Transpl Int (1998) 11 [Suppl 1]: S 367–S 371
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Hepatocyte transplantation – granulocytes and mononuclear cells recognize the surface of isolated autologous hepatocytes as non-self and destroy them

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Abstract Successful transplantation of hepatocytes (HC) is hampered by lack of a proper cellular (stromal) and humoral (cytokines) environment at the site of implantation. We have found that another factor responsible for the low survival rate of transplanted HC is their rapid destruction by host granulocytes. In this study we have investigated the mechanism of the rapid elimination of transplanted hepatocytes in a syngeneic and allogeneic recipient. Only 10% of the radioactivity of syngeneic (LEW-LEW) or allogeneic (BN-LEW) HC was recovered in recipient lymphoid tissues 6 h after i.v. infusion. Pretreatment of the recipient with Endoxan or sublethal irradiation brought about an increased accumulation of HC radioactivity in lungs and spleen, indicating the entrament of live cells in these organs. Only a few HC could be seen in the liver 6 h after intraportal infusion. Granulocytes were found to be cytotoxic to HC in vitro (39%). Monoclonal antibodies to class I and II antigens, CD 11/18 and 54, did not block the granulocyte cytotoxicity. Granulocyte and HC cluster formation could be seen on smears from a drop of mixed cultures, the rates of formation increasing with the time of incubation. Two out of 34 monoclonal antibodies to HC surface molecules partly blocked the granulocyte cytotoxicity. No evident differences in the elimination rate and in in vitro cytotoxicity were seen between the syngeneic and allogeneic HC.

Key words Hepatocyte · Transplantation · Granulocyte · Monocyte · Cytotoxicity

Introduction

Hepatocyte (HC) transplantation (tx) could be an alternative to whole liver tx. Also, for hepatic gene therapy, tx of HC is critical. Among the diseases to be treated with HC tx are alpha-1-antitrypsin deficiency, familial hypercholesterolemia (transduction of the low-density lipo-protein receptor gene), ornithine transcarbamylase deficiency, hemophilia B, and phenylketonuria. Successful tx of HC is hampered by lack of a proper cellular (stromal) and humoral environment, nutrient supply at the site of implantation, and by rapid destruction of most of the implanted cells. Only few transplanted HC survive of the large numbers of cells implanted [4–6, 11, 13]. This untoward reaction of the recipient makes the method clinically useless in patients requiring liver support. The destruction of both autologous and allogeneic HC takes place intravascularly as well as in the spleen, liver, subcutaneous tissue or the peritoneal cavity [8, 9], but at different rates. The molecular process of HC recognition and cytolysis remains obscure. It is likely that isolated HC, with their uncovered surface intercellular adhesion molecules (cadherins, ICAM) [14], are recognized after intravenous infusion or implantation into recipient tissues as non-self, preferentially by granulocytes and monocytes/macrophages, and subsequently lysed.

The question arises, what is the mechanism of the rapid destruction of HC transplanted intravenously, in-

traportally, intraperitoneally, subcutaneously, and into spleen and liver? An interesting question also remains why a few transplanted HC can survive and, if so, what conditions should be met to obtain this positive effect.

In this study we investigated the recipient cellular processes responsible for the destruction of transplanted syngeneic HC immediately after their grafting into various tissues.

Materials and methods

In vivo

Group 1

Lewis rat HC were isolated according to Seglen's method, labelled with ^{51}Cr, and infused i.v. Six hours after infusion, organ distribution of radioactivity was measured. Data were compared with the distribution of ^{51}Cr-labelled syngeneic lymphocytes resistant to local reaction of the recipient. Distribution studies were repeated in leukopenic (agranulocytic) rats after 7 days of Endoxan (20 mg/kg) administration, and after 7.5 Gy irradiation.

Group 2

FITC-labelled HC were injected intravenously, intraportally, intraperitoneally, and subcutaneously. Their distribution and morphology were evaluated after 6 and 24 h in tissue sections by staining with anti-FITC monoclonal antibody (mAb; M878 Dako). Some experiments were also performed in agranulocytic rats. In order to block HC membrane receptors, in some experiments transplanted HC were preincubated with HC surface-specific or anti-class I and anti-ICAM 1 mAbs and subsequently transplanted subcutaneously. The specimens were evaluated after 24 h on frozen sections. The phenotypes of host cells infiltrating HC transplants were identified with mAbs anti-ED1 (macrophages), W 3/13 (T lymphocytes), W 3/25 (helper cells), OX 8 (cytotoxic cells), and OX 6 (class II).

