Intercellular adhesion molecule-1 and MHC antigens on human intrahepatic bile duct cells: effect of pro-inflammatory cytokines

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Abstract

Human intrahepatic biliary epithelial cells were isolated from the livers of patients with primary biliary cirrhosis and from normal livers and established in primary culture. The in vitro expression of intercellular adhesion molecule-1, HLA class I, and HLA class II on biliary epithelial cells was studied in response to tumour necrosis factor- α (0-500 U/ml), interferon- γ (0-500 U/ml), and interleukin-1 (0-5 U/ml) by immunohistochemical staining and a semiquantitative scoring system validated by spectrophotometry and previously validated by laser confocal microscopy. The non-stimulated expression of intercellular adhesion molecule-1 and HLA class II was higher on cells derived from the primary biliary cirrhosis liver than on cells from normal liver, a difference not seen with HLA class I expression. A statistically significant increase in intercellular adhesion molecule-1 expression was seen with all three cytokines in cells derived from both primary biliary cirrhosis and normal liver. Increase in HLA class I expression was seen only with interleukin-1 5 U/ml for cells derived from both normal and diseased liver. Increase in HLA class II expression was seen only with interferon- γ 500 U/ml for cells derived from diseased liver and with interleukin-1 5 U/ml for cells derived from both diseased and normal liver. These data show that pro-inflammatory cytokines increase expression of intercellular adhesion molecule-1, HLA class I, and HLA class II on human intrahepatic biliary epithelial cells in vitro and are consistent with the hypothesis that these locally acting factors may play a part in the pathogenesis of immune mediated disorders such as primary biliary cirrhosis in which immune mediated bile duct damage occurs.

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Primary biliary cirrhosis is a disorder of unknown cause in which immune factors are believed to be involved. There is evidence to suggest that cell mediated damage to biliary epithelial cells may be important. The finding of portal tract infiltration by T cell subsets supports this hypothesis¹⁻⁵ along with ultrastructural studies showing cytotoxic T cells in intimate contact with epithelial cells in damaged bile ducts.⁵ Membrane expression of MHC molecules is important in immune mediated cytotoxicity being required for both antigen recognition and cytotoxic T lymphocyte mediated cytolysis. Intercellular adhesion molecule-1 (ICAM-1) dependent cell adhesion is a prerequisite step in these interactions.

Increased expression of HLA class I⁶⁻⁹ and class II⁶⁻¹⁰ and ICAM-1¹¹ on bile ducts has been shown in immunohistochemical studies in primary biliary cirrhosis and suggests that expression of these molecules may play a part in regulating the immune response in this disease.

In vitro studies of vascular endothelial cells, fibroblasts, and cell lines have shown increased expression of ICAM-1¹² and HLA molecules¹³ by pro-inflammatory cytokines. These locally acting factors may have a modulatory role in the immune response in vivo.¹⁴ The effect of cytokines, however, on in vitro expression of these molecules on human intrahepatic biliary epithelial cells has not been examined. We have therefore studied the in vitro effect of tumour necrosis factor α (TNF- α), interferon γ (IFN γ), and interleukin-1 (IL-1) on the expression of ICAM-1, HLA class I, and HLA class II molecules on primary cultures of human intrahepatic biliary epithelial cells. The effect of corticosteroids on unstimulated and maximally stimulated ICAM-1 expression was also assessed.

Methods

BILIARY EPITHELIAL CELL ISOLATION

Human intrahepatic biliary epithelial cells were harvested from the livers of patients with primary biliary cirrhosis having orthotopic liver transplantation, by differential density separation and immunopurification as described in detail elsewhere.^{15,16} Human intrahepatic biliary epithelial cells were also harvested from surplus normal liver tissue available from reduced paediatric grafts. The yield per 50 g liver tissue was $1-3 \times 10^6$ cells with above 95% purity and variability. Cells were plated onto collagen coated 96 well microtitre plates at 0.5×10^5 cells/well. Each liver preparation provided sufficient cells for 6-12 sets of triplicates for assay. Biliary epithelial cells were characterised by morphology under light and transmission electron microscopy and by staining characteristics using anti-cytokeratin 19 (DAKOPATTS), anti γ -glutamyl transferase (DAKOPATTS), and anti-HEA125 (PROGEN) monoclonal antibodies.

