Beta-2-microglobulin expression in the liver after liver transplantation

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SUMMARY The distribution of major histocompatibility complex (MHC) class I antigens was studied in the liver after transplantation by immunoperoxidase staining for β -2-microglobulin (β_2m), a subunit of the class I antigen system. Paraffin wax sections were examined from 25 "time zero" biopsy specimens, taken immediately after insertion of the graft, and 87 biopsy specimens taken after transplantation in seven diagnostic categories: acute cellular rejection ($n = 22$); resolving acute rejection ($n = 8$); chronic rejection ($n = 22$); pure cholestasis ($n = 14$); ischaemia/infarction ($n = 5$); biliary obstruction ($n = 8$); massive haemorrhagic necrosis ($n = 8$). Staining was graded semiquantitatively on a scale of 0-3+ in bile ducts, hepatocytes, sinusoidal lining cells and vascular endothelium. Using the "time zero" biopsy specimens as a baseline for comparison, increased expression of β_2m was seen in bile ducts, hepatocytes, and endothelial cells after transplantation. These changes were most pronounced in cases of rejection but also occurred in other graft conditions. The degree of hepatocyte and endothelial staining was significantly higher in cases of rejection and massive haemorrhagic necrosis than in the other categories.

These findings may have implications for the pathogenesis and diagnosis of rejection of the transplanted liver.

Although there is increasing agreement over the histological features that characterise liver allograft rejection,¹⁻⁶ the underlying mechanisms remain poorly understood. Previous studies have shown changed expression of class I major histocompatibility complex (MHC) antigens in the liver after transplantation.⁷⁻⁹ It is not clear, however, whether this is a specific feature of rejection or a non-specific finding seen in other circumstances following transplantation.

Retrospective analysis of biopsy material obtained during the first five years of transplantation procedures in Birmingham highlighted several "post-transplant" syndromes, each with distinctive clinical and pathological features.¹⁰ This study aimed at examining the distribution of class I antigens in biopsy specimens from each of these various syndromes and at seeing whether a specific pattern of expression was present in cases of rejection.

To assess the distribution of class I antigens we investigated the pattern of β_2 -microglobulin (β_2m) staining in paraffin wax embedded biopsy material using an immunoperoxidase method. β_2m is a low molecular weight protein which has been identified as the light chain of HLA A, B, and C antigens. It is non-

covalently attached to MHC class I heavy chains and is essential to their serological specificity and cell surface expression.^{11 12} Unlike other components of the class I antigen system, β_2m can be readily shown in paraffin wax embedded tissue sections.

Material and methods

CLINICAL AND HISTOLOGICAL DATA

One hundred and twelve specimens were examined from 32 transplantations carried out on 28 patients. These included 25 "time zero" biopsy specimens obtained immediately after insertion of the graft and 87 specimens taken between five and 876 days after transplantation. Transplant material comprised 78 percutaneous needle biopsy specimens, four wedge biopsy specimens taken at repeat laparotomy, and five surgically removed livers obtained at retransplantation. All tissues were fixed in formalin and processed routinely into paraffin wax blocks. Biopsy specimens were sectioned at four levels and stained with haematoxylin and eosin, together with other special stains as appropriate.

All of the "time zero" biopsy specimens showed minor histological abnormalities. These included sur-

gical hepatitis (n = 23), hepatocyte degeneration (cloudy swelling, fine cytoplasmic vacuolation, macrovesicular fat droplets; n = 21), hepatocyte necrosis (focal/confluent; n = 11), cholestasis (n = 11). These changes were generally confined to perivenular areas and peripheral acinar zones. Inflammation related to surgical trauma was more widespread.

Biopsy specimens taken from transplanted livers were designated one of seven diagnostic categories on the basis of characteristic histological features supported by compatible clinical, biochemical, and radiological findings.

Acute rejection (16 cases, 22 biopsy specimens, days 5–44). This was characterised by a combination of (i) portal inflammation, (ii) bile duct damage, and (iii) venous endothelial inflammation.

