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輪狀病毒在鼷鼠引發肝外膽道阻塞之致病機轉的研究

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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：輪狀病毒在鼷鼠引發肝外膽道阻塞之致病機轉的研究

英文：Studies on the pathogenesis of rotavirus induced extrahepatic biliary atresia in mice

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中文摘要

前人的研究已證實新生之鼷鼠感染輪狀病毒後會產生肝外膽管發炎及阻塞。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，我們先前已能將恆河猴輪狀病毒（Rhesus rotavirus，簡稱 RRV）繁殖於 MA-104 細胞中並加以定量，也已能將 BALB/c 鼷鼠肝外膽道之上皮細胞（MEBEC）分離出來並做初級培養。在本研究中我們將 RRV 與 MEBEC 共同培養後，以 FITC 標誌之輪狀病毒抗 NSP-4 血清染色後，在螢光顯微鏡下觀察再度確認 MEBEC 確可被 RRV 所感染。被 RRV 感染之 MEBEC 及未被感染之 MEBEC 分別與取自被 RRV 感染之同種鼷鼠之 T 細胞共同培養後，以 MTT 分析法測其在 O.D 550nm 之吸光度，則前者較後者呈有意義的減少，顯示 RRV 之感染確實增強了 MEBEC 對由 T 細胞所調控之細胞毒殺作用之敏感性。而去年我們也發現被 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。我們的結果支持 RRV 感染膽管上皮細胞後，會促進 MEBEC 表面之主要組織配合抗原及細胞黏著分子之表現，再加上 RRV 抗原在 MEBEC 之表現，可誘發對 RRV 敏感之 T 細胞對被 RRV 感染之 MEBEC 行細胞毒殺作用。綜言之，經由病毒感染誘發之免疫調控作用造成膽道上皮細胞之損傷之病有重要的意義。

關鍵字：輪狀病毒、肝外膽道閉鎖、主要組織配合抗原、細胞毒殺作用

ABSTRACT

Recent studies have shown that infection with rhesus rotavirus or group A human rotavirus can induce hepatobiliary inflammation leading to extrahepatic biliary obstruction in newborn BALB/c mice. To understand the role of rotavirus infection in the pathogenesis of extrahepatic biliary atresia (EHBA), rhesus rotavirus (RRV) passaged in MA-104 cells culture and the virus titer determined by plaque assay were done. Primary culture for murine extrahepatic bile duct epithelial cells (MEBEC) were also established. After cocultured with RRV, MEBEC was confirmed to have an expression of the NSP-4 antigen as assessed by immunofluorescence assay (IFA). MEBEC infected with RRV revealed no obvious cytopathic effect. MEBEC infected by RRV induced a significant decrease in the absorbance at O.D. 550 nm in a microtiter tetrazolium assay after these treated cells were cocultured with allogeneic T cells. Our recent study also showed that 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. Taken together these findings, we suggest that following upregulation of surface MHC antigens and adhesion molecules with cytokine treatment or RRV infection, the MEBEC can trigger the recognition of MEBEC by effector-T cell mediated cytotoxic responses. Our studies are ongoing to determine whether RRV-infected MEBEC can induce T cell proliferation. 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, murine extrahepatic bile duct cell, proliferation, cytotoxicity

INTRODUCTION

The pathogenesis of extrahepatic biliary atresia (EHBA) remains unknown.[1] EHBA occurs moe frequently in Chinese than in Caucasians. Evidence to date suggests several hypotheses for the development of EHBA. One of them 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

<u>Virus and virus titration</u>

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.

<u>Culture and characterization of murine extrahepatic biliary epithelial cells</u>

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.

Infection of MEBEC by RRV:

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.

Confirmation of RRV-infected MEBEC by immunofluorescence assay

After scrubbed from plastic culture tube and resuspended, MEBEC was then precipitated by centrifugation, washed three times with 1x PBSA, and fully suspended with 2% BSA-PBSA. One drop of this suspension was added to each circle on the open surface of the slide and reabsorbed out to allow only a monolayer of MEBEC left on the slide. After dry in room air, the slide was fixed with acetone for 20 min and preserved in 4° C or -80° C freezer.