STIMULATION AND STAINING OF CULTURES

Between 6-9 days after isolation, cultures were stimulated with either TNF α (TNF α human recombinant; SIGMA) 50-500 U/ml, IFN γ (IFN γ human recombinant; SIGMA) 50-500 U/ml, IL1 (IL1 β human recombinant; CISTRON) 0.5-5 U/ml or no cytokine (control) for 24 hours. In additional experiments of

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ICAM-1 expression, methyl-prednisolone 2.6×10^{-7} M was added to cultures maximally stimulated with one of the above cytokines. After 24 hours, cultures were labelled using one of the following mouse monoclonal antibodies; anti-human ICAM-1 (RR1/1: a gift from Dr R Rothlein, Department of Immunology, Boehringer Ingelheim Pharmaceuticals Inc, Ridgefield, CT, USA; $0.84 \mu\text{g/ml}$), anti-human HLA Class I (anti-HLA-class I; DAKOPATTS; $3.3 \mu\text{g/ml}$) or anti-human HLA Class II (anti-HLA DR, CR3/43; DAKOPATTS; $2.7 \mu\text{g/ml}$) using an alkaline phosphatase – anti alkaline phosphatase (APAAP) technique¹⁷ and visualised with Fast Red (SIGMA) colour substrate.

SCORING

At the time of immunocytochemical staining, cell cultures were seen as confluent islands of cells of varying size. It was therefore not possible to use an objective mechanised measure of staining intensity such as spectrophotometry as the measured intensity would vary with culture density as well as staining intensity. Attempts to produce a single cell suspension were avoided because of previous reports associating enzymatic dissociation of these cells with loss of viability.¹⁸ Cell staining was therefore scored visually using a semi-quantitative scale (0–3), by three independent 'blinded' observers (Fig 1). Mean scores were calculated from the three observers for each set of triplicates and the control value subtracted. This method of immunohistochemical scoring has been previously validated using laser confocal microscopy.¹¹

VALIDATION OF SCORING SYSTEM

To validate the scoring system further, human vascular endothelial cells were isolated and established in culture in two sets of eight triplicates as described elsewhere.¹⁹ Endothelial cells were used because they can be grown to confluence, permitting scoring of staining intensity by spectrophotometry. Cultures were labelled with a monoclonal antibody to factor VIII-related antigen (DAKOPATTS) at a range of titres from 0–1:10. One set of triplicates was stained by standard APAAP method with Fast

Red indicator and scored by eye. The second set was stained by the APAAP method but with staining intensity assessed by measuring the colour reaction in the supernatant spectrophotometrically. Linear regression was used to assess the relation between the two scoring systems and between each of the scoring systems individually and the log titre of primary antibody.

STATISTICS

The Kolmogorov-Smirnov test showed a normal distribution of results in all cases and parametric statistics were therefore used to compare results between stimulated cultures and controls. Student's *t* test was used to assess whether stimulation with the maximum dose of cytokine gave significantly enhanced staining intensity. Where this was found to be the case, analysis of variance and linear regression were used to assess whether there was a dose response relation.

Results

That human intrahepatic biliary epithelial cells remain viable at 6–9 days in lone culture was shown by light microscopy, trypan blue exclusion,²¹ chromium release, and release of intracellular enzymes as published elsewhere.²⁰ Human intrahepatic biliary epithelial cells continued to stain positively for cytokeratin 19, γ -glutamyl transferase, and HEA-125 during the study period.

VALIDATION OF SCORING SYSTEM

A sigmoid relation was seen between each of the scoring systems and the log titre of primary antibody. A similar relation was seen between the two scoring systems with a highly significant correlation ($r=0.995$; $p<0.001$).

HUMAN INTRAHEPATIC BILIARY EPITHELIAL CELLS FROM PRIMARY BILIARY CIRRHOSIS LIVER TISSUE ICAM-1

(Results are shown in Figs 2 and 3).

Unstimulated – mean staining intensity for unstimulated human intrahepatic biliary epithelial cells stained for ICAM-1 was 1.12 ($n=20$; $SD=0.34$).

TNF α – statistically significant increases in staining intensity were seen after incubation with TNF α 50 U/ml (mean=1.40; $n=3$; $SD=0.089$; $p<0.003$), 200 U/ml (mean=1.76; $n=3$; $SD=0.21$; $p<0.05$), 500 U/ml (mean=1.85; $n=11$; $SD=0.38$; $p<0.0001$). Analysis of variance for individual results showed $F=14.40$; $p<0.0001$ and linear regression $r=0.73$; $p<0.0001$.

IFN γ – statistically significant increases in staining intensity were seen after incubation with IFN γ 200 U/ml (mean=1.78; $n=3$; $SD=0.24$; $p<0.05$) 500 U/ml (mean 1.85, $n=11$; $SD=0.32$ $p<0.0001$). Analysis of variance for individual results showed $F=14.75$; $p<0.0001$ and linear regression $r=0.73$, $p<0.0001$.

