行政院國家科學委員會專題研究計畫成果報告

計畫名稱

中文：探討輪狀病毒在肝外膽道閉鎖致病機轉之角色

英文：Role of rotavirus in the pathogenesis of extrahepatic biliary atresia

計畫編號：NSC90-2314-B002-165

執行期限：90年8月1日到91年7月31日

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中文摘要

為探討輪狀病毒感染在肝外膽道閉鎖症致病機轉中所扮演的角色，我們先將購買來的恆河猴輪狀病毒(Rhesusrotavirus，簡稱 RRV)培養於 MA-104 細胞中使其繁殖，並建立病毒斑定量法（plaque assay）用以定量 RRV 之濃度。接著以我們以建立之方法將 BALB/c 鼷鼠肝外膽道之上皮細胞(MEBEC)分離出來並做初級培養。再將 RRV 與 MEBEC 共同培養後，以 RT-PCR 之方法可確認 MEBEC 確可被 RRV 所感染。藉單株抗體染色及流式細胞儀之分析，發現肝外膽道上皮細胞可表現低量的第一型細胞黏著分子（ICAM-1)，第一型及第二型主要組織配合抗原(MHC)。進一步發現被 RRV 感染之上皮細胞，ICAM-1、MHC class I 及 MHC class II 之表現呈有意義之增強。最近有研究顯示 RRV 感染可在 BALB/c 鼠造成膽道發炎及阻塞，我們的初步結果支持 RRV 感染膽管上皮細胞後可能經由免疫調控作用造成膽道上皮細胞之損傷。進一步的實驗仍持續進行中。預期會有非常有意義之成果。

關鍵字：輪狀病毒、肝外膽道閉鎖、細胞黏著分子、主要組織配合抗原

ABSTRACT

To understand the role of rotavirus infection in the pathogenesis of extrahepatic biliary atresia (EHBA), rhesus rotavirus (RRV) was purchased and passaged in MA-104 cells cultured in MEM medium. Virus pools was prepared and stored. The virus titer in the supernatant was determined by plaque assay in confluent monolayer of MA-104 cells. Murine extrahepatic bile duct epithelial cells (MEBEC) were isolated from extrahepatic bile ducts of BALB/c mice and established in primary culture. After cocultured with RRV, MEBEC was confirmed to have intracellular RRV genome by RT-PCR. By staining with monoclonal antibodies and flowcytometric analysis, MEBEC in culture constitutively express low levels of intercellular adhesion molecule (ICAM)-1, class I and class II major histocompatibility (MHC) antigens. The expression of ICAM-1 and MHC class I and II antigens on RRV-infected MEBEC were all significantly increased when compared with that of untreated MEBEC.. Recent studies showed that RRV infection can induce inflammation and obliteration of murine extrahepatic bile duct. Our preliminary results suggest that rotavirus infection on biliary epithelial cells may initiate an immune-mediated mechanism in the injury of biliary epithelial cells. Our studies are ongoing to determine whether RRV-infected MEBEC can induce T cell proliferation or induce T cell mediated cytotoxicity. These data are important in understanding the possible role of rotavirus infection in immune-mediated biliary epithelial cell injury disorders.

Key words: Rotavirus, MHC antigens, intercellular adhesion molecule, antigen presenting cells, murine extrahepatic bile duct cell

INTRODUCTION

Extrahepatic biliary atresia (EHBA), a complete occlusion or discontinuity of the bile duct at any point from the porta hepatis to duodenum, occurred more frequently in Chinese than in Caucasians. It pathogenesis remains unknown.[1] Evidence to date suggest several pathogenetic mechanisms for the development of EHBA. One of these hypotheses is that immunologic mechanisms initiated by some insults may cause inflammation of extrahepatic bile ducts. Such cholangitis is a chronic, fibrosclerosing process leading to progressive narrowing and complete obliteration of extrahepatic bile duct lumens, and ultimately liver cirrhosis and failure.[1] Immune-mediated injury to extrahepatic biliary epithelium initiated from viral infection has been proposed as a possible mechanism.[2] An establishment of a murine extrahepatic biliary epithelial cell (MEBEC) culture may provide a good *in vitro* method to study the pathogenetic mechanisms of both diseases.[3] Rotavirus infection has been considered as a possible cause of biliary atresia either in human or animal models.[4] After establishing a primary culture of MEBEC similar to that reported previously by Schreiber et al.[5], we are able to study the expression of class I and class II major histocompatibility antigen (MHC) and intercellular adhesion molecules (ICAM)-I on cultured MEBEC and their regulation by RRV infection, capacity of RRV-infected MEBEC to induce T lymphocyte proliferation or induce T cell-mediated cytotoxicity.

