Liver stem cells

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Abstract

The capacity of hepatocytes and cholangiocytes to contribute to their own maintenance has long been recognized. More recently, studies have indicated the presence of both intra-hepatic and extra-hepatic stem/progenitor cell populations. The intraorgan compartment probably derives primarily from the biliary tree, most particularly the most proximal branches, i.e. the canals of Hering and smallest ductules. The extra-organ compartment is at least in part derived from diverse populations of cells from the bone marrow. These three tiers of liver cell regeneration serve to maintain the normal organ and to regenerate damaged parenchyma in response to a variety of insults. The nature and extent of the insult determines the balance between these stem/progenitor compartments.

Abbreviations: FAH: fumaryl acetate hydroxylase; GFP: green fluorescent protein.

Introduction

The capacity of hepatocytes and cholangiocytes to contribute to their own maintenance has long been recognized. Studies of normal and diseased liver tissue readily demonstrate that hepatocytes and cholangiocytes can undergo mitotic division and, when injury is severe enough, show regenerative changes, such as hepatocyte binucleation, cell plate thickening, cholangiocyte hyperplasia. That hepatocytes could do this in a virtually stem cell like fashion was further shown by serial transplantation of mature hepatocytes from mouse to mouse with the fumaryl acetate hydroxylase (FAH) gene knockout, resulting in 1000 fold regeneration from the original cells, seemingly without limit (Overturf et al., 1997). This elegant experiment definitively established the ability of mature hepatocytes to repopulate an entire organ and self renew. Nevertheless, despite this finding that hepatocytes could essentially act as their own stem cell, candidates for a bipotent stem cell, which could also regenerate cholangiocytes, were still investigated and debated.

Intraorgan liver stem cells

In rodent models, the best candidate for hepatic stem cells has long been the 'oval cell' population which emerges in the context of toxic blocking of hepatocyte regeneration followed by additional liver damage, such as partial hepatectomy or carbon tetrachloride injury (Fausto et al., 1993; Grisham, 1995; Marceau, 1994; Sell, 1978; Sell et al., 1980; Sell and Leftert, 1982; Thorgeirsson, 1996; Wilson and Leduc, 1958). These cells were oval in shape, with a darkly staining nucleus and scant, basophilic cytoplasm. The source of these cells in the normal liver was felt to be a quiescent, facultative cell compartment, difficult to observe, lying either within the biliary tree (Faktor and Radaeva, 1992), directly adjacent to the biliary tree within portal tracts or in the periportal parenchyma (Dabeva and Shafritz, 1993; Sell et al., 1981). Though highly controversial for decades, consensus eventually formed that these were indeed facultative stem cells rather than merely a marker of severe hepatic injury (Sell, 2001).

In humans, the ductular reaction that is seen in massive hepatic necrosis was identified as a possible

human correlate to the oval cell. The morphologic appearance was different from the rodent oval cells: referred to as 'ductular hepatocytes' by Gerber et al. (1983), these clusters of cells often had cholangiocytic features at one end, hepatocytic features at the other, with cells of intermediate morphology and immunophenotyping between them (Fiel et al., 1997; Roskams and Desmet, 1998; Roskams et al., 1998; Thung, 1990). These features suggested activation of a stem cell compartment to many of the investigators, but biliary metaplasia in cholestatic, injured hepatocytes to others (Van Eyken et al., 1988).