Resolving acute rejection (six cases, eight biopsy specimens, days 12–62). Histological features of acute rejection resolved in these cases after treatment with high dose steroids and were accompanied by clinical and biochemical signs of recovery.

Chronic rejection (five cases, 22 biopsy specimens, days 10–164). This was characterised by recurrent episodes of portal inflammation, mainly mononuclear, with loss of bile ducts. Four cases progressed to end stage liver disease; two were retransplanted, the other two died. Both transplant hepatectomy specimens showed widespread foam cell arteriopathy as previously described in cases of chronic rejection.¹³ The fifth patient was alive at the time of writing with stable graft function 15 months after the original transplantation.

Pure cholestasis (four cases, 14 biopsy specimens, days 6–201). Severe cholestasis with no other histological abnormalities was seen in these cases. One patient was subsequently found to have sepsis as the likely cause for cholestasis. The cause of cholestasis in the other three was not known.

Ischaemia/infarction (five cases, four biopsy specimens, one hepatectomy, days 5–30). These cases all showed histological features of ischaemic necrosis.¹⁴ This was due to hypoperfusion or hypotension in three patients and hepatic artery thrombosis in the other two.

Biliary obstruction (four cases, eight biopsy specimens, days 45–876). These all had radiological or surgical evidence of bile duct obstruction with compatible histological changes.

Massive haemorrhagic necrosis (six cases, four biopsy specimens, four transplant hepatectomies, days 5–24). These cases were characterised clinically by fulminant graft failure of sudden onset and uncertain aetiology. The main histological findings were those of massive haemorrhage and hepatocyte necrosis with only a sparse cellular infiltrate. The cause of this lesion was not apparent histologically but there were no features

to suggest an ischaemic or infective aetiology.¹⁵

In each of the biopsy specimens included in this study a single cause or pattern of graft dysfunction was evident. Several cases, however, had biopsy specimens which fell into different diagnostic categories at different times—for example, many cases with early biopsy specimens showing features of acute rejection had subsequent biopsy specimens which showed other patterns of graft damage.

IMMUNOHISTOLOGICAL METHODS

Staining for β_2m was carried out using a three stage peroxidase-antiperoxidase (PAP) technique. Sections were dewaxed and trypsinised (0.1 g trypsin in 100 ml buffer) at 37°C for 30 minutes. These were then incubated with polyclonal rabbit anti-human β_2m (1 in 30 for one hour), swine anti-rabbit immunoglobulin (1 in 80 for 45 minutes), and rabbit-PAP (1 in 80 for 40 minutes). All antibodies and trypsin were diluted in phosphate buffered saline (PBS). The final reaction product was visualised with 3'-5' diaminobenzidine and hydrogen peroxide. All antisera used were obtained from Dakopatts, Denmark. Negative controls were obtained by substitution of the primary antiserum with PBS and by absorption with β_2m antigen (Sigma).

Examination of stained sections was carried out simultaneously by two observers (SGH and DHA). Staining was specifically assessed in the following four structures: bile ducts; hepatocytes (membranous staining); sinusoidal lining cells; and endothelial cells. It was often impossible to distinguish Kupffer cells from other sinusoidal lining cells, and endothelial staining in this study refers specifically to the endothelium of hepatic arteries, portal venules, and hepatic venules. The degree of positive staining was further graded semiquantitatively as follows: 0 = negative, +/- = minimal/only occasional cells positive; + = weak/focal; ++ = moderate/patchy in distribution; +++ = intense/generalised. In the case of membranous hepatocyte staining the extent of positive staining was graded thus: + = <10% of hepatocytes positive; ++ = 10–50% positive; +++ = >50% positive. Finally, a "mean staining score" was calculated for each histological category by scoring points for each of the staining grades described above (0 = 0 points, +/- = ½ point, + = 1 point, ++ = 2 points, +++ = 3 points) and dividing the total by the number of biopsy specimens containing the structure to be assessed. If a particular feature could not be assessed in one or more specimens (such as bile ducts in cases of chronic rejection, or hepatocytes in cases with extensive infarction) the total score was divided by the number of remaining specimens in which the given feature could still be assessed. The staining scores in different histological categories were compared statis-

