy and DC-based vaccines, have been applied in animal model of human diseases with excellent therapeutic outcome. However, when similar types of immune therapeutic approaches are applied in patients with chronic viral infections and cancers,

Causes underlying low efficacy of immune therapy

1. *Limitation of translation of immune therapy from mice to man:* Human is the only therapeutic model of human diseases
2. *Improper conception of immune therapy:* Immune therapy is not a replacement therapy
3. *Misunderstanding about nature of immunity:* Diminished immunity and distorted immunity
4. *Improper selection of immune interventional strategies:* Innate immunity or adaptive immunity
5. Restricted information about nature of antigen and epitope: Immunogenic or tolerogenic antigens
6. *Lack of proper protocol of cell-based therapy including dendritic cell-based therapy:* Study in patients with advanced diseases. Improper insights about antigen and method of preparation of antigen-pulsed dendritic cells
7. Restoration of immunity is not committed to therapeutic efficacy

Fig. 3: Major challenges about immune therapy in patients with chronic viral infections and cancers

the therapeutic efficacy is negligible. For example, different cytokines have shown potent antiviral effects in HBV-transgenic mice,[30,31] but not in patients with chronic HBV infection.[11] In HBV-transgenic mice, the replication cycle of the virus is completely different from that of patients with chronic HBV infection. Moreover, there are no liver damages in HBV transgenic mice, however, patients with chronic HBV infection exhibit variable degrees of liver damages. Finally, huge amounts of cytokines are given to mice, but that amount cannot be given in patients with chronic HBV infection due to concern about safety of patients.

In the context of cancer, animal models of cancers are developed by implantation of cancer cell lines in normal mice or by administration of some carcinogenic agents. Cancer develops in these animals in a short time. On the contrary, cancer development is a time-consuming matter in human. It takes several years for cancer development and the architecture of cancer tissues is altered. Moreover, cancers in human may have capsule, which hinders entry and activity of immune modulators. In line of this, the efficacy of immune therapy is excellent in animal model of human diseases, but it is extremely difficult to reproduce this in patients with cancers.

Challenges Related to Concept of Immune Therapy: Immune Therapy is not a Supplementary Therapy or Replacement Therapy

Studies have shown that the functions of different immunocytes, such as T-cells, B-cells, monocytes/macrophages and DCs, are diminished in patients with chronic viral infections and cancers. Some subjects also exhibit decreased levels of cytokines. Based on these findings, the strategy of immune therapy has previously been developed to upregulate the functions of different immunocytes or immune modulators. This principle of therapy may be followed during supplementary and replacement therapies. The immune intervention strategies should be designed in a manner so that a series of complex interactions among immunocytes, cytokines, chemokines and several other immune-related mediators occur *in vivo* without tissue damages.

Differences between Diminished and Distorted Immune Responses

There are some misconceptions about immune responses of patients with chronic viral carriers and cancers. It is usually noted that the immune responses of these subjects are diminished. This is not true. The immune responses of these patients are distorted. In most cases, the viral-specific and cancer-specific immune responses are diminished, whereas, antigen nonspecific immunity is exacerbated in these patients. Even, antigen-specific immunity to all types of antigens of the virus or cancers is not diminished. Accordingly, it becomes extremely difficult to design immune therapy in these diseases. If the immune responses are diminished in subjects with chronic infections and cancers, the target of immune therapy is simple because upregulation of host immunity will be the primary aim of this therapy. However, reshaping of distorted immune responses of patients with chronic viral infections and cancers is extremely difficult.

Selection of Interventional Strategies of Immune Therapy

Both innate and adaptive immunity can be activated by immune therapy. If the target of immune therapy is to activate one or more immunocytes, that can be accomplished by activating only innate immunity by immune modulators, like cytokines and growth factors. However, to have sustained antiviral and anticancer immune responses, immune therapy should induce long-lasting immunity and immune surveillance systems. This can be accomplished in chronic viral carriers and cancer patients by inducing optimum levels of adaptive immunity.

Limitations of Antigen-based or Epitope-based or DNA-based Vaccine Therapies

As it became evident that antigen-specific immunity has antiviral, anticancer as well as immune surveillance properties in virus-bearing and cancer-bearing hosts, the purpose of immune therapy is to induce antigen-specific immunity in these patients by antigen-based vaccines or epitope-based vaccines or DNA vaccines. Proper evaluation of these therapies has not been done yet. In fact, little is known about appropriate antigens, dose of antigen, and duration of vaccinations and route of vaccination. Application of immune therapy in more and more patients and randomized controlled-trials are needed to develop insights about the scopes and limitations of these approaches.

Limitations of Cell-based Therapy

To induce antigen-specific immunity in patients with chronic viral infections and cancers, mere administration of antigens or epitopes may not be effective. These patients have shown impaired functional capacities at all levels of immune cascades, such as at the level of antigen priming (antigen-presenting cell levels) and also in the context of functioning of effecter cells (T-cell and B-cell levels). Moreover, these patients harbor abundant amounts of antigen and basically

tolerant to virus-related and cancer-related antigens. Accordingly, antigen-based therapy and epitope-based vaccine therapy are not supposed to induce proper virus-specific and cancer-specific immunity in patients with chronic viral infections and cancers because the injected antigens or epitopes are not likely to be properly processed and presented for activation of T-cells and B-cells. These limitations can be overcome if cell-based therapy is employed. Adaptive transfer of activated T-cells has been used in several cancer patients to kill cancer cells. Although these T-cells can kill some cancer cells, they are unable to block growth of cancer cells and they can not induce anticancer immune surveillance mechanism.

Limitations of DC-based Therapies

Although little has been done to treat patients with chronic infection by antigen-pulsed or peptide-pulsed DCs, many clinical trials are going on regarding the utility of antigen-pulsed DCs and epitope-pulsed DCs in cancer patients. Meta-analyses have shown that the present regimen of DC-based therapy, in which antigen or epitope-loaded DCs are administered to these patients, has only limited therapeutic efficacy.[32] However, studies in animal models of human diseases and also in patients indicate that if these therapeutic approaches can be properly designed, their efficacies can be increased in patients with chronic viral infections and cancers.

The limited efficacy of DC-based therapy is related to improper understandings about (1) DCs that should be used (blood or bone marrow or lymph node derived), (2) antigens that should be chosen, (3) method of loading DCs with antigen, (4) method of administration of antigen-pulsed DCs (intradermal or subcutaneous or intramascular, or intravenous) and (5) characterization of antigen-pulsed DCs before administration to patients.

Induction of Immunity may not be Reflected in Therapeutic Efficacy

Immune therapy has been designed to treat patients with chronic viral infections and cancers by restoration of host immunity. These patients harbor abundant amount of viruses or cancer cells, they are tolerant to these antigens, exhibit tissue damages and complications. Immune therapy is targeted to induce and sustain immunity in these subjects. However, due to multifactorial problems of these patients, some patients may not exhibit therapeutic efficacy of immune therapy even if proper immunity is induced and sustained by immune therapeutic approaches.

ROAD MAP TO SOLUTION

The main purpose of immune therapy is to develop an immune surveillance mechanism in patients with chronic viral infections and cancers. The target of immune therapy in chronic viral carriers is to achieve sustained control of viruses with restoration of antiviral immunity. In patients with cancer, restoration and functioning of immune surveillance system is the principle aim of immune therapy.

We have discussed about the limitations of ongoing immune therapeutic approaches against chronic viral infections and cancers. Next, we will discuss how these problems can be solved (Fig. 4).

Re-evaluation of Immune Responses in Patients with Chronic Viral Infections and Cancers

Although important information about pathogenesis of disease processes and scope of immune therapy can be gathered by conducting experiments in animal models, the utility of these therapies can only be assessed by conducting investigations in patients with these diseases. In addition, it is unlikely that there is any universal immune therapeutic approach for all types of chronic viral infections and cancers. Accordingly, the interventional strategies should be ascertained on a case by case basis. In future, it may be

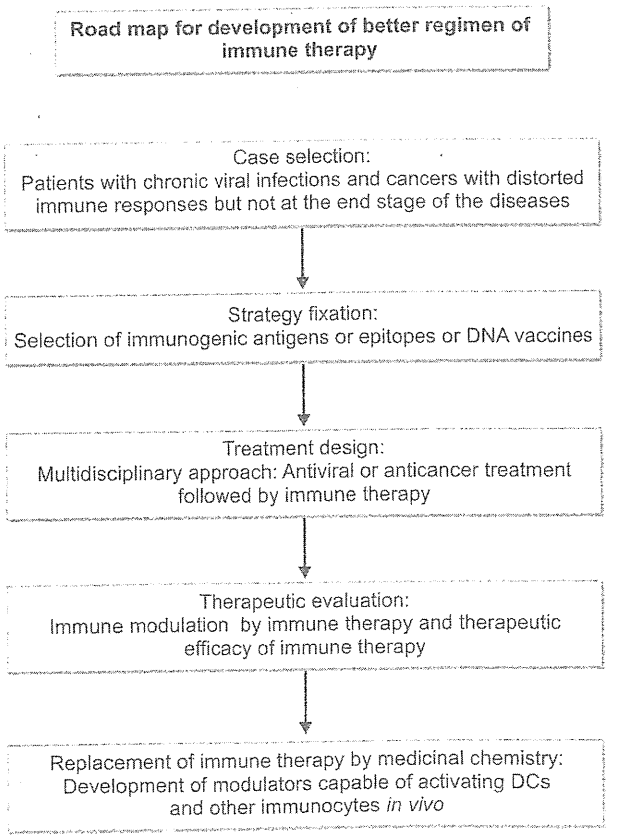

Fig. 4: Road map to develop better and effective regimen of immune therapeutic approaches against chronic viral infections and cancers

possible to get some common facts that will allow development of immune therapy for certain diseases. Also, it may be possible to assess the prognosis of immune therapy from the clinical background of these patients.