The question of which of these interpretations pertained was fairly well settled in two ways. In our own work we followed up on the work from Michael Gerber's laboratory. Immunostaining for biliary-type cytokeratins had identified small oval shaped cells in undisturbed hepatic parenchyma near to, but not within the portal tract stroma. They suggested that these might be the human equivalent to the oval cell (Theise et al., 1999). Three dimensional reconstruction of these cells and nearby bile ducts determined that they were in fact the small cholangiocytes lining the canals of Hering, thereby revising the accepted understanding of the architecture of these lesions, showing that they extend beyond the limiting plate, into the proximal third of the lobule. Similar three dimensional reconstruction of the ductular reactions in massive hepatic necrosis indicated that they were budding off of the pre-existent canal of Hering (Theise et al., 1999), which had become highly proliferative (Koukoulis et al., 1992). Thus, we concluded: 'the canal of Hering is comprised of, or at least harbors, facultative stem cells of the liver'. Following similar procedures, we have now also shown that small intra-septal hepatocyte nodules in cirrhosis derive from the biliary tree as well, though not just from the canal of Hering, but also from the interlobular bile ducts, thus the same statement may be said of more distal biliary structures (Author's unpublished data). The other convincing approach was that of Roskams et al. using serial sampling of livers with acute injury to extend their examination into the fourth dimension, i.e. over time (Faa et al., 1998). This added dimension again convincingly demonstrated that the ductular reactions are proliferative and regenerative, not merely a non-functional sign of damage.

Extraorgan liver stem cells

Two different lines of investigation led to the discovery that there were circulating cells, at least partially bone marrow-derived, which can also contribute to liver turn over and repair following injury. Bryon Petersen took note that he and several other investigators had identified markers on oval cell populations in rodents and in humans that were commonly thought of as hematopoietic markers, including Thy-1, CD34, and c-kit (Baumann et al., 1999; Omori et al., 1997; Petersen et al., 1998). Speculating that these marker profiles might be indicative of marrow origin he performed bone marrow transplantations and liver transplantations in rodents utilizing various markers to indicate cells of donor vs. recipient origin. Using the well-established protocol of AAF CCL₄ for liver injury he showed both oval cells and hepatocytes deriving from the marrow (Petersen et al., 1999).

On the other hand, we recognized that our hypothesis that the canal of Hering was the source of ductular/oval cell reactions could not explain the findings of Sell et al that in response to periportal necrosis from allyl alcohol toxicity in rats the oval cells were devoid of all usual markers of oval cells in the first day of proliferation, including the biliary cytokeratins (Yavorkovsky et al., 1995). This suggested an alternate source of cells to the canal of Hering. We performed bone marrow transplants in mice using both whole bone marrow and CD34⁺ lin⁻ marrow cells. In the absence of overt injury (i.e. without necrosis, regenerative changes, or an oval cell reaction) isolated hepatocytes were shown to be bone marrow derived (Theise et al., 2000a).

Thus, liver regeneration appeared, surprisingly, to be a three-tier process with mature cells, intraorgan stem cells, and extraorgan stem cells all contributing to liver turn over and regeneration after injury. It seemed that it was the proportional contribution of these compartments that shifted depending on the type and extent of injury. But the fact of these multi-tier processes led to more questions than ever.

Confirmation and elaboration

First, there was confirmation that circulating cells were not merely a rodent phenomenon. We examined human tissues that were correlates of both our own and Petersen's marrow-derivation studies, looking at tissue specimens from women with male bone mar-

row transplants with minimal injury and from men who received liver allografts from female donors, with subsequent injury severe enough to cause a ductular reaction (Theise et al., 2000b). In all of these specimens there was significant engraftment from outside the liver, and at much higher intervals than that seen in the rodents. In fact, in one patient with the most severe form of recurrent hepatitis C, the fibrosing cholestatic variant, up to nearly 40% of hepatocytes and cholangiocytes were derived from circulating cells. Moreover, as Petersen had shown in the rodents, the ductular reaction had significant numbers of cells also extrahepatically derived, suggesting a flow through of cells through the ductular intermediates. Virtually simultaneously, Alison et al similarly confirmed human hepatocyte derivation from marrow derived cells, using similar techniques of in situ hybridization for Y chromosomes in gender mismatched donor/recipient chimeric organs (Alison et al., 2000). Subsequent work from Korbly et al. (2002) revealed similar findings.

