Biliary expression of heat shock protein: a non-specific feature of chronic cholestatic liver diseases

E B G Martins, R W Chapman, K Marron, K A Fleming

Abstract

Aim—To analyse the expression of heat shock protein (HSP) 60 in biliary epithelium in auto-immune liver conditions and also in chronic cholestatic and other liver diseases.

Methods—Hepatic expression of HSP-60 in frozen liver biopsy specimens from patients with primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), auto-immune hepatitis (AIH), obstructive jaundice (LDO), alcoholic liver disease (ALD), and from normal controls was studied by immunohistochemistry using the APAAP technique and confocal laser scanning microscopy.

Results—Increased expression of HSP-60 was demonstrated in the biliary epithelium of patients with PBC, LDO and, to a lesser extent, with PSC. Focal, weaker, biliary epithelial expression of HSP-60 was observed in AIH, ALD and normal liver tissue. Increased expression was also seen on Kupffer cells in LDO and in hepatocytes in areas of piecemeal necrosis in AIH.

Conclusion—Enhanced biliary expression of HSP-60 is a common feature of chronic biliary disease irrespective of aetiology and is not specific to auto-immune diseases.

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Heat shock proteins (HSPs) are a highly conserved group of proteins present in all prokaryotic and eukaryotic cells. Their name derives from original experiments in which these proteins were induced in cells exposed to extreme heat.¹⁻⁵ It is now known that they respond to many environmental stresses such as temperature changes, fever, inflammation, irradiation, viral infection, malignant transformation, exposure to heavy metal ions, ethanol, and anoxia.³ It has been suggested, therefore, that they should be called stress related proteins. Their functions are related to protection of cells against the potential damage caused by noxious stimuli. They also have important functions in folding, assembly and oligomerisation of polypeptides, and are therefore essential for cell viability.⁵ The HSPs are classified into families according to their size, antigen cross reactivities and sequence homologies. Among the various families is the HSP-

60 family, which includes the mycobacterial 65 kilodalton HSP.^{4,6} This HSP shares homology with a human mitochondrial protein, and the GroEL protein of *Escherichia coli*,⁷ which corresponds to what was previously described as the bacterial "common antigen".⁸ HSP-60 has been associated with auto-immune diseases such as rheumatoid arthritis,⁹⁻¹¹ primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC)¹² and conditions such as alcoholic liver disease (ALD).^{13,14} It has recently been suggested that induced biliary expression of HSP-60 in PSC and PBC may be specifically involved in the auto-immune genesis of these conditions.¹² However, these studies did not exclude the possibility that this biliary expression of HSP-60 may represent a non-specific response to a variety of stresses. We therefore analysed the expression of HSP-60 in liver tissue from patients with PSC and PBC, ALD, auto-immune hepatitis (AIH), chronic extra hepatic bile duct obstruction (LDO), and from normal controls.

Methods

Liver biopsy specimens were obtained from seven cases of PBC (six with stage I-II disease), 13 of PSC (eight with stage I-II disease), seven of untreated AIH, six of LDO, and six of ALD. Six normal biopsy specimens served as controls. The biopsy specimen cores were embedded in OCT (BDH Ltd, Poole, Dorset, UK) and snap frozen in liquid nitrogen. Thin (5 µm) cryostat sections were fixed with acetone at 4°C for 10 minutes.

IMMUNOHISTOCHEMISTRY

HSP-60 was detected using ML30, an IgG1 monoclonal antibody, diluted 1 in 1000 (a gift from Professor J Ivanyi, Tuberculosis and Related Infections Unit, Hammersmith Hospital, London). This antibody reacts with the mycobacterial 65 kDa HSP and cross reacts with a human mitochondrial 60 kDa HSP, facilitating the study of HSP-60 expression in human tissue.¹⁵ An irrelevant antibody (mouse IgG1, Dako, High Wycombe, UK) was used as negative control. Immunohistochemistry was carried out using the APAAP technique, as described previously.¹⁶

ANALYSIS

The intensity of staining was assessed in septal, inter-lobular and proliferating bile ducts in the

Department of
Gastroenterology,
John Radcliffe
Hospital, Oxford
E B G Martins
R W Chapman

Nuffield Department
of Pathology
K A Fleming

Department of
Histochemistry, Royal
Postgraduate Medical
School, Hammersmith
Hospital, London
K Marron

Correspondence to:
Dr Kenneth A Fleming,
Nuffield Department of
Pathology, John Radcliffe
Hospital, Oxford OX3 9DU.