MATERIALS AND METHODS

Rhesus rotavirus (RRV) strain MMU 18006 was purchased from the American Type Culture Collection (Rockville, MD) and grown in MA-104 cells which was acquired from Dr. CN Lee (Department of Laboratory Medicine, NTUH). Virus pool were prepared from tissue culture supernatant. The inoculation for mock-inefcted controls was prepared from supernatants of uninfected MA-104 cell culture. The virus pool (1X10 11 pfu/ml) and control were stocked in liquid nitrogen. Virus was also maintained in MA-104 cells cultured in MEM medium supplemented with 0.5 ug/ml trypsin. CPE was confirmed when focal rounding of cells outlined by disintegration of the monolayer of MA-104 cells in a 24 well culture plates for one hour. Then the culture medium were removed and the cells were washed, added with agarose medium (serum-free MEM medium containing 0.5 ug/ml trypsin and 0.3% agarose), cultured for 5 days, and finally stained with 0.01% Neutral red. The plaques were counted.

After inducing proliferation of extrahepatic bile duct epithelial cells[6], murine common bile ducts were harvested by microdissection and placed in cold HBSS. After being minced into fine pieces on a petri dish by surgical blades, the bile ducts were digested with trypsin in the presence of DNase and were further digested with collagenase. After centrifugation, the cells in suspension were transfered to plastic tissue culture petri dish and incubated for 45 minutes to allow adjacent fibroblasts to adhere. The floating cells in the supernatant

after the last incubation were mostly epithelial cells and were seeded in a 6-well culture plate containing DMEM with 10% FBS.

Cells on culture plates were released and stained with CK-19, linked with FITC-conjugated anti-mouse IgG fragment, and subjected to flow cytometric analysis for the purity of cultured MEBEC. Anti-CD45, anti-CD34 and anti-vimentin were also used to exclude leukocyte or endothelial cell or fibroblast contamination.

MEBEC were treated with murine recombinant INF-γ or murine recombinant TNF-α or infected with RRV and examined for the expression of class I and class II antigen and ICAM-I.

$5X10^5$ MEBEC single cells (resting or RRV infected MEBEC) were suspended in 100 ul PBS and incubated with 20 ul fluorescein isothiothionate (FITC) labeled H-2 Kd monoclonal antibody (class II, PharMingen, Sandiego, CA) or I-Ad monoclonal antibody (class I, pharMingen), or anti-mouse CD54 (ICAM-1) monoclonal antibody (Pharmingen) and CK-19 monoclonal antibody in dark at 4° C for 30 min. After centrifugation, the precipitates was resuspendedin 0.5 ml FACScan buffer and analyzed by flow cytometry (FACScan, Becton Dickinson).

Freshly harvested MEBEC were incubated in 6-well culture plates at an initial concentration of 10^6 cells/well for 3 days before infection. Culture medium was removed and cells in each well were exposed to 10^6 pfu of RRV inoculum for adsorption at 37°C for 6 hours in humidified CO_2. The MOI was 1. Mock-infected cells were exposed to a virus–free supernatant from non-infected MA-104 cell culture. After adsorption, the cells were washed thrice with DMEM and incubated again in complete culture medium for another 3 days and then used for assay of class I antigen, class II antigen and ICAM-1 expression.

MEBEC on culture was extracted for cellular RNA. The extracted RNA was reverse transcribed to c-DNA and subjected to PCR. PCR primers were selected from the RRV VP-7 genes from published sequence data. The base sequence were the following: primer VP7-1L-GGCTAGCGGTTAGCTCCTC and primer VP 7-1R-GATTCACACCATCGACAACG. The PCR solutions were placed for an initial 3 min incubation at 94°C followed by 35 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1.5 min.