Table 1 β_2 m staining in bile ducts

Histological diagnosis	No of specimens	Degree of staining					Mean staining score
		O	+/-	+	++	+++	
Time zero	25	2	16	7	0	0	0.60
Acute rejection	22	0	1	5	10	6	1.98
Resolving acute rejection	8	0	0	4	4	0	1.50
Chronic rejection	16*	0*	0	5	10	1	1.75
Pure cholestasis	14	0	2	6	5	1	1.43
Ischaemia/infarction	5	0	0	3	2	0	1.40
Biliary obstruction	8	0	0	1	5	2	2.12
Massive haemorrhagic necrosis	8	0	0	1	5	2	2.12

*Six biopsy specimens had no residual bile ducts

Table 2 β_2 m staining in hepatocytes (membranous staining)

Histological diagnosis	No of specimens	Degree of staining					Mean staining score
		O	+/-	+	++	+++	
Time zero	25	17	4	4	0	0	0.24
Acute rejection	22	0	0	7	12	3	1.82
Resolving acute rejection	8	0	2	3	3	0	1.25
Chronic rejection	22	0	0	5	10	7	2.09
Pure cholestasis	14	0	0	6	8	0	1.57
Ischaemia/infarction	3*	0*	0	3	0	0	1.00
Biliary obstruction	8	0	1	7	0	0	0.94
Massive haemorrhagic necrosis	8	0	0	1	2	5	2.50

*Two biopsy specimens had no viable hepatocytes

Table 3 β_2 m staining in endothelium

Histological diagnosis	No of specimens	Degree of staining					Mean staining score
		O	+/-	+	++	+++	
Time zero	25	0	9	14	2	0	0.90
Acute rejection	22	0	0	6	14	2	1.82
Resolving acute rejection	7*	1	1	5	0	0	0.79
Chronic rejection	22	0	0	8	9	5	1.86
Pure cholestasis	14	0	1	8	5	0	1.32
Ischaemia/infarction	5	0	2	3	0	0	0.80
Biliary obstruction	8	0	3	4	1	0	0.94
Massive haemorrhagic necrosis	8	0	0	1	7	0	1.87

*One biopsy specimen had no blood vessels

Table 4 β_2 m staining in sinusoidal lining cells

Histological diagnosis	No of specimens	Degree of staining					Mean staining score
		O	+/-	+	++	+++	
Time zero	25	0	2	7	15	1	1.64
Acute rejection	22	0	1	3	18	0	1.80
Resolving acute rejection	8	0	0	1	7	0	1.87
Chronic rejection	22	0	0	3	15	4	2.05
Pure cholestasis	14	0	0	5	9	0	1.64
Ischaemia/infarction	5	0	1	2	2	0	1.30
Biliary obstruction	8	0	0	1	5	2	2.12
Massive haemorrhagic necrosis	7*	0	0	2	5	0	1.71

*Not possible to assess properly in one biopsy specimen

Fig 1 Bile duct β_2m expression in acute rejection: (day 8 biopsy specimen) bile ducts (arrowed) show strongly positive membranous and cytoplasmic staining for β_2m . Diffuse staining is also present in hepatic sinusoids.

Fig 2 Bile duct β_2m expression in early chronic rejection: (day 12 biopsy specimen). This shows intense biliary epithelial staining for β_2m . Bile ducts are effaced by inflammatory cells and were difficult to identify on sections stained by haematoxylin and eosin.

tically using the Mann-Whitney U test.

Results

Tables 1–4 summarise the staining pattern for β_2m in each of the four structures that were specifically assessed. Positive immunoperoxidase staining was completely abolished by replacing the first antiserum with PBS and by absorption with β_2m antigen.