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Summary of bile duct expression of HSP-60

Disease	Septal ducts	Interlobular ducts	Proliferating ducts
PBC	+++ (100%)	+++ (100%)	+++ (100%)
LDO	+++ (100%)	+++ (100%)	+++ (100%)
PSC	+++ (100%)	+ / +++ (70%)	+ / +++ (80%)
AIH	++ (100%)	+ / ++ (60%)	- / ++ (80%)
ALD	+ / ++ (100%)	- / ++ (50%)	-
Normal liver tissue	+ / ++ (100%)	- / ++ (50%)	-

The results are expressed as (%) positive ducts in each group. The semiquantitative score for intensity of staining is as follows: -, negative staining; +, weak staining; ++, moderate staining; + + +, strong staining.

different diseases. A semiquantitative scoring system for the staining intensity was used: no staining, -; weak staining, +; moderate staining, ++; and strong staining, + + +. The percentage of positive bile ducts was also assessed.

Figure 1 A bile duct (arrow) in a biopsy specimen from a patient with LDO, in which all of the bile duct epithelial cells were strongly positive for HSP-60. (Original magnification, $\times 100$.)

Figure 2 The same bile duct (arrow) as in fig 1, studied using CLSM. Note the granular perinuclear staining pattern. (Original magnification, $\times 120$.)

CONFOCAL LASER SCANNING MICROSCOPY
Bile ducts strongly positive for HSP-60 were analysed further using confocal laser scanning microscopy (CLSM). The light microscopy sections were used as alkaline phosphatase-fast red is a good fluorescent label for CLSM.¹⁷ This technique permits the study of epitope distribution by focusing on a single plane within the tissue section with enhanced contrast and resolution, compared with light microscopy. Individual planes may then be reconstructed to give an impression of the entire cellular distribution of HSP-60 immunoreactivity.

This study conformed to the guidelines of the Central Oxfordshire Health Authority Research Ethics Committee (COREC). Informed consent for liver biopsy was obtained from each patient.

Results

EXPRESSION OF HSP-60 IN BILE DUCTS
Epithelial cytoplasmic expression of HSP-60 was present in the septal bile ducts in all biopsy specimens, including normal liver; in normal liver, however, the staining intensity was much weaker than that observed in the diseased tissue (table). HSP-60 was expressed strongly in liver tissue from patients with PBC, LDO and PSC. Increased expression of HSP-60 was observed in all interlobular bile ducts in PBC and LDO samples (fig 1). In PSC samples there was increased expression of HSP-60 in 70% of interlobular ducts, with weak or no staining in others. These findings did not correlate with histological stage. Focal staining for HSP-60 was noted in some interlobular bile ducts in AIH, ALD and normal liver tissue samples, but the intensity of staining was weak, particularly in the normal samples. In PBC and LDO samples all proliferating bile ducts exhibited increased expression of HSP-60, and again enhanced expression was seen focally in PSC. Focal positive staining was also observed in proliferating ductules in AIH samples.

On light microscopy, epithelial staining of the bile ducts was cytoplasmic and granular, and all cells were diffusely positive. Nuclear staining was not detected. On CLSM, the pattern of staining was confirmed to be granular and cytoplasmic. The nuclei did not stain (fig 2). The HSP-60 staining pattern was similar in all of the diseased tissue studied.

HEPATOCYTE STAINING

Cytoplasmic expression of HSP-60 was observed in hepatocytes in all of the patient groups studied. Expression was focal and without any specific pattern of distribution, except for AIH where there was noticeably strong expression of HSP-60 in hepatocytes in the areas of piecemeal necrosis and adjacent to inflammatory infiltrate. These hepatocytes seemed to be undergoing degeneration or biliary transformation. The overall pattern of hepatocyte staining was slightly less granular than in the bile ducts. This was confirmed by CLSM, which demonstrated a more homogeneous cytoplasmic distribution of HSP-60. As in bile

ducts, nuclear staining was not observed. Staining intensity was stronger in patients with liver disease than in normal controls, where negative hepatocytes were often present.

KUPFFER CELLS

HSP-60 was strongly expressed in Kupffer cells in LDO tissue samples. This staining pattern was not observed in the other liver diseases studied nor in the normal controls.

INFLAMMATORY INFILTRATE

Infiltrating portal and lobular cells were strongly positive for HSP-60, as were mononuclear cells and granulocytes. Again, the staining pattern was cytoplasmic and intensely granular. This was confirmed on CLSM. In some areas the staining intensity of the inflammatory infiltrate was stronger than that of the bile ducts and hepatocytes.