Case Selection for Immune Therapy

It is needless to say that no therapeutic approach can be available that is effective in all patients with chronic viral infections and cancers. In the context of antiviral therapy, all patients with chronic HBV infection without liver damages are not given any antiviral drugs because these patents do not respond to these drugs. In case of cancers, different types of therapeutic approaches are not given to patients with advanced cancers. Unfortunately, immune therapy has mainly been applied in patients with advanced cancer. This is not an exception because any new type of therapy is done in these types of patients due to concern of safety. All patients with advanced cancers are immune compromised. Accordingly, immune therapy is unlikely to achieve its goal in such patients. Due to safety concern, there are few studies with DC-based immune therapy in patients with chronic viral infections. As immune therapy is in its infancy, the real potentials of this therapy should be assessed in immune competent patients with cancers and in patients with chronic infection without complications. It would be non-scientific if we conclude about the efficacy of immune therapy without performing clinical trials in proper subjects.

Strategy of Immune Therapy

Induction of innate immunity can be counter productive as a therapeutic approach in these patients because this can induce inflammation in patients with chronic viral infections and cancers. When a chronic viral infection is established or a cancer is clinically detected, it means that the innate immunity of these subjects could not inhibit the disease processes. Attempt should not be taken to induce innate immunity in these patients. On the contrary, adaptive immunity can kill virus infected cells and cancer cells by a noncytopathic mechanism. Thus, the possibility of tissue destruction is minimized. Accordingly, the purpose of immune therapy should be to induce adaptive immunity.

Selection of Antigens

In all types of viruses and cancers, there are several virus-related antigens and cancer-related antigens. However, the functions of these antigens differ considerably. Some of these antigens are immunogenic, whereas, others may be tolerogenic. For example, many antigens of HBV or HCV down regulate host immunity. Thus, immunogenic antigens should be selected for immune therapy. Again, some antigens may induce T helper 1 immunity, whereas, others can induce T helper 2 immunity. Some antigens may induce humoral immunity, whereas, others can give a cytotoxic T cell response. Based on the virological and immunological status of the patients, antigens should be carefully selected for successful immune therapy.

Production of Immunogenic Antigen-Pulsed DCs

Antigen-presenting DCs loaded with antigen (antigen-pulsed DCs) are now used for treatment of cancers[32] and their safety has recently been confirmed in noncancerous subjects.[33] Extensive use of antigen-pulsed DCs is expected in cancer patients as well as in chronic viral infections in near future. There are major limitations regarding the protocol of production of antigen-pulsed DCs or epitope-pulsed DCs. Antigen-pulsed DCs should be prepared by culturing DCs with immunogenic antigens. Now, it is prepared by culturing DCs with whole tumor products or tumor RNAs or exosomes. The immunogenic nature of these products is not clear. Whole tumor may contain immunogenic and tolerogenic antigens and it is really elusive if the injected DCs would induce immunity or tolerance. Viral antigen-pulsed DCs have mainly been administered to animals with chronic viral infections. Many viral antigens are not immunogenic, rather, they suppress immune responses. The protocol for preparing immunogenic antigen-pulsed DCs should be confirmed by conducting preliminary studies in man and mice.

Immune Therapy as a Multidisciplinary Approach

The utility of immune therapy as an independent therapeutic approach against chronic viral infection or cancer is not so inspiring at this point. This is mainly because it is extremely hard to induce and sustain antiviral immunity in subjects with chronic viral infection with very high viral load. Also, antitumor immunity may not be induced in patients with cancers with abundant amounts of cancer cells. Abundant amounts of viruses and cancer cells induce immunogenic tolerance in these patients. Antigen excess always hinders induction of antigen-specific immune responses. To address this issue, immune therapy may be applied after decreasing the amounts of virus and cancer cells. This can be done by treating patients with chronic viral infections by antiviral drugs and patients with cancers by conventional antitumor therapeutic therapies. These therapeutic approaches would reduce viral and cancer burden and thus a file may be prepared for proper activity of immune therapy. In fact, combination of antiviral and immune therapy has shown potent therapeutic effect compared to monotherapy with

either antiviral drugs or with only immune therapeutic approach.[34] Studies have shown that immune therapy is effective in cancer patients after ablation of cancer mass.

Replacement of Immune Therapy by Medicinal Chemistry

The purpose of immune therapy is noble, induction and maintenance of immune surveillance mechanisms against viruses and cancer cells. Vaccine therapy and DC-based vaccine therapy seems to be safe therapeutic approaches and capable of inducing the proper therapeutic efficacy against chronic viral infections and cancers, if applied after conventional therapeutic approaches. However, all types of cell-based therapy, including DC-based therapy, need special techniques, trained manpower and highly sophisticated facilities. These types of therapies can be effective but their mass usage is not expected. DCs are basically adjuvants that allow antigens to be properly presented to T-cells and B-cells. Antigen-pulsed DCs carry immunogenic forms of the antigens and directly stimulate the immunocytes for induction of antigen-specific immunity. Studies with DC-based vaccines for more than a decade in cancer patients have provided important insights about the method of induction of antigen-specific immunity. Now, it is known that antigen-pulsed DCs cause activation of cytokines and chemokines *in vivo*. Also, they ensure inflammatory microenvironments for immune responses.

The time is mature to start investigations about the methods of replacing DC-based vaccines by products of medicinal chemistry. The key factors that antigen-pulsed DC provides *in vivo* should be identified and characterized. It may be possible to active DCs of patients with chronic infections and cancers by administrating products of medicinal chemistry. Further development of medicinal chemistry can replace a cell-based therapy with drugs.

Understanding of philosophy and strategy of immune therapy and collaboration between immunologists and scientists of medicinal chemistry may lead to the development of magic bullets for treatment of chronic viral infections and cancers in near future.

CONCLUSION

Immune therapy, especially DC-based therapy seems to be an alternative therapeutic approach for treating patients with chronic viral infections and cancers. Immune therapies have mainly been started as pilot study and open clinical trials have shown that these types of therapies can be adopted in future. Recently only, antigen-pulsed DCs have been used in noncancerous human. The safety of antigen-based vaccine therapy and DC-based vaccine therapy has been confirmed in several studies. Now, there is a need to increase their efficacy. More and more clinical trials should be conducted in these patients. Especially, immune therapy should be done at the primary stages of the diseases to assess the real efficacy of this therapy. The real therapeutic potentiality of immune therapy is yet unknown because immune therapies have been applied mainly in patients with advanced diseases and also, there is no appropriate therapeutic protocol of immune therapy. It seems that immune therapy should be conducted as part of multidisciplinary therapeutic approaches. Finally, immune therapy should be replaced by products of medicinal chemistry. However, further progress of immune therapy is dependent on proper understandings about immune pathogenesis of different diseases and also on development of proper interventional strategies.

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10.5005/jp-journals-10018-1012
REVIEW ARTICLE

Immunotherapy for Chronic Hepatitis B: Will This Lead to Rome?

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ABSTRACT

The entity and concept of immune therapy against chronic infections and cancers is still elusive. Credible preclinical studies and clinical trials are scare in this field due to improper understating about cellular and molecular mechanism of these diseases. In addition, immune interventional strategies against these diseases have not been properly formulated. A sketch about immune pathogenesis of these diseases and interventional strategies has been formulated in this communication.

Keywords: Immune therapy, Chronic infection, Cancers, Strategy.

INTRODUCTION

Not only hepatitis B virus (HBV), but also several other viral, bacterial and protozoal infections, and many noninfectious chronic diseases, have effectively been controlled or strategies have been developed to control these diseases in developed and rich countries of the world through implementation of several prophylactic and therapeutic approaches. Although these measures were initiated almost simultaneously in both developed and developing countries, the beneficial effects of the global fighting against microbial agents have not been reproduced or duplicated in developing and resource-constrained regions and countries.[1,2] However, developing countries have been flooded with mutant microbial agents those are difficult to control and treat.