Group 3

In order to study whether granulocytes and blood mononuclear cells will damage HC in situ, $3–5 \times 10^6$ granulocytes or $15–25 \times 10^6$ mononuclear cells were injected into hepatic tissue. Six hours later, a biopsy was taken from the site of implantation and the specimen was snap-frozen for histological processing.

In vitro

Group 4

Cell-mediated cytotoxicity (CMC) of buffy coat granulocytes, splenocytes, mesenteric lymph node lymphocytes, and adherent and nonadherent peripheral blood mononuclear cells (PBM) to syngeneic HC was measured in a standard 6-h ^{51}Cr test. Blocking of CMC with mAbs against class I and II antigens, adhesion molecules (ICAM 1), and HC surface-specific antigens (Hepa 33 and 34 raised in the Institute of Biotechnology, Warsaw) was performed. CMC of PBM in the presence of decomplemented rat serum (ADCC) was carried out. In some experiments, the cytotoxicity of granulocytes and PBM was evaluated on cell smears. Briefly, a suspension of hepatocytes and leukocytes was incubated for 6 h at 37 °C. A drop of cellular suspension was placed on a microscope slide, dried, and stained with Giemsa dye. The number of hepatocyte-leukocyte clusters was counted per 100 HC. HC with damaged membranes usually stain dark blue with Giemsa stain. The percentage of dark-blue HC in clusters and as separate cells was counted after a 6-h incubation and compared with controls.

Results

Group 1

As in a previous investigation [5], only around 10% of transplanted HC-bound radioactivity was recovered after 6 h in lymphoid tissues, whereas the rate for transplanted lymphocytes was around 30%. Also, low levels of radioactivity were found in spleen, mesenteric lymph node, and bone marrow, but high levels were found in kidney and serum. For comparison, the radioactivity of transplanted lymphocytes was high in spleen, lymph nodes, liver, and bone marrow, but low in serum and kidney. In agranulocytic rats after Endoxan therapy, a high level of radioactivity was recovered in lungs and after 7-Gy irradiation in lungs and spleen.

Group 2

FITC-labelled HC behaved differently, depending on the route of tx. Six hours after i.v. tx only HC debris could be detected in lungs. Twenty-four hours later, intraportal transplanted clusters of HC with preserved structure but surrounded by granulocytes and monocytes were seen in portal tributaries. Only a few HC which migrated into sinusoids could be visualized (Fig. 1). Two and six hours after intraperitoneal HC tx, the peritoneal fluid aspirate showed HC with blebs in clusters with granulocytes and macrophages (Fig. 2). On the smear of peritoneal exudate, the percentage of granulocytes adhering to HC was 55.0 ± 8.7 and 42.2 ± 9.3, and the number of granulocytes adhering per HC was 1.1 ± 0.5 and 1.0 ± 0.5, respectively. The percentage of granulocytes in peritoneal fluid rose from 24.1 ± 4.8 to 53.4 ± 5.5 and 56.5 ± 22.1 at 2 and 6 h after HC tx. After 24 h no HC were found in the peritoneal fluid. In subcutaneous tissue, the transplanted HC were found infiltrated by granulocytes (Fig. 3). They evoked a a strong chemotactic reaction with granulocytes and later with PBM (Fig. 3). Transplanted HC released FITC and lost their normal structure. Among the infiltrating cells, some OX 6-positive and ED 1-positive cells were seen among the granulocytes. Agranulocytic rats with subcutaneously transplanted HC revealed only minor granulocytic infiltrates. The structure of transplant-

