Hepatocytes isolated from neoplastic liver-immunomagnetic purging as a new source for transplantation

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Abstract

AIM: To investigate whether hepatocytes isolated from macroscopically normal liver during hepatic resection for neoplasia could provide a novel source of healthy hepatocytes, including the development of reliable protocols for malignant cells removal from the hepatocyte preparation.

METHODS: Hepatocytes were procured from resected liver of 18 patients with liver tumors using optimised digestion and cell-enrichment protocols. Suspensions of various known quantities of the HT-29 tumor cell line and patient hepatocytes were treated or not with Ep-CAM-antibody-coated immunomagnetic beads in order to investigate the efficacy of tumor-purging by immunomagnetic depletion, using a semi-quantitative RT-PCR method developed to detect tumor cells. Immunomagnetic bead-treated or bead-untreated tumor cell-hepatocyte suspensions were transplanted intra-peritoneally in Balb/C nude mice to assess the rates of tumor development.

RESULTS: Mean viable hepatocyte yield was 9.3 x 10^5 cells per gram of digested liver with mean viability of 70.5%. Immunomagnetic depletion removed tumor cells to below the RT-PCR detection-threshold of 1 tumor cell in 10^6 hepatocytes, representing a maximum tumor purging efficacy of greater than 400,000-fold. Transplanted, immunomagnetic bead-purged tumor cell-hepatocyte suspensions did not form peritoneal tumors in Balb/C nude mice. Co-transplantation of hepatocytes with tumor cells did not increase tumorigenesis of the tumor cells.

CONCLUSION: Immunomagnetic depletion appears to be an effective method of purging contaminating tumor cells to below threshold for likely tumorigenesis. Along with improved techniques for isolation of large numbers of viable hepatocytes, normal liver resected for neoplasia has potential as another clinically useful source of hepatocytes for transplantation.

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Key words: Hepatocyte transplantation; Immunomagnetic purging; Isolation of hepatocytes

INTRODUCTION

While advances in supportive medical care have improved survival, liver failure in its most severe form continues to carry a high mortality rate unless orthotopic liver transplantation (OLT) is performed. Nonetheless, there is a limit to the number of patients that can be treated in this way. The rapidity with which the clinical syndrome often progresses and a worldwide shortage of donor organs are significant limiting factors and many patients listed for OLT die or develop contraindications to transplantation before a donor liver becomes available. Further, the presence of co-morbidities in a substantial number of patients often precludes listing for OLT altogether. Consequently, there is considerable ongoing interest in the provision of other means of liver support, including extracorporeal artificial or bioartificial devices and hepatocyte transplantation (HT).

The feasibility of HT as a therapeutic tool has been demonstrated in studies performed in animals with liver-
based metabolic defects such as analbuminaemic and Gunn rats, hypercholesterolaemic rabbits and dogs with impaired purine metabolism. Experience with HT in experimental animals with liver failure due to chemically-induced hepatic necrosis, surgical models of hepatic ischaemia or resection has also been favourable, with evidence of improved survival even when small numbers of cells (0.5% to 3% of normal hepatocyte mass) are used.

Transplantation of human hepatocytes has, to date, been performed in only a small number of paediatric and adult patients with liver failure. Instances of mostly short-term improvement in encephalopathy and some metabolic parameters have been recorded both in OLT and non-OLT candidates. Potential advantages of HT over OLT include its minimal invasiveness, ease of treatment, substantially lower cost and the possibility of on-demand use following the establishment of a cell bank. Limited availability of primary human hepatocytes for transplantation however, represents a major limitation. Currently, the standard approach is to isolate hepatocytes from livers rejected for liver transplantation due to excessive steatosis, cirrhosis and prolonged ischemia. However, availability of hepatocytes from this source is in increasingly short supply and concerns with the functional capability of such cells have been raised.

Our group recently reported the feasibility of harvesting tumor-free hepatocytes from macroscopically normal liver unavoidably removed during hepatic resection for malignancy. Here we report further improvements in our hepatocyte isolation and tumor-purging techniques, resulting in the harvest of sufficiently large numbers of viable hepatocytes from resected liver specimens to offer the prospect of effective clinical support and a high degree of tumour-purging efficacy. Furthermore, we demonstrate the safety of transplantation of hepatocytes harvested in this way, in terms of lack of complicating tumor development in an athymic mouse model.