In the context of HBV infection, new cases of HBV infection are negligible, chronic HBV infection is a treatable and emergence of mutant HBV has effectively been controlled in rich and developed countries during last three decades. On the contrary, about 50 million healthy people are infected by HBV each year in developing countries in spite of availability of potent prophylactic vaccines against HBV. Also, the therapeutic regimen against chronic hepatitis B (CHB) has not been optimized in developing countries that would be able to provide benefit to majority of treated patients and reduces adverse effects. Health care delivery system of developing countries, inadequate resources and defective strategies is partially responsible for these unfortunate situations.[3] However, elusive regimen of antiviral therapy in CHB, elicit use of drugs and lack development of society-friendly therapeutic approaches are equally accountable for miserable conditions of developing countries to fight HBV.[4,5]

Out of 160 million people of Bangladesh, about 50 million or more are supposed to be infected with the HBV at some point of their life. Also, conservative estimates show that about 8 million people of this country are chronic HBV carrier, a condition that implies that these patients would continue to harbor HBV for the rest of their life even being treated with all types of antiviral drugs. To treat millions of patients with CHB at Bangladesh, antiviral drugs are available in the market. Out of several forms of HBV, available antiviral drugs can only reduce growth of replicating HBV, but these drugs are unable to control cccDNA, a form of HBV DNA that is integrated in the liver and can act as template for future replication. Be sure that none of these drugs can eliminate HBV completely from any patient with CHB. Immune modulatory capacity of these drugs is not so promising. Thus, these drugs can control liver damage in one-third or lesser numbers of CHB patients for certain duration. Taken together, the role of these drugs in delaying liver cirrhosis and liver cancer is still elusive.[6] Although these antiviral drugs can induce positive therapeutic outcome in one-third of treated patients, these benefit would come if the following preconditions are fully complied: (1) The patients should be properly assessed by a trained physician about his/her liver disease activity prior to starting antiviral drug therapy, (2) the patients should take drug on a regular basis for prolonged period or even for years or for life, (3) the patient should be properly followed up and several virological, biochemical and immunological parameters of liver function tests should be done periodically, and (4) the patient should be checked for mutant HBV and flare of liver diseases regularly. It is natural to ask how many CHB patient of Bangladesh can

fully adhere to these preconditions and how many CHB patients are informed of scopes and limitations of antiviral drug therapy by attending physicians. Out of about 2 million treatable patients of CHB in Bangladesh, these conditions may be addressed by few thousands patients of big cities of Bangladesh. However, if treatment of CHB patients is done with antiviral drugs without adhering to these conditions, the patients will develop several complications of HBV infection that would compromise his/her quality of life tremendously and the country will be filled up with mutant HBV. Control of mutant HBV may not accomplished by health care delivery system of Bangladesh even with global support.

Dissecting the present situations, we found that: (1) Present regimen of antiviral therapy is not applicable in its present form in Bangladesh and most other developing countries, and (2) the surrogate markers of antiviral therapy in CHB patients that have been shown by several eminent scholars may be misleading or untrue.[7] Based on evidences, a new approach of therapeutic maneuvers for CHB patients has been developed that seems to be: (1) Safe in short and long perspective, (2) cheap for patients of developing countries, (3) moderately effective, (4) devoid of inducing mutant HBV strains and (5) adjusted with the socio-economic and cultural behaviors of developing countries. The principle of immune therapy of CHB patients is based on a concept of 'paradise lost' to 'paradise gain' phenomenon. About 100% patients with acute HBV infection and 80% of chronic HBV infection effectively control HBV replication and liver damages by HBsAg and HBcAg-specific immunity. However, patients with CHB with progressive liver damages are unable to inducing and maintaining protective immunity efficiently. To establish an antigen-specific immune therapy in CHB patients, we have provided evidences: (1) Countering self/nonself theory of immunity and (2) discarding neonatal tolerance theory. HBV-based vaccine has been applied in an animal model of HBV-carrier stage, HBV-transgenic mice, for about two decades to assess: (1) Safety, (2) immunogenecity and (3) antiviral and liver supporting potentiality using several adjuvants and therapeutic designs. During last one decade, we have reproduced some of our studies of HBV-transgenic mice in patients with CHB, an approach to bring information of the laboratory benches to patient's bedsides.[8] Finally, during last 3 years, we have accomplished a phase I/II clinical trial in CHB patients in which HBsAg/HBcAg combined vaccine was given to CHB patient by nasal as well as by parental route.[9] Two years after therapy termination, all patients are safe and none have developed mutant HBV. The vaccine reduced HBV DNA in majority of patients and HBV DNA negativity was detected in about 40 to 50% CHB patients. Most of the patients have been showing a normal alanine aminotransferase (ALT) level, a fact that imply that liver damages have effectively been controlled. A phase III clinical trial has been planned with this vaccine in CHB patients. Although more studies are needed to optimize immune therapy in CHB patients, our concept of immune therapy may also be applied to treat hepatitis C, malaria, tuberculosis, HIV, autoimmune diseases, allergic conditions and all sorts of cancers. What we need is the development of appropriate viral, bacterial, fungal or cancer antigen(s) and a group of people who assume that 'we should follow the road that should be followed, but not one that have been followed by our traditional predecessors'. If people of allied fields of developing countries become interested to develop these therapeutic approaches, therapy with low side effects and moderate efficacies can be developed for combating microbial infections and managing cancers in developing and resource-constrained countries. Basically, these therapeutic regimens act on restoration of one's self-resistance to fight pathological diseases, a philosophy that has been unfolded in this subcontinent and ancient Chinese, Egyptian and Greece civilizations, but has not been properly optimized and updated for medical science of 21st century.

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Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2011.04445.x

Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus

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Accepted for publication 2 June 2011
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Summary

The immunosuppressive state of tumour-bearing hosts is attributable, at least in part, to myeloid-derived suppressor cells (MDSC). However, the role of MDSC in physiological conditions and diseases other than cancer has not been addressed. As the liver is a tolerogenic organ, the present study attempted to localize and assess functions of hepatic MDSC in a normal liver and in a murine model of chronic hepatitis B virus (HBV) infection. MDSC was identified in the liver of normal mice and HBV transgenic mice (TM) as $CD11b^+$ $Gr1^+$ cells by dual-colour flow cytometry. Highly purified populations of MDSC and their subtypes were isolated by fluorescence-activated cell sorting. The functions of MDSC and their subtypes were evaluated in allogenic mixed lymphocyte reaction (MLR) and hepatitis B surface antigen (HBsAg)-specific T cell proliferation assays. Normal mice-derived liver MDSC, but not other myeloid cells ($CD11b^+$ $Gr1^-$), suppressed T cell proliferation in allogenic MLR in a dose-dependent manner. Alteration of T cell antigens and impaired interferon-γ production seems to be related to MDSC-induced immunosuppression. In HBV TM, the frequencies of liver MDSC were about twice those of normal mice liver ($13{\cdot}6 \pm 3{\cdot}2\%$ *versus* $6{\cdot}05 \pm 1{\cdot}21\%$, $n = 5$, $P < 0{\cdot}05$). Liver-derived MDSC from HBV TM also suppressed proliferative capacities of allogenic T cells and HBsAg-specific lymphocytes. Liver MDSC may have a critical role in maintaining homeostasis during physiological conditions. As liver MDSC had immunosuppressive functions in HBV TM, they may be a target of immune therapy in chronic HBV infection.

Keywords: hepatic immunity, hepatitis B virus, homeostasis, immunosuppression, myeloid-derived suppressor cells

Introduction

Myeloid-derived suppressor cells (MDSC) are a phenotypically heterogeneous cell population that includes mature myeloid cells as well as immature myelomonocytic precursors [1]. Excessive numbers of MDSC have been detected in the blood of patients with head and neck squamous cell carcinoma [2] and mice with lung tumours [3]. These cells have been detected in bone marrow, spleen and peripheral blood, within primary and metastatic solid tumours and in lymph nodes in tumour-bearing mice [1,4–6]. Functionally, MDSC suppresses the function of T cells, block natural killer (NK) cell cytotoxicity [7], modulate macrophages to an immunosuppressive M2 phenotype [8,9] and induce the development of regulatory T cells [10] in tumour-bearing hosts.

Although considerable insight has been developed into the immunosuppressive functions of MDSC in cancers, little is known about these cells in physiological conditions. However, many parenchymal organs of the body maintain an immune tolerogenic state in physiological conditions. The liver is a typical example of a tolerogenic organ in physiological condition. Different food products, inflammatory substances, allergens and drug metabolites constantly enter the liver through the gut or bloodstream. Under physiological conditions, the liver induces immunological tolerance to these substances to prevent detrimental immune reactions [11,12]. The inherent tolerogenic property of the liver is attributable to a unique hepatic microenvironment. However, unexplored immunocytes such as MDSC may have a role in this regard.

This study was performed to detect MDSC in normal mice liver by dual-colour flow cytometry. The subtypes of liver MDSC and expressions of surface antigens on different liver MDSC subtypes were elucidated. Functional assessment was accomplished to assess whether MDSC are immunosuppressive in normal mice. Also, the extents of immunosuppressive potentials of two subtypes of liver MDSC were compared. Finally, we evaluated functions of liver MDSC from hepatitis B virus (HBV) transgenic mice (TM), an animal model of virus-induced immunosuppression [13], to determine the clinical implications of MDSC in chronic viral infections.

Materials and methods

Mice

Seven-week-old male C57BL/6JJcl and C3H/HeNJcl mice were purchased from Clea Japan, Inc. (Tokyo, Japan). HBV-TM (official designation: l.2HB-BS10) were produced by microinjecting a partial tandem duplication of the complete HBV genome into fertilized eggs of C57BL/6 mice [14]. HBV TM produced hepatitis B surface antigen (HBsAg), hepatitis B e antigen, and HBV DNA in sera. HBV-related mRNAs were expressed in the liver, kidney and testis [14]. Normal C57BL/6JJcl (7-week-old male) mice were immunized twice with intraperitoneal HBsAg (10 μg, Heptavax-II, subtype adw; Banyu Pharmaceutical, Osaka, Japan) at an interval of 4 weeks to induce HBsAg-specific lymphocytes. Normal mice and HBV TM were housed separately in polycarbonate cages in a temperature-controlled room (23 ± 1°C) with a 12-h light/dark cycle in a pathogen-free animal housing facility at Ehime University Graduate School of Medicine. All animals received humane care, and study protocols were in compliance with the institution's guidelines. An animal experimental board of Ehime University approved the study.