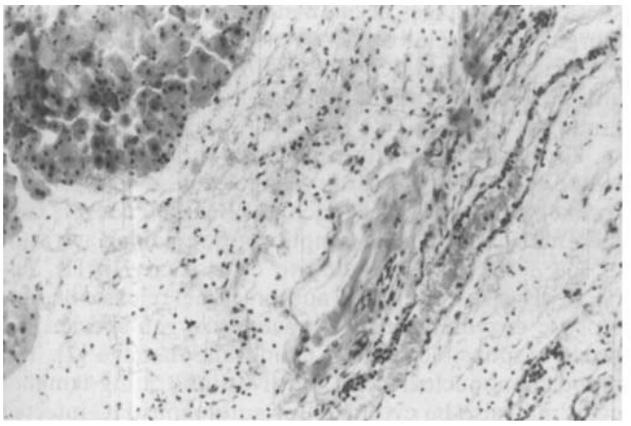

Fig. 1 Rat syngeneic hepatocytes (HC) were isolated and injected subcutaneously into rat paw. Six hours after transplantation they are infiltrated by granulocytes and mononuclear cells *(left upper corner)*. Note emigration of host leukocytes from a blood vessel *(right lower corner)* and migration toward the graft. Hematoxylin-eosin stain, × 400

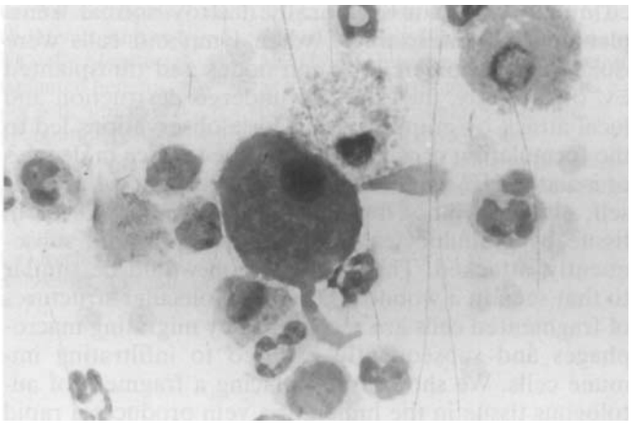

Fig. 3 A transplanted HC after 6 h in the peritoneal cavity. Its membrane has been damaged (blebs). Granulocytes, a macrophage, and small mononuclear cells adhere to its membrane. Anti-FITC mAb stain, counterstain hematoxylin, × 1000

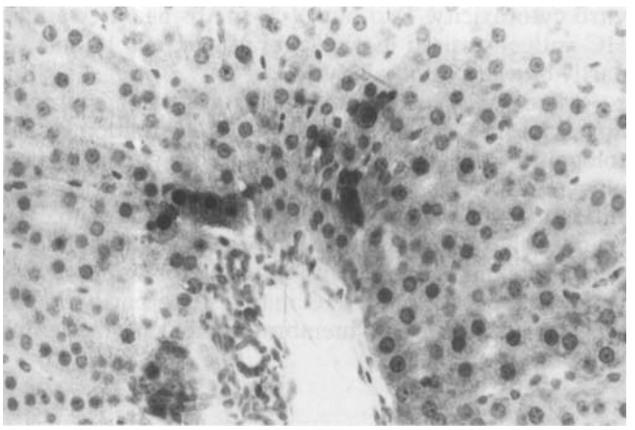

Fig. 2 Isolated HC were injected intraportally and 6 h later they could not be found in portal tributaries. A few HC entered sinusoids, but showed distorted morphology *(dark stained)*. Anti-FITC monoclonal antibody (mAb) stain, counterstain hematoxylin, × 400

ed HC was largely preserved. Preincubation of HC with anti-ICAM 1 and anti-HC-specific mAbs before subcutaneous tx protected a few HC against destruction. Small islands of relatively normal HC could be seen after 24 h.

Group 3

On histology, 4–6 h after intrahepatic injection of granulocytes, the adjacent HC stained more weakly and coalesced with each other. Less of that pattern could be seen after injection of PBM. The control, saline-injected sites did not reveal such patterns.