Monolayers of MEBEC fixed on the slide were incubated with diluted anti-NSP4 (full) mouse serum at 37°C in humidified chamber for one hour, washed three times with 1x PBSA, stained with diluted FITC-conjugated goat-anti-mouse serum at 37°C for 30 min, mounted with 90% glycerol, covered with cover slide and observed by immunofluorescence microscope.

Isolation and preparation of primed or unprimed murine T cells

Murine spleens were removed from RRV-infected or non-infected BALB/c mice and minced. After centrifugation and lysis of RBC, the leukocyte pellet was subjected to immunomagnetic separation of common T cells using BDTM IMag ANTI-MOUSE CD90.2 (Thy1.2) PARTICLES-DM. More than 95% of the isolated cells were identified as T cells by αβ TCR MoAb (pharmingen) staining followed by flow cytometric analysis.

Cellular cytotoxicity assays

To determine the cytotoxic effect of murine T cells on MEBEC, a MTT-based colorimetric assay (Cell proliferation kit I, Boehringer Mannheim GmbH, Germany) was used. The assay is useful for the quantification of viable cells because the yellow

tetrazolium salt MTT is cleaved to formazan dye only by metabolic active cells. Briefly, freshly isolated MEBEC were seeded onto 96-well, flat bottomed culture plates ($1X10^5$ cells/well) and cultured for 3 days. Cells in each well were then incubated with 100 ul complete medium containing murine TNF-α (100 U/ml), INF-γ (100 U/ml) or RRV ($1.5X10^4$ pfu) for another 3 days. Then purified T cells from RRV-infected mice at a concentration of $5X10^5$ cells/100 ul/ well was added and incubated for 3 days. Each well then was treated with MTT labeling reagent followed by solubilization solution (10% SDS in 0.01 M HCl) and allowed to stand overnight at 37° C. The absorbance of the samples was measured by an ELISA reader at O.D. 550 nm.

Cytotoxicity of MEBEC was determined by reduction of absorbance in MTT assay. Since the absorbance revealed strongly correlates to the viable cell numbers, a lower O.D. value of residual viable epithelial cells indicated a higher T cell-mediated cytotoxicity to MEBEC.

Statistics

All results are expressed as mean ± s.e.m.. Statistical comparison was performed by using Student's t-test

RESULTS

Flow cytometric analysis revealed 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 coculture with RRV, MEBEC was studied for the expression of NSP-4 antigen by IFA and was found that about 80% of MEBEC was positive for NSP-4 whereas only about 5% of untreated MEBEC was positive for NSP-4 staining.

Cytotoxic effect mediated by T cells in culture was measured as absorbance at O.D. 550 nm in MTT assay. The absorbance of each measurement of residual viable epithelial cells from T cells in culture with untreated MEBEC was 0.423±0.040 (mean± s.e.m, N=6, range 0.38-0.46). The average absorbance of residual viable MEBEC in different experimental conditions were the following: T cells in culture with TNF-α stimulated MEBEC was 0.340±0.030 (range 0.31-0.374) ($P<0.05$); T cells in culture with INF-γ stimulated MEBEC was 0.345±0.080 (range 0.268-0.422) ($P<0.05$); T cells in culture with RRV-infected MEBEC was 0.280±0.060 (0.221-0.34) ($P<0.05$); Thus RRV infection did significantly increase the susceptibility of MEBEC to T cell mediated cytotoxic injury.

DISCUSSION

A previous study showed that 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.

We have also shown 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].

From our results, RRV-primed T cell-mediated cytolysis could be observed in INF-γ treated or RRV-infected MEBEC with enhanced class I MHC antigen expression, or even in TNF-α activated MEBEC with increased ICAM-I expression. We could demonstrate in MTT assays the significantly higher cytotoxic effect induced by primed T cells on RRV-infected MEBEC. This indicates that following infection with RRV in vitro, MEBEC may express viral specific antigens at their cell surface, which in association with class I antigen expression can sufficiently function as target cells for effector T cells mediated cytotoxic responses. Further studies are still needed to study the role of RRV infection in the production of immune-mediated injury in inflammatory and fibroobliterative change of RRV-induced EHBA in mice

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