“TIME-ZERO” BIOPSY SPECIMENS

A moderate degree of staining was present in sinusoidal lining cells. Weaker staining was seen in endothelial cells and bile ducts. Hepatocyte staining was absent in 70% of cases. The remaining 30% of biopsy specimens showed faint focal membranous staining, usually around central venules. The presence of membranous hepatocyte staining correlated with the presence and severity of cholestasis and hepatocyte necrosis in sections stained with haematoxylin and eosin.

BIOPSY SPECIMENS AFTER TRANSPLANTATIONS

Bile ducts

Bile duct expression of β_2m was significantly increased in all transplant histological groups in comparison with the time-zero biopsy specimens ($p < 0.001$ for each category). Biliary epithelial staining was strongest on the basolateral membrane but was also present throughout the cytoplasm of bile ducts in most cases (figs 1 and 2). Staining of bile ducts in acute rejection was associated with positive staining of lymphoid cells in portal inflammatory infiltrates. Although increased bile duct expression of β_2m was a feature of acute and chronic rejection (figs 1 and 2), it often persisted after portal inflammation had resolved and also occurred in non-rejection biopsy specimens (fig 3). Overall, there was no significant difference in the degree of staining among cases of rejection (acute or chronic) and non-rejection biopsy specimens (pure cholestasis, ischaemia, biliary obstruction). The degree of bile duct β_2m expression was slightly higher in massive

Fig 3 Bile duct β_2m expression in pure cholestasis: (day 19 biopsy specimen). Portal inflammation has resolved following a previous episode of acute rejection. Bile ducts continue to display β_2m antigens strongly. Conventional histological examination showed persistent perivenular cholestasis.

Fig 4 Membranous hepatocyte β_2m expression: (day 12 biopsy specimen). Positive membranous staining for β_2m is seen in numerous perivenular hepatocytes and a few periportal hepatocytes. (From a case of early chronic rejection) (P = portal tract, C = central vein).

haemorrhagic necrosis than in non-rejection biopsy specimens ($p < 0.05$).

Hepatocytes

Membranous expression of β_2m in hepatocytes was significantly increased in all post-transplant groups in comparison with time-zero biopsy specimens ($p < 0.0001$ for all categories except ischaemia— $p < 0.005$). Hepatocyte staining was most prominent in perivenular areas but occasional periportal hepatocytes also stained positively (fig 4). It was commonly associated with cholestasis, many bile plugs also staining positively for β_2m (fig 5). The overall degree of hepatocyte staining was higher in cases of rejection than in non-rejection biopsy specimens when these were considered as a whole ($p < 0.005$ for acute rejection, $p < 0.0005$ for chronic rejection). There was still an overlap between rejection and other post-transplant syndromes, however, when

individual categories (such as pure cholestasis) were considered separately. Hepatocyte expression was also greater in cases of massive haemorrhagic necrosis than in non-rejection biopsy specimens ($p < 0.0005$). An unusual and striking pattern of membranous hepatocyte staining was seen in two of the six cases of massive haemorrhagic necrosis. In both of these there was panacinar membranous staining entailing almost 100% of hepatocytes (fig 6). This degree of staining was not seen in any of the other post-transplant biopsy specimens.

Endothelium

Endothelial staining for β_2m was significantly increased in cases of acute rejection ($p < 0.00005$), chronic rejection ($p < 0.00001$), pure cholestasis ($p < 0.01$), and massive haemorrhagic necrosis ($p < 0.0001$) compared with time-zero biopsy specimens. Endothelial staining was not significantly affected in

Fig 5 Membranous hepatocyte β_2m expression associated with cholestasis: (day 13 biopsy specimen). This case of resolving acute rejection shows membranous hepatocyte staining associated with bile plugs, many of which also stain positively for β_2m .

the other three transplant categories (resolving acute rejection, ischaemia, biliary obstruction). Increased β_2m staining in endothelium was mainly seen in portal and hepatic venules and was much less evident in hepatic arterioles. In cases of acute rejection β_2m expression in venular endothelium was associated with infiltration by lymphoid cells, many of which also showed strongly positive staining for β_2m (fig 7). Endothelial expression of β_2m was significantly greater in cases of acute rejection ($p < 0.0001$), chronic rejection ($p < 0.0005$), and massive haemorrhagic necrosis ($p < 0.001$) than in non-rejection biopsy specimens. These differences remained significant ($p < 0.02$) when each of the non-rejection groups was considered separately.