Discussion

HSPs are among the most conserved proteins in nature. They are found in all eukaryotic and prokaryotic cells. Their functions vary among different families, with HSP-60 being responsible for protein folding and oligomerisation. HSPs are also involved in removing damaged proteins from cells and in mediating receptor activation, as in the case of HSP-90 and the steroid receptor. In all cells they are essential for maintaining viability under unfavourable circumstances. Most HSPs exist in small quantities in each cell, but they can be induced by various forms of stress, when their intracellular content is greatly increased. In these instances they can be detected using immunohistochemical methods. Most antibacterial defences include reactions directed against HSPs. Accordingly, because of their shared homology with bacterial proteins—HSP-60 shares more than 50% sequence homology with the GroEL protein of *E coli*—it has been hypothesised that this may result in auto-immune reactions due to molecular mimicry.¹⁸ This hypothesis is supported mainly by data from studies on rheumatoid and reactive arthritis.^{9,10,19} This evidence, however, is still inconclusive.²⁰

In the present study increased expression of HSP-60 was detected in the biliary epithelium of patients with PSC, PBC and LDO. The human homologue of mycobacterial HSP-60 is a mitochondrial protein,⁷ and the pattern of staining we detected in biliary epithelium was granular and cytoplasmic. Nuclear staining was not observed. This distribution is consistent with the previously described mitochondrial location of HSP-60. A similar pattern of staining has been reported in other studies using the ML30 antibody.¹⁵

Recently, Broomé *et al*¹² reported induced expression of HSP-60 in biliary epithelium in patients with PSC and PBC. In their study there was also some degree of biliary expression of HSP-60 in ALD and chronic hepatitis C, but to a much lesser extent than in PSC and

PBC. The same group reported the absence of biliary expression of HSP-60 in normal liver tissue. In view of these results it was suggested that this phenomenon could be directly involved in the auto-immune genesis of PSC and PBC. As bile ducts in PSC and PBC also aberrantly express HLA-DR,^{21,22} it is possible that these cells act as antigen presenting cells and HSP-60 could be the putative auto-antigen. In the present study, however, enhanced expression of HSP-60 was detected in the bile ducts of patients with LDO as well in those with PSC and PBC. These findings suggest that enhanced expression of HSP-60 in bile ducts may not have a primary role in biliary auto-immunity. In the patients with LDO presented herein, extra hepatic duct obstruction was caused by a variety of conditions, none of which were immune mediated. Moreover, non-specific aberrant expression of HLA-DR has also been demonstrated in LDO.^{21,22} If HLA-DR positive bile ducts present HSP-60 as an auto-antigen, one would expect manifestations of auto-immunity to occur in LDO as well as in PBC and PSC. As yet, there is no evidence to suggest that auto-immune bile duct damage occurs in LDO. Therefore, as HSPs are known to respond to a variety of stresses, it is likely that HSP-60 expression is induced non-specifically in bile ducts exposed to a variety of chronic noxious stimuli, such as chronic cholestasis, and that this is a secondary phenomenon, rather than a primary event. The intensity of the staining of the inflammatory infiltrate and Kupffer cells in LDO also suggests that the HSP-60 response is non-specific. If HSP-60 is an auto-antigen, one would expect that these cells, in view of their high HSP content, would become targets of an auto-immune reaction. At present, this does not appear to be the case. Another possibility is cross-reaction between the ML30 antibody and bacterial HSP, as these patients are more prone to biliary colonisation. This is unlikely, however, as although the ML30 epitope in mycobacteria and humans is homologous, it is not widely shared among bacteria.²³

Expression of HSP-60 may be involved in the pathogenesis of PSC via an alternative mechanism. Anderson *et al*²⁴ demonstrated that cross-reactivity in the immune response to the mycobacterial HSP-60 can be directed against self proteins, such as HLA-DR2. Also, human CD8+ T cells have been shown to recognise mycobacterial antigens in a HLA-B8 restricted presentation.²⁵ Interestingly, both HLA-B8 and DR2 are strongly associated with PSC. Therefore, although HSP-60 expression was not unique to PSC, this HSP may induced auto-immune liver damage in genetically susceptible individuals. If this was the case, the auto-immune response would therefore be constrained by the expression of HLA antigens. This may also be the case for PBC but, as yet, this condition has not been closely associated with expression of a particular HLA antigen.

HSP-60 was highly expressed by hepatocytes in tissue samples from patients with LDO, PBC, PSC, AIH, and ALD. This expression is also presumably non-specific, a supposition

supported by the non-specific increase in circulating antibodies directed against HSP-60 in ALD, PBC and PSC.¹⁴ Similar data has been reported by Lohse *et al*,²⁶ who found increased hepatocytic expression of HSP-60 in fatty livers, and viral and auto-immune hepatitis, diseases with a very different aetiopathogenesis.

In conclusion, the results reported here suggest that the enhanced biliary expression of HSP-60 is a non-specific phenomenon, characteristic of a variety of cholestatic liver diseases, and is not a primary event solely associated with auto-immune liver diseases. The role of HSP-60 in biliary auto-immunity has yet to be determined.

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