The next confirmation of the marrow to liver pathway was a paper by Lagasse et al using the FAH knockout mouse described above (Hatch et al., in press). Using different subpopulations of marrow-derived cells instead of hepatocytes, the investigators once again achieved metabolic rescue. However, the rescue was slower and less efficient than that seen in the original hepatocyte transplantation work, suggesting that for some types of therapeutic interventions hepatocytes might prove a better option.

Questions and controversies

Now that these various phenomena have been well established in experimental animals and in humans, research efforts are now turning to more finely detailed elucidation of the mechanisms whereby cells enter the liver from the circulation.

Which circulating cells can regenerate hepatocytes?

The initial animal studies primarily used whole, unfractionated bone marrow to accomplish the experiments. However, in our own study, two archival mice initially transplanted with 200 CD34⁺ lin⁻ cells also demonstrated the same engraftment potential (Theise et al., 2000a). Thus, CD34 positive cells are capable of hepatic engraftment. In the FAH knockout model, the authors' emphasized the complete restitution of

function only by their favored hematopoietic subpopulation: c-kit⁺ sca1⁺ lin⁻ (Lagasse et al., 2000). While it is true that this population is the most efficient and successful at metabolic rescue, it is not the only population – as the authors claim – that can regenerate hepatocytes. Indeed they cite data that shows hepatocyte regeneration from all fractionated subpopulations: positive and negative for c-kit, sca1, and mature lineage markers. Thus, while these other subpopulations are less efficient, they still are capable of plasticity in the direction of hepatocytes, detected under the extreme pressure of the injury model. The data thus solidly demonstrates the stochastic nature of these cell lineage phenomena and points up the likely folly of definitive statements as to which cells are capable of hepatocyte differentiation. The answer is that all cells are, it just depends on how they are conditioned and what environment encourages the process (Theise and Krause, 2002; Theise, in press).

Other mesoderm-derived cells have experimentally been demonstrated to give rise to hepatocytes, even if they are less likely to contribute to physiologic processes. Experimentally, human cord blood stem cells have engrafted as albumin and alpha-1-antitrypsin producing hepatocytes in SCID mice (Beerheide et al., 2002). 'Multipotent adult progenitor cells', obtained from long term culture and conditioning of marrow-derived stromal cells, as per Verfaillie et al., can give rise to hepatocytes *in vitro* and *in vivo* (Jiang et al., 2002; Lagasse et al., 2000). These findings further emphasize the far greater possibilities for plasticity than imagined until recently, and indicate that sources of cells for therapeutic options are not limited to those which play important physiological rolls.

What signaling regulates hepatic engraftment from marrow cells?

Recent work by Petersen demonstrates that in severe toxic injury to the liver, hepatocytes produce SDF-1 (Hatch et al., in press). This chemokine binds to CXCR4, present on some circulating cells, and acts as a chemoattractant. Originally defined as a signaling pathway whereby hematopoietic cells are summoned to the stem cell:stromal cell niche in the marrow, this work suggests that it is part of a more ubiquitous engraftment modulating pathway. Interestingly, in Petersen's experiments, partial hepatectomy did not result in the same production of SDF-1 and in our own experiments (author's unpublished data), partial

hepatectomy did not upregulate engraftment from the marrow.

A second signaling pathway that may be important is that of c-kit and its ligand, SCF (stem cell factor). Direct experimental evidence for this is, as yet, lacking. However, the canals of Hering are c-kit positive as are the proliferating ductular reactions in massive hepatic necrosis (Theise et al., 1999) and c-kit positive cells isolated from human livers have been demonstrated *in vitro* to behave like an hepatic progenitor population (Baumann et al., 1999; Crosby et al., 2001). Moreover, recent work implicates MMP-9-stimulated SCF production in activation and recruitment of hematopoietic stem cells in the marrow (Heissig et al., 2002). These isolated findings are suggestive that c-kit/SCF is another important pathway for multiorgan engraftment processes.