Isolation of spleen cells and liver non-parenchymal cells (NPCs)

To produce a single cell suspension from the spleen, spleens were cut into pieces and passed through a 40-μm pore-size nylon filter (BD Falcon, Durham, NC, USA); the resulting cells were collected and suspended in a culture medium (RPMI-1640 medium; GIBCO® Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (GIBCO® Invitrogen) [15,16].

To retrieve liver NPCs, liver tissues were cut into pieces, homogenized, passed through 70-μm pore-size steel meshes (Morimoto Yakuhin Co., Matsuyama, Japan) and suspended in 35% Percoll (Sigma Chemical, St Louis, MO, USA). After centrifugation for 15 min at 450 *g* at room temperature, a high-density cell pellet was collected and suspended in a culture medium [15,16].

Isolation of T lymphocytes and dendritic cells (DC)

T lymphocytes and DC were isolated from mouse spleen, as described previously [15,16]. T lymphocytes were isolated from C3H/He mice spleen single-cell suspension by a negative selection column method using a mouse pan T isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). DC were isolated from C57BL/6J mice spleen by positive selection column method using a mouse CD11c Microbeads (Miltenyi Biotec) based on the manufacturer's instructions.

Flow cytometry and cell sorting

To identify MDSC and their subtypes, allophycocyanin (APC) anti-mouse Gr-1 (clone RB6-8C5) and phycoerythrin (PE) anti-mouse CD11b (clone M1/70) were used (BD Biosciences, San Jose, CA, USA). In order to assess the expressions of surface antigens on subtypes of MDSC, fluorescein isothiocyanate (FITC) anti-mouse Ly-6G (clone 1A8), Ly-6C (clone AL-21), CD31 (clone 390) were purchased from BD Biosciences, and F4/80 (clone BM8) from eBioscience (San Diego, CA, USA). PE anti-mouse Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) (clone UC10-4F10-11), PD-1 (clone J43), CD62L (clone MEl-14) and CD40L (clone MR1) (BD Biosciences) were used to evaluate expressions of activation/exhaustion markers on T cells. For intracellular cytokine staining, cells were lysed using Fixation and Permeabilization Kit (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions, and stained with APC anti-mouse interferon (IFN)-γ (clone XMG 1·2) (eBioscience). The corresponding isotype antibodies were used with all the samples as controls. Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter (FACS)Calibur using CellQuest Software (Becton Dickinson, Franklin Lakes, NJ, USA). Data analysis was performed by using FlowJo software (TreeStar Corporation, Ashland, OR, USA).

To isolate MDSC and MDSC subtypes, spleen cells and liver NPCs were stained with monoclonal antibodies to CD11b and Gr1 and were sorted with the BD FACSAria™ Cell Sorting System (Becton Dickinson). $CD11b^+$ $Gr1^-$ cells were also sorted from liver NPCs and spleen cells suspensions by similar methods. All sorted cells were of purity above 98%.

The expressions of different surface antigens on MDSC and T cells were shown as relative frequencies among total cell populations or mean fluorescence intensity (MFI).

T cell suppression assay

C3H/HeN spleen T lymphocytes were mixed with C57BL/6J spleen DC and co-cultured in the absence or presence of sorted MDSC at different ratios to evaluate the suppressive function of MDSC in allogenic mixed leucocyte reaction (MLR). Spleen cells were also stimulated with concanavalin A (ConA, 1 μg/ml; Sigma). Spleen cells from HBsAg-injected

C57BL/6J mice were cultured with or without HBsAg in the absence or presence of MDSC to assess the role of MDSC on HBsAg-specific lymphocyte proliferation. The culture conditions are described in detail elsewhere [15–17]. All cultures were performed in 96-well U-bottomed plates (Corning Inc., New York, NY, USA). [^{3}H]-thymidine (1·0 μCi/ml; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile RPMI-1640 and added to the cultures for the last 16 h and harvested automatically by a multiple cell harvester (Labo Mash; Futaba Medical, Osaka, Japan) onto a filter paper (Labo Mash 101–10; Futaba Medical). The levels of incorporation of [^{3}H]-thymidine were determined in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA). The levels of T cell proliferation were enumerated as counts per minute (cpm). The level of cpm in culture containing only T cells was considered background proliferation and expressed as a stimulation index (SI) of 1·0. The levels of T cell proliferation in allogenic MLR were estimated by dividing the cpm in cultures containing T cells with DC or MDSC or other myeloid cells with the cpm of control cultures containing only T cells. The levels of proliferation of HBsAg-specific lymphocytes were estimated by dividing the cpm in cultures containing lymphocytes/HBsAg or MDSC with the cpm of control cultures containing T cells and an irreverent antigen, pyruvate dehydrogenase complex (Sigma Aldrich Corporation, St Louis, MO, USA) [17].

Statistical analysis

Data were expressed as mean ± standard deviation (s.d.). In all statistical analysis, data of two groups were analysed by Student's *t*-tests if they were normally distributed and by the Mann–Whitney rank-sum test if they were skewed. Differences were considered significant at $P < 0·05$ between two groups.

Results

Enumeration of MDSC and their subtypes

MDSC was detected in the liver and spleen of normal mice and HBV TM as cells expressing both CD11b and Gr1 (Fig. 1a). In normal mice, the proportions of liver MDSC were significantly higher than in the spleen (6·05 ± 1·21% *versus* 1·33 ± 0·50%, respectively; $P < 0·05$, $n = 5$). MDSC consisted of two main subtypes: CD11b$^+$ Gr1^{high} (shown by circle in Fig. 1a) and CD11b$^+$ GR1^{dim} (shown by square in Fig. 1a). In the spleen, the proportions of both subtypes of MDSC were almost comparable (Fig. 1b). However, the main subtype of MDSC in the liver was CD11b$^+$ Gr1^{dim} (Fig. 1c). This was seen in both normal mice and HBV TM (Fig. 1c).

The expression of different surface antigens showed considerable variations between MDSC subtypes of the liver (Fig. 2). The relative ratio of different surface antigens on two subtypes of liver MDSC was shown in Fig. 2b. Ly6G was expressed in most CD11b$^+$ Gr1^{high} MDSC, whereas CD31 and F4/80 were detected mainly in CD11b$^+$ Gr1^{dim} MDSC (Fig. 2b). The levels of expression of Ly6G (assessed by MFI) were significantly higher in CD11b$^+$ Gr1^{high} MDSC than in CD11b$^+$ Gr1^{dim} MDSC (Fig. 2c). Conversely, the levels of expression of Ly6C were significantly higher in CD11b$^+$ Gr1^{dim} MDSC compared to CD11b$^+$ Gr1^{high} MDSC (Fig. 2c).

Immunosuppressive capacities of normal mouse liver-derived MDSC, but not by non-MDSC myeloid cells

MDSC from normal liver suppressed T cell proliferation in allogenic MLR in a dose-dependent manner. In allogenic MLR, the levels of T cell proliferation were 87·0 ± 5·3 SI ($n = 5$). As shown in Fig. 3a, when 1×10^4 MDSC were added to the culture, the levels of blastogenesis decreased to 73·4 ± 7·8 SI ($n = 5$) ($P < 0·05$). The levels of blastogenesis decreased further to 60·7 ± 5·6 SI ($n = 5$) ($P < 0·05$) and 45·3 ± 2·6 SI ($n = 5$) ($P < 0·05$) when 2×10^4 and 3×10^4 MDSC were added to the cultures, respectively (Fig. 3a). However, increased proliferations of T cells were seen in allogenic MLR when non-MDSC myeloid cells (CD11b$^+$Gr1$^-$ cells) from normal mouse liver were added to the cultures (Fig. 3a).

As MDSC from mouse liver suppressed T cell proliferation, it was important to assess if there is any difference in T cell suppressive capacity between MDSC subtypes. In allogenic MLR, the levels of T cell proliferation were 86·0 ± 8·7 SI ($n = 5$) when 2×10^5 T cells from C3H/HeN mice were cultured with 1×10^4 DC from C57BL/6J mice. When 3×10^4 CD11b$^+$ Gr1^{high} MDSC were added to the culture, the levels of blastogenesis decreased to 62·1 ± 9·2 SI ($n = 5$) ($P < 0·05$, compared to cultures without MDSC). The levels of blastogenesis decreased to 40·2 ± 4·1 SI ($n = 5$) when 3×10^4 CD11b$^+$ Gr1^{dim} MDSC were added to the culture ($P < 0·05$, compared to cultures containing CD11b$^+$ Gr1^{high} MDSC). Taken together, CD11b$^+$ Gr1^{dim} MDSC exhibited significantly higher T cell suppressive capacities than CD11b$^+$ Gr1^{high} MDSC (Fig. 3b).