IL1 – statistically significant increases in staining intensity were seen after incubation with

Figure 1: Photomicrographs of human intrahepatic biliary epithelial cells stained for ICAM-1 with staining intensity from 0 (left side) to 3 (right side).

Figure 2: In vitro staining intensity (mean SD) of ICAM-1 (top row), HLA class I (middle row), and HLA class II (bottom row) on human intrahepatic biliary epithelial cells derived from primary biliary cirrhosis (solid line) and normal (dotted line) liver induced by tumour necrosis factor α (TNF) (left column), interferon γ (IFN) (middle column), and interleukin-1 (IL1) (right column).

Figure 3: In vitro staining intensity dose response (mean SD) of ICAM-1 on human intrahepatic biliary epithelial cells derived from primary biliary cirrhosis liver induced by tumour necrosis factor α (TNF), interferon γ (IFN), and interleukin-1 (IL1).

IL1 2 U/ml (mean=2.19; n=1; $p < 0.0001$), 5 U/ml (mean=1.73; n=8; SD=0.18; $p < 0.0001$). Analysis of variance for individual results showed $F=35.82$; $p < 0.0001$ and linear regression $r=0.63$; $p < 0.001$.

Prednisolone – prednisolone at 2.6×10^{-7} M did not significantly affect the staining intensity for ICAM-1 in either unstimulated human intrahepatic biliary epithelial cells cultures or when stimulated with any of the chosen cytokines.

Unstimulated without prednisolone mean=1.12; n=20; SD=0.34; with prednisolone mean=1.46; n=5; SD=0.56; NS.

TNF α – a 500 U/ml without prednisolone mean=1.95; n=11; SD=0.48 with prednisolone 2.6×10^{-7} M mean=2.1 n=4; SD=0.23, NS.

IFN γ – 500 U/ml without prednisolone mean=1.95; n=11; SD=0.33; with prednisolone 2.6×10^{-7} M mean=1.83; n=4; SD=0.68; NS.

IL1 – 5 U/ml without prednisolone mean=1.83; n=8; SD=0.40; with prednisolone 2.6×10^{-7} M mean=1.48; n=2; SD=0.05; NS.

HLA Class I (Results are shown in Fig 2).

Unstimulated – the mean staining intensity for unstimulated human intrahepatic biliary epithelial cells stained for HLA class I was 0.83 (n=9; SD=0.34).

TNF α – statistically significant increases in staining intensity were not seen after incubation with TNF α 500 U/ml.

IFN γ – statistically significant increases in staining intensity were not seen after incubation with IFN γ 500 U/ml.

IL1 – statistically significant increases in staining intensity were seen after incubation with IL1 5 U/ml (mean=1.25; n=6; SD=0.21; $p < 0.0005$).

HLA Class II

(Results are shown in Fig 2).

Unstimulated – the mean staining intensity for

unstimulated human intrahepatic biliary epithelial cells stained for HLA class II was 0.83 (n=13; SD=0.66).

TNF α – statistically significant increases in staining intensity were not seen after incubation with *TNF α* 500 U/ml.

IFN γ – statistically significant increases in staining intensity were seen after incubation with *IFN γ* 500 U/ml (mean=1.20, n=8; SD=0.19; p<0.0001).

IL1 – statistically significant increases in staining intensity were seen after incubation with *IL1* 5 U/ml (mean=1.38; n=8; SD=0.37; p<0.005).

HUMAN INTRAHEPATIC BILIARY EPITHELIAL CELLS FROM NORMAL LIVER TISSUE

ICAM-1 (Results are shown in Fig 2).

Unstimulated – mean staining intensity for unstimulated human intrahepatic biliary epithelial cells stained for ICAM-1 was 0.93 (n=12; SD=0.26). This was significantly lower than that seen in human intrahepatic biliary epithelial cells derived from primary biliary cirrhosis liver (mean=1.12; n=20; SD=0.34; p<0.05).

TNF α – statistically significant increases in staining intensity were seen after incubation with *TNF α* 500 U/ml (mean=1.58; n=6; SD=0.42; p<0.0005).

IFN γ – statistically significant increases in staining intensity were seen after incubation with *IFN γ* 500 U/ml (mean 1.56; n=6; SD=0.25 p<0.0005).

IL1 – statistically significant increases in staining intensity were seen after incubation with *IL1* 5 U/ml (mean=1.31; n=6; SD=0.40; p<0.05).

HLA Class I (Results are shown in Fig 2).

Unstimulated – the mean staining intensity for unstimulated human intrahepatic biliary epithelial cells stained for HLA class I was 0.64 (n=8; SD=0.15). This was not significantly different from that seen in human intrahepatic biliary epithelial cells derived from primary biliary cirrhosis liver (mean=0.83; n=9; SD=0.34; NS).