RESULTS

By flow cytometric analysis, we found that over 90% of the isolated and cultured cells were stained positively for CK-19. Contaminated, fibroblast-like cells that were negative for CK-19 but positive for vimentin were always less than 5% of total cell populations in each analysis of MEBEC preparation. In addition, there was no significant contamination by cells expressing CD45 or CD34.

Morphologic changes characteristic of RRV infection were observed by phase contrast

microscopy. Both RRV-infected MEBEC and mock-infected MEBEC did not have altered morphology over the observation period. After cocultured with RRV, MEBEC were washed thoroughly with DMEM and RNA was extracted and subjected for amplification of the sequence of RRV by RT-PCR. RRV was not detectable in the last wash of DMEM, but was detectable in the MEBEC, confirming the inoculation of RRV into the MEBEC.

MEBEC constitutively expressed ICAM-1, class I and class II MHC antigens at a low level. RRV infection of MEBEC strongly provoked class I and class II antigen as well as ICAM-1 expression levels on MEBEC. By using rotavirus antigen monoclonal antibody, the percent expression of RRV protein on cultured MEBEC and RRV-infected MEBEC are currently under investigation in our laboratory.

DISCUSSION

The role of rotavirus in immune-mediated biliary epithelial cell injury is unclear. In a previous study, infection of newborn mice with RRV or group A human roravirus can cause a fibro-obliteration of the murine extrahepatic bile duct.[4] In this animal model, viral infection induces an inflammatory process in the liver and in the intrahepatic and extrahepatic bile duct. This pancholangitis are similar to the histopathological findings in infants with EHBA.

Our previous study showed that cytokines can upregulate the expression of MHC antigens or adhesion molecules on our MEBEC. Those results suggest that alterations of cytokines secreted by local inflammatory cells in inflammatory microenvironment may be important in regulating the interaction between lymphocytes and MEBEC and thus may be implicated in the pathogenesis of immune mediated extrahepatic biliary epithelial injury.[7] In the present study, we further showed that the expression of class I and class II antigen as well as ICAM-1 on MEBEC was significantly increased by in vitro rotavirus infection of BEBEC. Recently, It has been suggested that biliary epithelial cells may act as an APC or as a target of T cell-mediated cytotoxic response in immune-mediated biliary epithelial injury disorders.[8-10] On the assumption that rotavirus infection may trigger an immune-mediated injury of MEBEC, further studies are needed to clarify the role of RRV infection in the production of inflammatory and fibroobliterative change in RRV-induced EHBA in mice.

REFERENCES

1.Mowat AP 1987 liver Disorders in Childhood, Butterworths London, pp 72-88.
2.Desmet VJ 1987 Cholangiopathies: past, present, and future. Semin Liver Dis 7:67-76
3.Chapman RWG, Jewell DP 1985 Primary sclerosing cholangitis- an immunologically mediated disease? West J Med 143: 193-195

4.Riepenhoff-Talty M, Schaekel K, Clark HF, Mueller W, Uhnoo I, Rossi T, et al. Group A rotavirus produce extrahepatic biliary obstruction in orally inoculated newborn mice. Pediar Res 1993;33:394-99

5.Schreiber RA, Huang JQ, Kleiman RE 1992 Isolation and culture of biliary epithelial cells from extrahepatic bile ducts. Gastroenterology 102:A 3336.

6.Glaser JH, Morecki R, Fallon-Friedlander S, Horwitz MS 1987 An extrahepatic bile duct growth factor: in vivo effect and preliminary characterization. Hepatology 7: 272-276.

7.Hsu HY, Chang MH, Ni YH, Huang SF. Cytomegalovirus infection and proinflammatory cytokine activation modulate the surface immune determinant expression and immunogenicity of cultured murine extrahepatic bile duct epithelial cells. Clin Exp Immunology 2001;126:84-91

8.Allison JP. 1994 CD28-B7 interaction in T -cell activation. Curr Opin Immunol 6:414-419

9.Leon MP, Bassendine MF, Wilson JL, Alis S, Thick M, Kirby J 1996 Immunogenecity of biliary epithelium: Investigation of antigen presentation to CD4+ T cells. Hepatology 24:561-567.

10.Schreiber RA, Kleinman RE 1993 Genetics, immunology and biliary atresia: an opening or diversion? J Pediatr Gastroenterol Nutr 16:111-113