Mechanism of T cell suppression by MDSC

To develop insights into the mechanism of MDSC-induced T cell suppression, we checked the expressions of CD40L, CD62L, CTLA-4 and PD-1 on T cells cultured without or with MDSC (Fig. 4a). The frequencies of T cells expressing CD40L were decreased and those expressing CTLA-4 were increased due to culture with MDSC ($n = 5$, $P < 0·05$) (Fig. 4b,c). However, the frequencies of CD62L and PD-1 were not altered significantly due to cultures with MDSC (Fig. 4b). The levels of expression of CTLA-4 and PD1 increased significantly on T cells due to culture with MDSC ($n = 5$, $P < 0·05$). In addition, the frequencies of

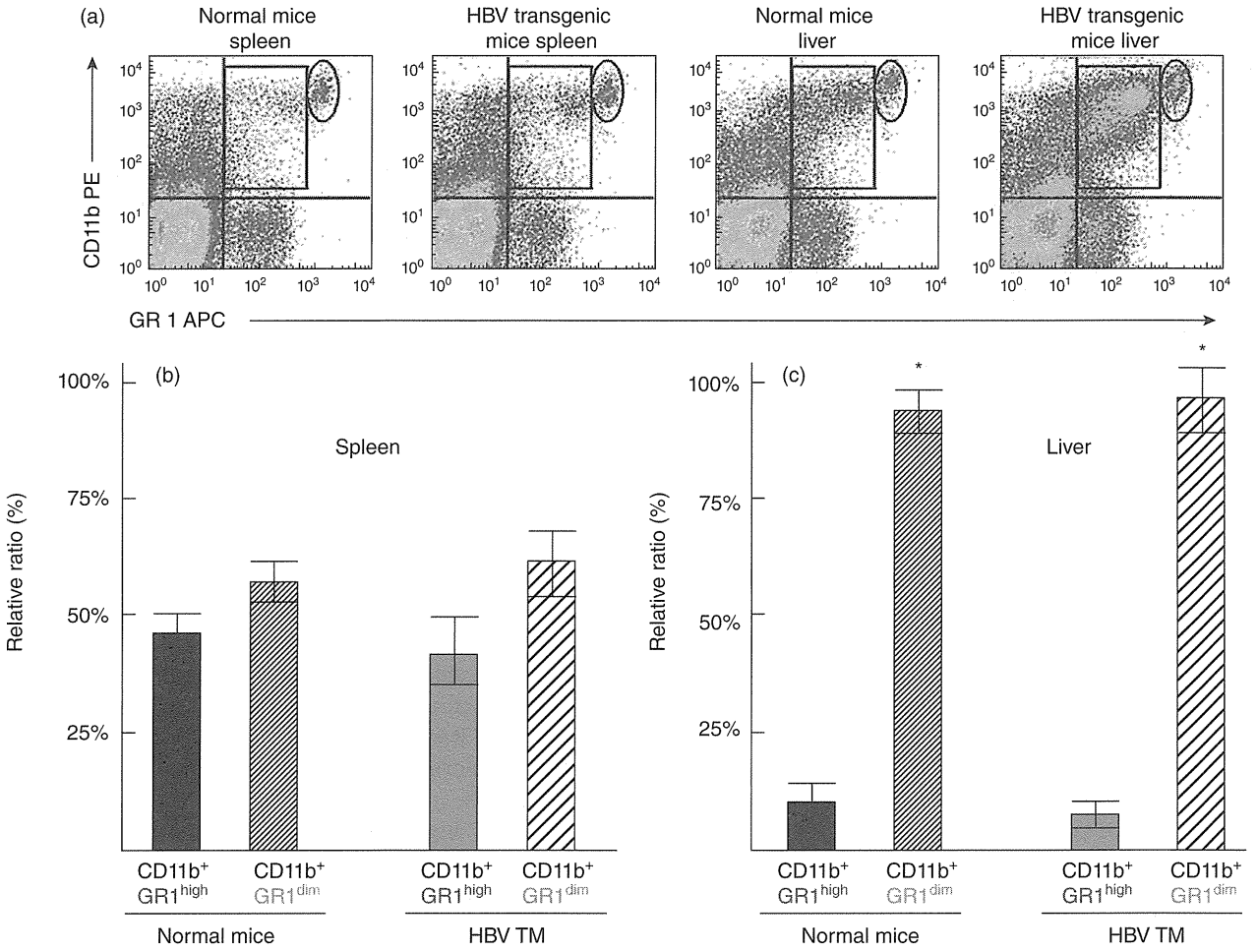

Fig. 1. Dual-colour flow cytometry shows myeloid-derived suppressor cells (MDSC) in the spleen and liver of normal mice and hepatitis B virus (HBV) transgenic mice (HBV TM) (a). MDSC in the upper right quadrant expressed both CD11b and Gr1 antigens. Depending on the expression of Gr1 antigen, two subtypes of MDSC were detected: one expressing high levels of Gr1 ($CD11b^+Gr1^{high}$) (shown by circle) and another expressing low levels of Gr1 ($CD11b^+Gr1^{dim}$) (shown by square). (b) Comparable frequencies of $CD11b^+Gr1^{high}$ MDSC and $CD11b^+Gr1^{dim}$ MDSC in the spleen of normal mice and HBV TM. (c) Significantly higher frequencies of $CD11b^+Gr1^{dim}$ MDSC compared to $CD11b^+Gr1^{high}$ MDSC in the liver of normal mice and HBV TM (c). * $P < 0{\cdot}05$, compared to $CD11b^+Gr1^{high}$ MDSC.

IFN-γ-producing T cells among total T cells were decreased significantly due to the addition of MDSC in T cell cultures compared to cultures without MDSC (1·81 ± 0·49% *versus* 6·11 ± 2·21%, respectively; $n = 5$, $P < 0{\cdot}05$) (Fig. 4d,e).

Immunosuppressive function of liver MDSC in a murine model of chronic HBV infection

To assess the immunosuppressive capacity of MDSC in chronic viral infection, we checked the frequencies and functions of MDSC in HBV TM, a murine model of chronic HBV carrier state [15]. The proportions of liver MDSC were significantly higher in HBV TM compared to normal mice (13·6 ± 3·2% *versus* 6·05 ± 1·21%, respectively; $P < 0{\cdot}05$, $n = 5$). In functional analyses, liver MDSC from HBV TM suppressed T cell proliferation in allogenic MLR in a dose-dependent manner (Fig. 5a).

To assess the role of MDSC on antigen-specific immune responses, lymphocytes from HBsAg-immunized normal C57BL/6J mice were stimulated by HBsAg without or with MDSC. As shown in Fig. 5b, MDSC from both normal mice and HBV TM suppressed proliferation of HBsAg-specific lymphocytes. Moreover, the levels of suppression were significantly higher in HBV TM-derived MDSC compared to normal mice-derived MDSC ($n = 5$, $P < 0{\cdot}05$) (Fig. 5b).

Discussion

Under physiological conditions, the liver maintains a state of immunological tolerance to various noxious substances to prevent extreme and detrimental immune reactions, although the liver harbours abundant amounts of immunocytes capable of inducing inflammation and cell damage. The immunosuppressive properties of the normal

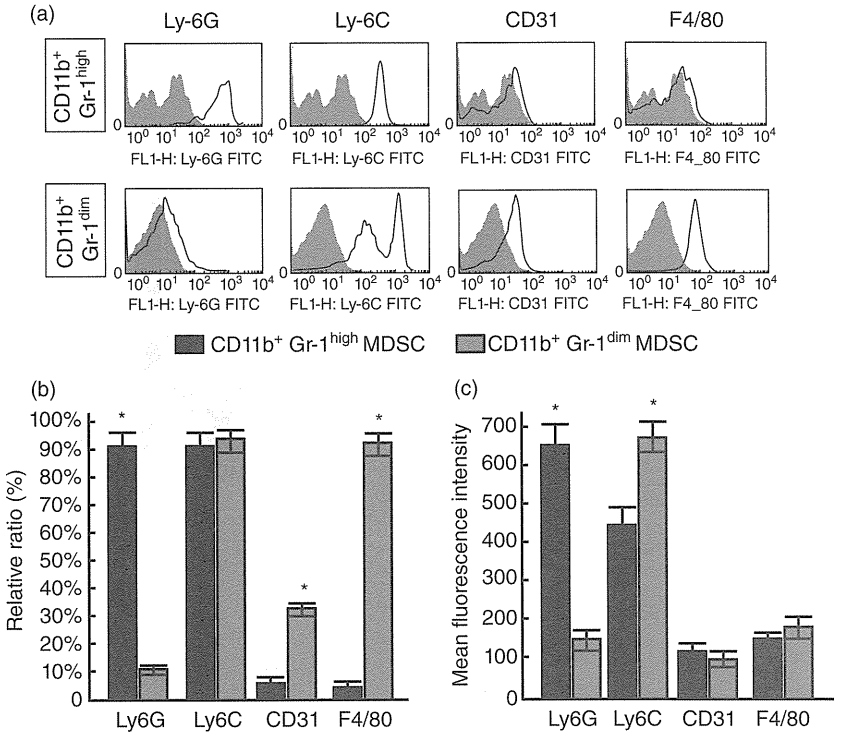

Fig. 2. Phenotypic analyses of myeloid-derived suppressor cells (MDSC) subtypes of normal mice liver. (a) Representative histograms of phenotypic profiles of $CD11b^+Gr1^{high}$ MDSC and $CD11b^+Gr1^{dim}$ MDSC. (b) Proportions of Ly6G, Ly6C, CD31 and F4/80 in $CD11b^+Gr1^{high}$ MDSC and $CD11b^+Gr1^{dim}$ MDSC of liver from normal mice. (c) Levels of expression of Ly6G, Ly6C, CD31 and F4/80 [shown by mean fluorescence intensity (MFI) on $CD11b^+Gr1^{high}$ MDSC and $CD11b^+Gr1^{dim}$ MDSC. * $P < 0{\cdot}05$, compared to other subtypes.