Group 4

A high level of cell-mediated cytotoxicity by buffy coat and isolated granulocytes was observed (22.3 ± 15.1 %). Splenocytes, mesenteric lymph node lymphocytes, and PBM revealed only marginal cytotoxicity (1–7 %). Blocking of target HC with mAbs against MHC class I, CD11 a/18, and CD54 did not inhibit granulocyte cytotoxicity. In contrast, blocking with mAbs against HC-specific antigens Hepa 10 and 34 decreased the level of cytotoxicity.

In the HC-granulocyte cluster formation test, the percentage of clusters rose from 9.1 % to 19.2 % after 4 h, and the viability dropped from 67 % to 43 %.

Discussion

Our studies revealed that isolated syngeneic HC transplanted i. v. or into tissues undergo a rapid destruction. A number of factors may be responsible for this phenomenon. Isolation of HC from liver fragments with enzymatic methods may damage the cellular membrane and make it fragile. After tx these cells are carried in the bloodstream to lymphoid organs where the damaged membrane will be recognized. After topical tx the subcellular structures released from damaged HC may be chemoattractive for macrophages and granulocytes. Granulocytes, once they become activat-

ed in situ, may non-specifically destroy normal transplanted HC. Interestingly, when lymphoid cells were isolated from spleen or lymph nodes and transplanted i.v. or topically, they did not undergo destruction and local attack by granulocytes. These observations led to the formulation of a concept that the surface molecules of isolated HC (cadherins) [14] are recognized as non-self, either in blood by circulating granulocytes or in tissue by granulocytes and macrophages, and, subsequently, attacked. This phenomenon would be similar to that seen in a wound where the molecular structures of fragmented cells are recognized by migrating macrophages and subsequently exposed to infiltrating immune cells. We showed that placing a fragment of autologous tissue in the lumen of a vein produces a rapid accumulation of granulocytes on the implant and its subsequent lysis [7].

Leukopenic rats after Endoxan therapy and irradiation destroyed i.v.-transplanted HC in at a slow rate. HC, rather, accumulated in lungs and spleen. These data strongly support our concept that destruction of transplanted HC is a recipient cell-mediated process.

We noticed that the speed of elimination of transplanted HC depends on the route of transplantation. FITC-labelled HC transplanted i.v. could not be recovered after 24 h in lungs and those transplanted intraperitoneally were not detected in the peritoneal fluid, whereas HC transplanted intraportally were found in portal areas or sinusoids. Interestingly, they formed clusters with many adherent granulocytes and large mononuclear cells. Most remarkable were histological pictures of HC transplanted subcutaneously where clusters of HC were infiltrated by granulocytes. This was already evident 2 h after transplantation and reached its peak at 6 h. After 24 h, HC debris could be seen. Agranulocytic rats did not show granulocytic infiltrates but, instead, macrophages accumulating first after 24 h at the margin of the relatively well preserved clusters of HC. Preincubation of HC with anti-CD54 (ICAM 1) and anti-HC-specific mAbs partly blocked infiltration by granulocytes.

The in vitro CMC tests evidently showed high granulocyte cytotoxicity toward syngeneic HC. The cytotoxicity exerted by other cells such as PBM, splenocytes, and mesenteric lymph node lymphocytes remained at a rather low level. The granulocyte cytotoxicity to HC has been observed by other authors. The release of proteolytic enzymes and not reactive oxygen species was found responsible for HC membrane damage [2]. The blood mononuclear cells and liver-infiltrating lymphocytes may also be cytotoxic for autologous HC infected with virus but not for normal HC [1, 12]. Peritoneal macrophages destroy tx HC intraperitoneally within 24 h [3, 10].

HC express MHC class I, CD18, and CD54 (ICAM 1) antigens. These molecules did not play any role in contact with granulocytes as blocking procedures with mAbs directed against them did not inhibit the in vitro cytotoxicity. Two out of 34 mAbs against various HC antigens raised in our laboratory, Hepa 10 and 34, partly blocked cytotoxicity.

Taken together, isolated HC transplanted intravenously or into tissues undergo rapid destruction by autologous granulocytes. According to our concept, granulocytes recognize exposed surface molecules (cadherins) on HC previously hidden and inaccessible to them in the liver tissue, and initiate the cytotoxic process. Blocking experiments with mAbs support this concept. Destruction of HC may also be partly due to the fragility of the HC membrane after enzymatic isolation.

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