The work of Orlic et al, regarding mouse models of myocardial infarction and marrow derived cardiac myocytes implicates a third pathway (Orlic et al., 2001). By administering G-CSF to their experimental mice, thereby promoting mobilization of 'hematopoietic stem cells' (classically speaking) from the bone marrow, they achieved higher levels of cardiac engraftment from the marrow. Whether this was a purely artificial effect, or reflects a physiological pathway of control of mobilization of cells for healing of injury in distant organs remains unclear.

Questions of quantification

There are two primary methods so far employed by researchers in this growing field. The first uses *in situ* hybridization to detect Y-chromosomes that indicate derivation of hepatocytes in female animals from a male donor pool. The second employs marker transgenes such as beta-galactosidase (Lagasse et al., 2000; Wang et al., 2002), green fluorescent protein (GFP) (Wagers et al., 2002), and bcl-2 (Mallet et al., 2002). The studies that employ these different methods yield different estimates of the extent of the response, resulting in controversy.

On the one hand, in the transgender model, virtually every cell that is donor derived will certainly contain a Y chromosome. Thus, identification of the donor-derived hepatocytes will not be subject to variations in gene expression. On the downside, it is difficult in tissue sections to completely exclude the possibility of some Y-chromosomes from neighboring, non-hepatocyte cells are appearing to be within the overlying hepatocyte. Analysis of thin tissue sec-

tions (3 microns or less), confocal microscopy, and examination of cell isolates should avoid this pitfall.

On the other hand, dependence on marker transgenes may underestimate the number of engrafted cells. For example, in the beta-galactosidase transgenic ROSA mice, the protein is located downstream from a 'ubiquitous' promoter, so that it should theoretically be present in all cells. However, in our own experiments, stainable beta-galactosidase (either by histochemistry or immunohistochemistry) was present in a minority of splenic mononuclear cells, despite >98% engraftment of the spleen from the male ROSA donor, as determined by Y chromosome. Similarly, in the experiments by Wagers et al, the authors did not rigorously show that the GFP expression was truly 'ubiquitous', either in the original donor mice they created or in transplanted populations of cells (Wagers et al., 2002).

If one instead tries to use marker transgenes under an hepatocyte specific promoter, one should theoretically achieve higher consistency of transgene expression. However, at any given time, hepatocytes do not uniformly produce the 'usual' hepatocyte specific markers: not all hepatocytes produce albumin or alpha-1-antitrypsin, for example. Better reliability may be found by using promoters for proteins truly present in all hepatocytes, such as biliary glycoprotein-1 or DPPIV (canalicular antigens) or cytoskeletal elements such as cytokeratins 8 or 18.

Conclusion

We are at the beginning of a new era in understanding of liver regeneration. One main avenue of investigation will explore the physiological pathways by which liver tissue is maintained or repaired after injury. The other avenue will involve elucidation of the technical aspects of therapeutic interventions, from which cells can be employed, to methods of cell application (transplantation, construction of artificial organs, growth of new organs, targets for gene therapy). Undoubtedly, the latter avenue will exploit details elucidated in the former; however, there will certainly also be creative use of non-physiological possibilities. In this latter category we may consider hepatic progenitor sources such as cord blood or embryonic stem cells.

Ultimately, therapeutic success will be achieved when the combinations of techniques yield efficient and cost-effective production and expansion of cells *in vitro*, or of the cytokine/chemokine elements that

could stimulate our own bodies to accomplish therapeutic outcomes. On the other hand, despite the occasional ineffectiveness of the system – such as in fulminant hepatic failure – the majority of us have livers that function steadily, without significant defect, over a lifetime and which recover from even severe insults. Therapeutics will focus on rescuing the comparatively rare failures of this system, but the rest of us will rely on the rather wondrous ability of the body to keep our liver and us alive in that long period stretching from birth to death (from non-hepatic causes).

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