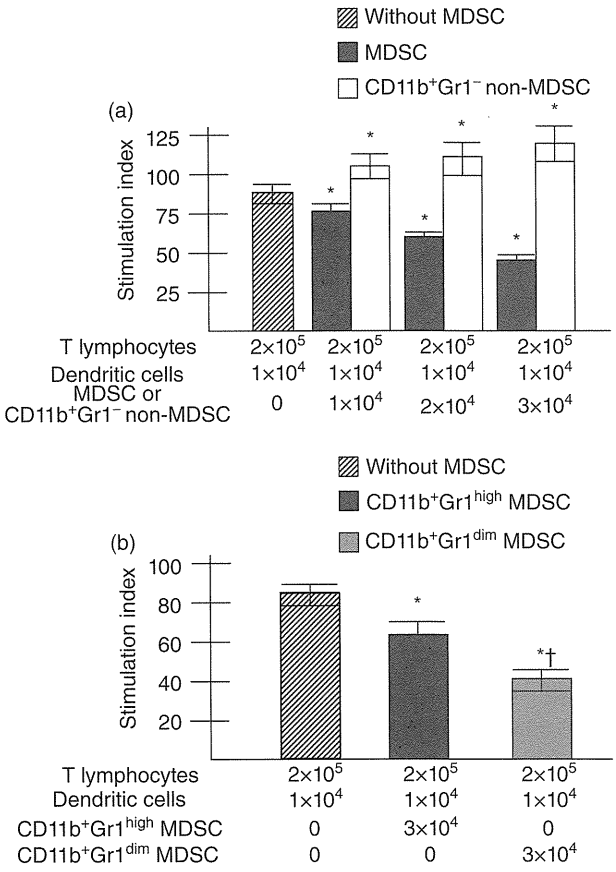

Fig. 3. (a) Suppression of T cells proliferation by liver myeloid-derived suppressor cells (MDSC), but not by liver non-MDSC. T cells (2×10^5) and dendritic cells (1×10^4) from spleen of normal mice were cultured in allogenic mixed leucocyte reaction (MLR). MDSC or $CD11b^+Gr^-$ non-MDSC were added to allogenic MLR, as mentioned. The levels of T cell proliferation are shown as the stimulation index (SI), as described in the Methods. The levels of SI in allogenic MLR without MDSC are shown as hatched bar and those in presence of MDSC and $CD11b^+Gr^-$ non-MDSC are shown as black bar and clear bar, respectively. Data of five separate experiments are shown, with means and standard deviations. *$P < 0{\cdot}05$ compared to levels of T cell proliferation in allogenic MLR without MDSC. (b) Increased T cell suppressive capacity of $CD11b^+Gr1^{dim}$ MDSC compared to $CD11b^+Gr1^{high}$ MDSC in allogenic MLR. The experimental conditions of allogenic MLR are similar to that described in Fig. 3a. *$P < 0{\cdot}05$ compared to levels of T cell proliferation in allogenic MLR without MDSC. †$P < 0{\cdot}05$ compared to levels of T cell proliferation in allogenic MLR containing $CD11b^+Gr1^{high}$ MDSC.

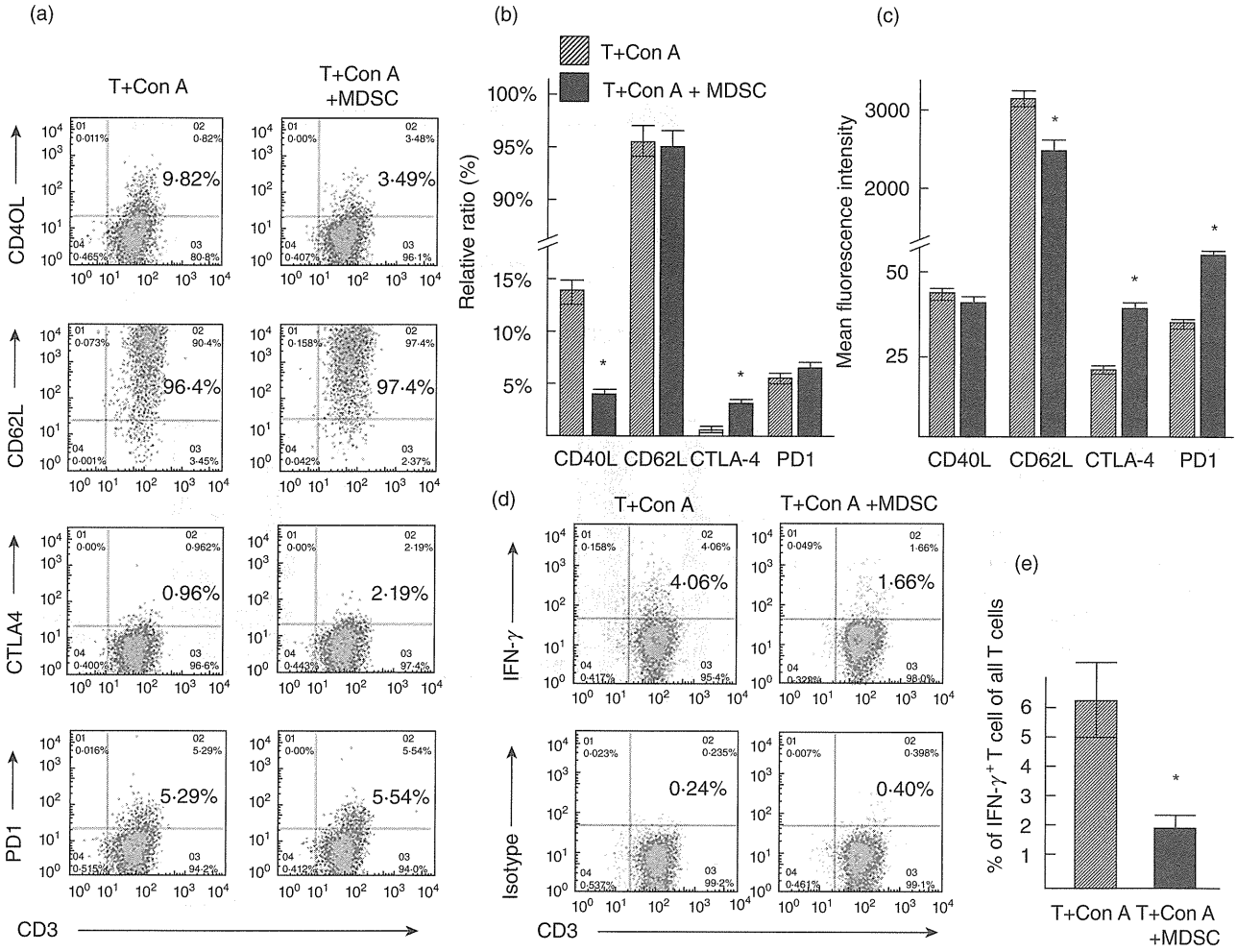

Fig. 4. (a) Representative dot-plots of CD40L, CD62L, CTLA-4 and PD1 antigens on T lymphocytes cultured without or with myeloid-derived suppressor cells (MDSC). (b) The ratio of T cells expressing CD40L, CD62L, CTLA-4 and PD1 antigens cultured without or with MDSC. (c) The levels of expression (shown by mean fluorescence intensity) of CD40L, CD62L, CTLA-4 and PD1 antigens cultured without or with MDSC. (d) A representative staining of intracellular interferon (IFN)-γ in T cells among all T cells cultured without or with MDSC. (e) Data of five separate experiments about intracellular IFN-γ production by T cells among all T cells are shown. $n = 5$, $^*P < 0{\cdot}05$, compared to without MDSC.

liver are regulated by several suppressor immunocytes, such as regulatory DC [18], regulatory NK cells [19] and regulatory T cells [20]. Interestingly, these suppressor cells of the liver also have immunogenic counterparts; immunogenic DC for regulatory DC, immunogenic T cells for regulatory T cells and immunogenic NK cells for regulatory NK cells. It seems that comprehensive functions of immunosuppressive immunocytes and immunogenic immunocytes maintain normal homeostasis under physiological conditions.

In this regard, there is a paucity of information about hepatic $CD11b^+$ myeloid cells and their functional implications. Generally, it is assumed that $CD11b^+$ myeloid cells are capable of producing inflammatory cytokines and play a role as immunogenic cells. Recently, it has been shown that MDSC, a subpopulation of $CD11b^+$ myeloid cells that express both CD11b and Gr1 antigens, are endowed with immunosuppressive properties in cancer patients and in mice models of different cancers [1,2,4,5]. However, there is a paucity of information about localization, frequencies and functions of MDSC in the normal liver.

The studies presented here showed that MDSC were present in the liver of normal mice and suppressed non-antigen-specific (Fig. 3) as well as antigen-specific T cell proliferation (Fig. 5). To be more confident about the immunosuppressive properties of liver MDSC, we compared the functional capacities of liver MDSC and non-MDSC myeloid cells of the liver (cells expressing $CD11b^+Gr1^-$) in the same run. The data showed conclusively that MDSC, but not other myeloid cells, were immunosuppressive (Fig. 3).