Sinusoidal lining cells

A slightly increased expression of β_2m was observed in

Fig 6 Panacinar hepatocyte β_2m expression in massive haemorrhagic necrosis: (day 5 biopsy specimen). This biopsy shows an early lesion of massive haemorrhagic necrosis with perivenular congestion and early disruption of liver cell plates. There is striking panacinar membranous hepatocyte β_2m display. Nearly all hepatocytes stain positively for β_2m . (P = portal tract, C = central vein).

cases of chronic rejection ($p < 0.05$) and biliary obstruction ($p < 0.05$) when compared with time-zero biopsy specimens. No significant increase was seen in the other post-transplant groups. β_2m expression was also increased in cases of acute rejection compared with ischaemia ($p < 0.05$), and in cases of chronic rejection compared with pure cholestasis ($p < 0.05$) and ischaemia ($p < 0.05$). No significant differences

Fig 7 Endothelial β_2 m expression: (day 7 biopsy specimen). In this case of acute rejection hepatic venular endothelium shows strongly positive staining for β_2 m. There is infiltration by lymphoid cells which also stain positively for β_2 m.

were observed when cases of rejection (acute and chronic) and of massive haemorrhagic necrosis were compared with non-rejection biopsy specimens as a whole.

Discussion

The pattern of β_2 m expression in the "time-zero" biopsy specimens from the present study is similar to that previously described for MHC class I antigen expression in normal human liver.^{16,17} Staining was present most strongly in sinusoidal lining cells, more weakly in bile ducts and vascular endothelium, and was generally absent in hepatocytes. Focal membranous staining of hepatocytes was present in 30% of our baseline biopsy specimens and correlated with the presence and severity of cholestasis and hepatocyte necrosis in these specimens. While some previous studies failed to show the presence of class I antigens on normal hepatocyte membranes,^{16,17} others have

shown weak staining in normal livers.^{7,18} Increased expression of β_2 m has also been described in association with intra- and extra-hepatic cholestasis^{7,19} and hepatocyte necrosis,⁸ these observations having been made both in transplanted livers and non-transplant biopsy specimens.

After liver transplantation we found a noticeably increased expression of β_2 m in bile ducts, hepatocytes and endothelial cells, with changes being present in biopsy specimens from all of the diagnostic categories examined. Only minor and mostly insignificant changes were observed in the staining of sinusoidal lining cells.

Increased expression of β_2 m in bile ducts occurred in cases of acute and chronic rejection, both of which are associated with histological evidence of bile duct damage.¹⁻⁶ Increased display of class I antigens on cells renders them more susceptible to cytotoxic T cell damage and is an attractive hypothesis to explain the bile duct damage that occurs in acute and chronic rejection. Bile duct expression often persisted in cases of resolving acute rejection, however, and was also a prominent finding in other post-transplant syndromes, particularly biliary obstruction. There was no significant difference in the degree of bile duct β_2 m staining among cases of rejection, resolving rejection, and non-rejection syndromes.