TNF α – Statistically significant increases in staining intensity were not seen after incubation with *TNF α* 500 U/ml.

IFN γ – statistically significant increases in staining intensity were not seen after incubation with *IFN γ* 500 U/ml.

IL1 – statistically significant increases in staining intensity were seen after incubation with *IL1* 5 U/ml (mean=1.08; n=4; SD=0.33; p<0.01).

HLA Class II (Results are shown in Fig 2).

Unstimulated – the mean staining intensity for unstimulated human intrahepatic biliary epithelial cells stained for HLA class II was 0.58 (n=8; SD=0.19). This was significantly lower than that seen in human intrahepatic biliary epithelial cells derived from primary biliary

cirrhosis liver (mean=0.83; n=13; SD=0.66; p<0.001).

TNF α – statistically significant increases in staining intensity were not seen after incubation with *TNF α* 500 U/ml.

IFN γ – statistically significant increases in staining intensity were not seen after incubation with *IFN γ* 500 U/ml.

IL1 – Statistically significant increases in staining intensity were seen after incubation with *IL1* 5 U/ml (mean=1.16; n=4; SD=0.22; p<0.0005).

Discussion

This study provides evidence that locally acting cytokines may play an important part in regulating the expression of ICAM-1 and HLA antigens on biliary epithelial cells. This in turn may be of relevance in facilitating lymphocyte mediated damage to these cells.

The results of this study are in keeping with previous immunohistochemical studies showing a weak expression of ICAM-1 and HLA II on biliary epithelium in normal liver but increased expression in disorders associated with immune damage to bile ducts.⁷⁻¹¹ Our results show that both *IFN γ* and *IL1* can increase HLA class II expression on human intrahepatic biliary epithelial cells from primary biliary cirrhosis patients whereas only *IL1* increased expression in normal human intrahepatic biliary epithelial cells. Increased expression of ICAM-1 was seen with all three cytokines used in human intrahepatic biliary epithelial cells from both normal and primary biliary cirrhosis related liver. These data are compatible with the hypothesis that local release of pro-inflammatory cytokines might be responsible for increased expression of HLA antigens and ICAM-1 on human intrahepatic biliary epithelial cells in vivo. Antigen presentation to helper T cells is a crucial step in T lymphocyte activation and cytotoxic damage. Although well recognised, antigen presenting cells such as macrophages have been implicated in primary biliary cirrhosis and other authors have suggested that biliary epithelial cells may act as antigen presenting cells.²¹ As cells must express ICAM-1 and HLA class II if they are to present antigen,^{22,23} our data suggest that human intrahepatic biliary epithelial cells might be capable of antigen presentation. The effector limb of the immune system also requires adhesion and expression of ICAM-1 on target structures bearing class I MHC products may facilitate MHC restricted cytotoxicity.²⁴

The relevance of these in vitro findings to disease mechanisms in vivo remains unclear. There are several limitations regarding the methodology of the study. It is not known whether the method of biliary epithelial cell isolation leaves surface structures and the means of their production intact although the results of this study suggests that it does. That human intrahepatic biliary epithelial cells cultures remain viable at 6–9 days has been found in other studies.²⁰ Activation of human intrahepatic biliary epithelial cells during isolation may also be a confounding factor. That increased expression was shown with cytokines, however,

and suggests they can still be further stimulated.

We have attempted to validate our scoring system but problems remain. A non-linear relation is shown between our scoring system and spectrophotometry. Similarly the relation between either of these scoring methods and antigen density may not be linear. It is not possible to compare results quantitatively between assays for the three antigens assessed in the study as the monoclonal antibodies have different binding affinities and are used in different concentrations within the assay.

Statistical analysis show a significant increase in ICAM-1 expression with all three cytokines used with increased intensity of staining ranging up to +0.73. In the endothelial cell/factor VIII-related antigen model this corresponds to a 17-fold increase in antibody concentration. With HLA, however, although significant rises were seen in some assays the increase in staining enhancement is small (+0.37 to 0.55) and may be of little or no functional significance.

That ICAM-1 expression showed the greatest increase after incubation with cytokines supports a regulatory role for this adhesion molecule in immune mediated disease such as primary biliary cirrhosis. Increased ICAM-1 expression will facilitate leukocyte adhesion to human intrahepatic biliary epithelial cells thereby allowing interaction with HLA class II for antigen presentation and class I for cytotoxic T cell responses. The clinical significance of these findings is unclear but the potential for externally influencing these mechanisms by either modulating cytokine production or the use of monoclonal antibodies to block ICAM-1 expression is an exciting possibility as therapeutic antibodies to ICAM-1 have been shown in animal studies to reduce immune reactions in graft rejection.^{25 26}

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