We detected two subtypes of MDSC in the liver on the basis of expression of Gr1 antigen. Further analyses revealed that these two subtypes showed significant differences regarding expressions of Ly-6G, Ly-6C, CD31 and F4/80

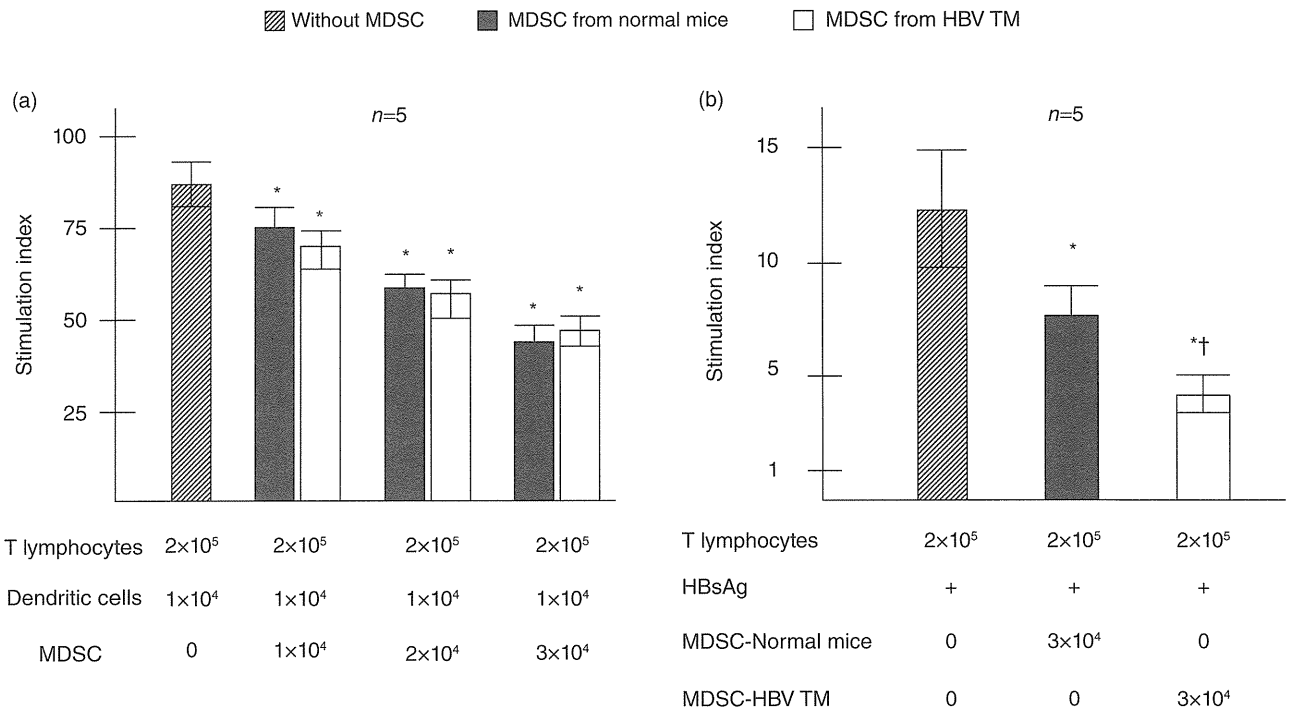

Fig. 5. T cell suppressive capacity of hepatitis B virus transgenic mice (HBV TM)-derived MDSC. (a) T cell suppression by HBV TM-derived MDSC in allogenic MLR. (b) Suppression of antigen-specific lymphocyte proliferation by liver MDSC. T cells (2×10^5) from hepatitis B surface antigen (HBsAg)-injected mice were cultured with or without HBsAg. MDSC from normal mice and HBV TM were added to the cultures. The levels of T cell proliferation are shown as the stimulation index (SI), as described in the Methods. The levels of SI in lymphocyte proliferation without MDSC are shown as hatched bar and those in presence of MDSC from normal mice and HBV TM are shown as black bar and clear bar, respectively. Data of five separate experiments are shown, with means and standard deviations. $^*P < 0{\cdot}05$ compared to levels of HBsAg-specific lymphocyte proliferation without MDSC. $\dagger P < 0{\cdot}05$ compared to levels of T cell proliferation in HBsAg-specific lymphocyte proliferation containing normal mice-derived MDSC.

antigens (Fig. 2). In addition, the magnitudes of T cell suppressive capacities of $CD11b^+$ $Gr1^{dim}$ MDSC were significantly higher than $CD11b^+$ $Gr1^{high}$ MDSC, the clinical implications of which should be assessed in a future study. Although both subtypes are now regarded as MDSC, further studies would be required to gain more insight into their roles in hepatic immunity in normal as well as in pathological conditions.

Various tumour-derived factors as well as arginase, nitric oxide and reactive oxygen species play a role in MDSC accumulation and their immunosuppressive functions in cancers [7–10]. In addition, MDSC-mediated immunosuppression is mediated, at least in part, through the regulation of functions of NK cells and DC [21,22] in cancers. Using a model of Con-A-induced T cell proliferation, this study pointed that MDSC may have some effects on expression of T cell antigen (Fig. 4). However, this should be confirmed in other models of T cell proliferation, especially in antigen-specific T cell proliferation. Addition of MDSC in T cell cultures down-regulated production of IFN-γ in T cells (Fig. 4).

In addition to exploring the functional capacities of MDSC in normal mice liver, we also checked MDSC function in a pathological condition other than cancer. The proportions of MDSC in the liver were significantly higher in HBV TM compared with those in the liver of normal mice. MDSC from HBV TM suppressed T cell proliferation in allogenic MLR. In addition, MDSC from HBV TM revealed significantly increased the capacity to suppress proliferation of HBsAg-specific lymphocytes compared to normal mice-derived MDSC (Fig. 5).

Immunosuppressive activity of MDSC in HBV TM, especially suppression of antigen-specific T cell proliferation by MDSC, is worthy of further study because manipulation of MDSC may have therapeutic implications. It has been shown that treatment that reduces MDSC levels, such as antibody depletion of Gr1 cells, or treatments that down-regulate MDSC, such as chemotherapeutic drugs or retinoic agents, improve the efficacy of cancer vaccines or other immunotherapy *in vivo* [23–27]. At present, there is no curative therapy against chronic HBV infection [28]. Immune therapy has been accomplished in patients with chronic hepatitis B, but an effective immune therapeutic regimen has yet to be developed. The therapeutic effects of different agents that deplete MDSC in HBV TM remain to be elucidated for the development of novel therapeutic approaches against chronic HBV infection.

In conclusion, this is one of the first reports to show that MDSC are present in the liver of normal mice. In addition, these cells were shown to suppress T cell immunity. We also showed that in contrast to $CD11b^+$ $Gr1^{high}$ MDSC, $CD11b^+$ $Gr1^{dim}$ MDSC were significantly higher in the liver and had increased immunosuppressive functions. Furthermore, we provided credible evidence about a role of MDSC in chronic HBV infection. Further studies into liver MDSC and their subtypes would provide more insight into the maintenance of hepatic homeostasis in the normal liver and information about immunosuppression after infection with hepatotrophic viruses. Finally, it may be possible to develop novel therapeutic strategies against these diseases by targeting MDSC.

Acknowledgements

We thank the Integrated Center for Science, Shigenobu Station, Ehime University for animal management and cell sorting.

Disclosures

The authors declare that there are no conflicts of interest related to the publication of this manuscript.

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Prevalence and Risk Factors of Asymptomatic Hepatitis C Virus Infection in Bangladesh

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ABSTRACT

Objectives: There are paucity of information about prevalence and risk factor of hepatitis C virus (HCV) in Bangladesh.
Methods: Blood was collected from 1018 randomly selected subjects from a semi-urban area of Bangladesh. Anti-HCVs were checked in the blood twice using a third-generation commercial kit. The data of the questionnaires were analyzed to find possible risk factors.
Results: Nine of the 1018 subjects (88%) were tested positive for anti-HCV. The HCV-positive subjects were >28 years old. Major risk factors for HCV infection were treatment by unqualified and traditional practitioners, history of mass-vaccination against smallpox, hair cutting and shaving by barbers, and body piercing. However, known risk factors such as blood transfusion, surgery, invasive therapy, and intravenous drug use were not detected in any HCV-infected subjects.
Conclusion: Control of HCV infection in Bangladesh may be difficult because the risk factors are related to normal tradition and culture of Bangladeshi people.

INTRODUCTION

Hepatitis C virus (HCV) is notorious for causing chronic infection, and about 170 million people of the world are infected with this virus. HCV-infected persons mostly develop chronic hepatitis and complications like liver cirrhosis and HCC.[1,2] In the absence of valid population-based and nation-wide surveys, it is assumed that the prevalence of HCV is low in developing countries of Asia and Africa. However, recent studies have shown that developing countries like Pakistan and Egypt harbor high percentages of HCV-infected subjects.[3,4]

Keywords: Bangladesh, hepatitis C virus, prevalence, public health, risk factors, semi-urban
Received: 13.04.2011; *Accepted:* 06.06.2011
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Abbreviations: HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HIV: human immunodeficiency virus
doi: 10.1016/S0973-6883(11)60111-4

Bangladesh is a developing country of Southeast Asia, with a population of 140 million. There is no population-based data on HCV prevalence in Bangladesh. In 1993, Khan et al[5] have reported zero prevalence of HCV among blood donors in Bangladesh. However, this was contradicted when Akbar et al[6] reported that about 5% apparently healthy subjects of Bangladesh were harboring HCV RNA. Recently, a study conducted in rural Bangladesh has shown that 0.5% apparently healthy subjects were infected with HCV.[7] On the other hand, another study from Bangladesh has shown that HCV is highly prevalent among injectable (24.8%) as well as in noninjectable drug users (5.8%).[8] These figures indicate that more studies should be conducted in different regions of Bangladesh to develop insights about considerable variations in HCV prevalence in the country. Also, risk factors related to HCV infection should be explored, since this is yet to be done in Bangladesh.

The study presented here was performed to assess the prevalence of HCV in a semi-urban area of Bangladesh. It was carefully conducted after considerable mass campaigns among study population to ensure active participation of the local people. A questionnaire was developed to identify risk factors associated with HCV transmission in Bangladesh.