Expression of β_2 m on hepatocyte membranes was first reported as a feature of liver allograft rejection by Nagafuchi *et al.*⁷ Increased MHC class I antigen expression on hepatocytes has also been observed in acute rejection by So *et al.*,⁸ who claimed this to be a specific feature of rejection. In contrast, Gouw *et al.*⁹ found no significant difference in class I antigen expression on hepatocytes when comparing cases of rejection with other graft pathologies. We found an increased expression of β_2 m on hepatocytes in cases of acute rejection which partially resolved after successful immunosuppression treatment but which failed to return to baseline concentrations. Increased hepatocyte staining was also seen in several other graft states including chronic rejection. Overall, hepatocyte β_2 m staining was significantly higher in cases of rejection (acute and chronic) than in non-rejection syndromes. Interestingly, β_2 m expression in hepatocytes was often associated with cholestasis—membranous staining and bile plugs being present in the same hepatocytes. Increased class I antigen expression on hepatocytes has been previously described in various non-transplant cholestatic syndromes including large duct obstruction,⁷ drug induced cholestasis,¹⁹ and primary biliary cirrhosis.¹⁹ Cholestasis is a prominent feature of many post-transplant syndromes including acute and chronic rejection, and membranous hepatocyte staining may therefore be an epiphenomenon related to cholestasis rather than a

specific feature of rejection. The observation that bile plugs often stain positively for β_2m and the correlation between cholestasis and membranous hepatocyte β_2m display shown in "time zero" biopsy specimens might further support this hypothesis.

Previous studies of MHC expression after liver transplantation have concentrated mainly on hepatocytes, bile ducts, and sinusoidal lining cells,⁷⁻⁹ and have not specifically examined vascular endothelium. It is therefore interesting to note that our study showed vascular endothelium (particularly of portal and hepatic venules) to be the site in which the degree of β_2m expression correlated most closely with histological features of rejection. This is not surprising in cases of acute rejection where inflammation of venular endothelium is a well recognised phenomenon.^{2,4,5} Venous endothelial inflammation has not been a prominent feature in our experience of acute rejection,⁶ but the relatively few lymphoid cells seen often displayed strong cytoplasmic and surface staining for β_2m . The finding of strong endothelial staining in cases of chronic rejection could also reflect continued endothelial damage. Histologically this is seen mainly in arteries which show a characteristic subendothelial foam cell proliferation¹³ rather than in veins which are histologically normal at this stage.

An interesting pattern of β_2m expression was observed in six cases of massive haemorrhagic necrosis. We have recently described this as a specific syndrome of fulminant graft failure with distinctive clinical and histological features.¹⁰ The mechanisms underlying this condition are uncertain but several aetiologies including ischaemia,²⁰ infection,²¹ rejection,²² and a Schwartzmann mechanism²³ have been suggested. Five of our six cases showed extensive membranous hepatocyte staining, and in two there was a panacinar distribution which was not seen in any other post-transplant biopsy specimens. These two cases also showed extensive deposits of immunoglobulins in hepatocytes and sinusoids (unpublished observations). The clinical importance of these observations is uncertain but they could reflect a state of accelerated humoral mediated graft damage. A similar picture of massive haemorrhage and hepatocyte necrosis with only mild cellular infiltration has recently been described in an animal model of hyperacute humoral mediated rejection.²⁴ Increased expression of β_2m was also present in bile ducts and endothelial cells in our cases of massive haemorrhagic necrosis. Whether this represents evidence of immune mediated damage is uncertain, but these changes correlate with our histological findings which show endothelial inflammation and bile duct damage in some cases of massive haemorrhagic necrosis (unpublished observations).

The functional importance of increased β_2m expression in the liver after transplantation is uncertain.

Increased MHC class I antigen expression in bile ducts and endothelial cells may have a role in the pathogenesis of rejection as these structures are the main targets of immune mediated damage. The considerable overlap that exists between rejection and other post-transplant syndromes in terms of β_2m display in bile ducts, however, makes this unlikely to be the only mechanism involved. Several factors probably play a part in regulating the surface expression of MHC antigens,²⁵ and the possibility that changed class I expression is an epiphenomenon related to non-immunologically mediated events such as cholestasis and ischaemia cannot be excluded.

Although increased expression of β_2m in the liver following transplantation is not confined to cases of rejection, clear differences exist between rejection syndromes, massive haemorrhagic necrosis, and other graft conditions in terms of β_2m staining in hepatocytes and vascular endothelium. These observations suggest that staining for β_2m may be of some value as an adjunct to conventional histological analysis in the interpretation of needle biopsy specimens of transplanted material.

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