METHODS

Study Design

The study was conducted in the outskirts of Dhaka city. The area has a large industrial base. Nature and purpose of the study was informed to the people of the community by undertaking mass campaigning for 1 month to get their support and active participation. Prior to the study, meetings were held with people's representatives of the area to request their cooperation. Especially, the nature and purpose of the study were discussed with Imams (religious leaders), local leaders, and social representatives. Extensive broadcasting was done about the study, and posters and banners were erected in key locations of the locality. The study was carried out in August 2010.

Twenty graduate physicians were given training for the collection of epidemiological data and blood samples.

Printed questionnaire were supplied to collect relevant data from the study subjects. Demographic data such as age, sex, religion, and education levels of each participant were registered. In addition, information about possible risk factors of HCV infection such as history of blood transfusion, dental procedure, jaundice, surgery, abscess drainage, urinary catheterization, blood donation, gastrointestinal endoscopy, immunization, tattooing, intravenous drug use, multiple sexual partners, acupuncture, vaccination for cholera and small pox, treatment by nonqualified village doctors, shaving and hair cut in barber shop, circumcision, and ear piercing were also included in the questionnaire. The study area was divided into 10 blocks, and the subjects were selected randomly. A total of 1018 blood samples were collected. Patients suffering from acute or chronic liver disease and those who were already aware of their HCV status from previous investigations were excluded from the study. Five milliliter of blood was collected and transported to our Dhaka laboratory by using a cold-chain system. Sera were stored at −20°C, and a commercial third-generation anti-HCV enzyme-immunoassay method (Abbott Laboratories, Abbott park, IL, USA) was employed to assess the presence of anti-HCV in the sera. All subjects or their guardians gave informed consent to the study. The samples that tested positive for anti-HCV were further tested for HCV RNA (COBAS AmpliPrep/TaqMan; kit Roche HCM for COBAS AmpliPrep, USA; lower limit of detection 15 IU/mL), and HCV genotyping (real-time PCR with TaqMan-MGB probes, USA) was also done. Ethical approval was taken from Viral Hepatitis Foundation Bangladesh to conduct this study. The study was performed according to the principle of the "Declaration of Helsinki of 1975."

RESULTS

Sera were collected from 1018 apparently healthy persons of Savar area of Bangladesh. The area is situated 20 Km from Dhaka metropolitan area and is a typical semi-urban area. People with different professions such as farmers, industrial labors, businessmen, and small and mid-level manufacturers live here. The subjects were aged between 1 and 60 years. Questionnaire was filled in by the attending physicians. In case of adults, the responses were given by the subjects, and in case of minors, the legal guardians helped to fill the questionnaire. None of the subjects were suffering from acute or chronic liver disease. Those who were already aware of their HCV status from previous investigations were excluded from the study.

Demographic data of the patients such as age, sex, religion, and educational status of the subjects have been shown in Table 1.

Nine (0.88%) of the 1018 subjects tested positive for anti-HCV in the sera. Analyses of the data of the questionnaire revealed that the HCV-infected subjects were 28–60 years old.

Table 1 Demographic characteristics of study population.

	Numbers	Percentage
Age distribution (years)		
0–16	411	40.37
17–50	574	56.39
50+	33	3.24
Sex distribution		
Male	584	57.37
Female	434	42.63
Religion		
Muslim	950	93.32
Hindu	61	5.99
Christian	7	0.69
Education level		
None	113	11.10
Primary	322	31.63
High School	432	42.44
College	114	11.20
University	37	3.63

Two-thirds of them (N=6) were males and the rest 3 were females. All of them had either primary or secondary education. On further analysis of the anti-HCV-positive serum samples, 7 had detectable HCV RNA. Of them, 5 had genotype 3, 1 had HCV genotype 2, and the rest 1 had HCV genotype 1.

One of the main objectives of this study was to develop insight into the risk factors related to HCV infection in this area, and a questionnaire was developed to materialize this. Co-infection with hepatitis B virus (HBV) was not detected in any subject. Of the 9 HCV-infected subjects, history of jaundice in family members was reported by 3. All 3 female HCV-infected subjects had history of pregnancy, and one of them had previous experience of abortion.

The prevalence of HCV-related possible risk factors is shown in Table 2. None of the patients had previous history of blood transfusion, dental procedures, or any other invasive procedure that might predispose to HCV infection. None of the HCV-infected subjects were injection-drug user, and none had multiple sexual partners.

Possible risk factors for HCV transmission in this cohort included: (1) previous vaccination for small pox and cholera, (2) circumcision, (3) treatment by nonqualified village doctors, and (4) body piercing for cosmetic purpose (Table 2).

The prevalence of risk factor in 1009 HCV noninfected subjects was also evaluated to develop insights about HCV infection in this area. The prevalence of risk factors (Table 2) was higher in non-HCV-infected subjects compared with the HCV-infected subjects (Tables 1 and 2), because none of these factors were detected in any HCV-infected subjects in this study.

DISCUSSION

This study, conducted in a semi-urban area of Bangladesh supports what Khan et al[7] reported about HCV prevalence

Table 2 Risk factors in 1018 subjects at Savar area of Bangladesh.*

S. no.	Risk factor	Hepatitis C virus-infected subjects (n = 9)	Hepatitis C virus-noninfected subjects (n = 1009)
1	Blood transfusion	0	122
2	Dental procedure	0	153
3	History of jaundice	0	374
4	History of surgery	0	102
5	Intravenous infusion	0	226
6	Abscess drainage	0	24
7	Urinary catheterization	0	29
8	Blood donation	0	31
9	Gastro-intestinal endoscopy	0	23
10	Tattooing	0	30
11	Intravenous drug use	0	4
12	Multiple sexual partners	0	33
13	Acupuncture	0	0
14	Vaccination for cholera and small pox	6	350
15	Treatment by nonqualified village doctors	6	723
16	Shaving and hair cut in barber shop	3	504
17	Circumcision	3	607
18	Ear piercing	3	130

*Nine of the 1018 subjects were positive for anti-HCV in their sera.

in rural Bangladesh; 0.88% apparently healthy subjects were infected with HCV. It seems that these studies have apparently contradicted the data about high HCV prevalence in urban Bangladesh reported by Akbar et al[6] (about 5% HCV positivity among general population of Dhaka) and by Shirin et al[8] (5.8% HCV positivity among nonintravenous drug users of Dhaka). These discrepancies about HCV prevalence among rural, semi-urban, and urban areas of Bangladesh deserve careful analyses. The study by Akbar et al[6] showed that the prevalence of HCV among Dhaka's population was 5%. However, only 2.3–2.6% of the service holders and businessmen were infected with HCV, respectively. On the other hand, 9.5% of the day laborers were harboring the HCV RNA in their blood. Shirin et al[8] also documented high prevalence of HCV (5.8%) among nonintravenous drug users (5.8%) in Bangladesh. Thus, HCV prevalence may vary from 0.5% to 2–3% among general population. It remains to be evaluated why 9.2% of the day laborers were HCV infected, as mentioned by Akbar et al.[6] Accordingly, a population-based study would be required to develop proper insight into the prevalence of HCV and to design control strategy against HCV in Bangladesh.

One of the major contributions of the present study is the analysis of various risk factors related to HCV infection.[9,10] The common risk factors (Table 2), which are usually related to HCV transmission, were not detected in any of the 9 HCV-infected subjects in this cohort. On the other hand, factors related to normal lifestyle of Bangladesh people may be related to HCV infection in these subjects (Table 2). Treatment by nonqualified village doctors, shaving at barber shops, circumcision by traditional practitioners popularly known as "hajams," and ear and nose piercing by females are part of common tradition and culture of our people. These practices would continue for decades in Bangladesh. In fact, role of ethnic and cultural characteristics as risk factors of HCV transmission has previously been reported.[11]

The wide diversity in the prevalence of HCV in Bangladesh deserves special attention. Observation of 0.5% prevalence of HCV in apparently healthy subjects in the rural area by Khan et al[7] represents "relatively low prevalence" of HCV in Bangladesh. Considering the pathological process and virology of HCV, even 0.5% or 1% HCV prevalence constitutes a major public health problem. Most of the HCV-infected subjects develop chronic liver disease and its complications. Treatment of HCV infection is also extremely costly and endowed with serious side-effects. Also, contrary to hepatitis B virus, no prophylactic vaccine is available against HCV.

In this context, the study by Siddiqui et al is important. They observed HCV positivity at a hospital of India for 7 years from 2001 to 2007 on a yearly basis.[12] They found a progressive increase in HCV positivity among voluntary blood donors in their university hospital. No HCV-infected cases were recorded in 2001, and only 1 HCV-infected person was identified in 2002; whereas, HCV prevalence among volunteer blood donors increased by 18-folds from 2002 to 2007.[12]

Taken together, HCV prevalence of 1% or less should not be regarded as low HCV prevalence in Bangladesh. Rather, this should be regarded as a serious public health problem. We should learn important lessons from the epidemiological studies and government declarations about HIV infection from developing countries in the 1980s and 1990s. At that time, it was considered that HIV was not a dominant problem for the developing world.[13–15] Even zero prevalence of HIV was declared among 15,700 prostitutes in Thailand.[13] However, now, large numbers of HIV-infected individuals have been detected in these countries. Understanding the risk factors allows control of infection,[4] which has now occurred in Egypt and Bangladesh, and we can follow this example for containment of HCV.

ACKNOWLEDGMENT

The author is grateful to Square Pharmaceuticals Ltd., Dhaka, Bangladesh, for an unrestricted grant for this study.