**MATERIALS AND METHODS**

**Hepatocyte isolation and cryopreservation**

The human isolation study was approved by the South East Sydney Area Health Service Ethics Committee (approval No. 01/123). Hepatocytes were harvested from liver resection specimens of consenting patients undergoing partial hepatectomy for neoplasia. Patients with hepatocellular carcinoma were excluded. Patient demographic and disease details are outlined (Table 1). The bulk of specimens (61%) arose from livers with colorectal metastases.

Following resection, the specimen was transferred to a sterile table where the diseased portion of the liver was dissected with a 1cm margin, leaving the remaining macroscopically normal liver for hepatocyte harvest. The tissue was placed immediately into a sterile cooling solution (< 4°C) and 2-4 of the largest vessels were cannulated. Warm ischemic time, defined as clamping time of hepatic inflow and/or outflow during resection plus specimen processing time before being cooled, was recorded. Cannulae ranging from 0.95 mm to 2 mm diameter were suture-ligated around the respective vessels and capped with one-way valved bungs to prevent backflow. Histoacryl (Braun, Germany) was used to seal most of the remaining liver surface, in addition to the cannula entry points. This was done to prevent leakage of perfusate and to maximize perfusion of the microcirculation during isolation, having been previously shown to increase hepatocyte yield and viability. The specimen was then flushed with 500-1000 mL of heparinized (5000 units per litre) Custodiol™ histidine-tryptophan-ketoglutarate (HTK) solution (Kohler Chemie, Germany) to prevent obstruction of the hepatic microcirculation by thrombus and facilitate organ preservation. The specimen was then transported under sterile conditions to the laboratory where it was re-warmed and digested by a modified version of Seglen's original 2-step technique. Cold ischemic time, defined as time that the specimen had been on ice prior to re-warming, was recorded. Pre-warmed (37°C) buffers were perfused in the following order: (1) Wash buffer-HBSS without Ca²⁺/Mg²⁺ (Gibco, Auckland, New Zealand) + 208.1 mg/L EDTA (Sigma, St Louis, MO, USA) + ascorbic acid 50 mg/L (Sigma, St Louis, MO, USA) + bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) 0.5% w/v-10 min perfusion and then discarded. (2) EDTA washout-HBSS + BSA 0.5% w/v; 5 min perfusion and then discarded. (3) Digestion buffer-HBSS + 0.05% w/v Collagenase P (Roche, Germany) + 0.5% w/v BSA-re-circulated for 20-30 min.

Perfusion was carried out manually due to the varying size of the specimens (range, 55-690 g) at 20-40 mL/min per cannula depending on the weight of the specimen and cannula size. Digestion buffer was perfused until the specimen was soft and friable. A cell suspension was obtained by gentle mechanical dissociation of the digested specimen in 500 mL of ice-cold suspension buffer DMEM (Gibco, Auckland, New Zealand) + 10% (v/v) FCS (Gibco, Auckland, New Zealand) +

| Parameters                      | n     |
|---------------------------------|-------|
| Age (yr; mean ± SD, range)      | 59 ± 11 (33-78) |
| Male                            | 10    |
| Female                          | 8     |
| Primary tumor                   |       |
| Colorectal cancer               | 12    |
| Benign liver lesions            | 2     |
| Renal carcinomas                | 1     |
| Prostate carcinoma              | 1     |
| Cholangiocarcinoma              | 1     |
| Pseudopapillary pancreas tumor  | 1     |
| Operation                       |       |
| Right hemi-hepatectomy          | 7     |
| Left hemi-hepatectomy           | 4     |
| Extended right hemi-hepatectomy | 2     |
| Right lateral sectorectomy      | 2     |
| Extended left hemi-hepatectomy  | 1     |
| Left lateral sectorectomy       | 1     |
| Non-anatomic resection          | 1     |

**Table 1  Patient demographic, clinical and operative details**

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1% antibiotic-antimycotic Penicillin 10000 units/mL + streptomycin 25µg/mL + amphotericin B as Fungizone® (Gibco, Auckland, New Zealand) and sequentially filtered through three sterile stainless steel filters of decreasing pore size (425 µm, 150 µm and 75 µm) (Endecotts, UK). The raw fraction was pelleted by centrifugation at 50 g for 3 min at 4°C and washed three times in the suspension buffer. Cells were counted in quadruplicate and viability assessed by Trypan blue exclusion, using a hemocytometer (Neubauer, Germany). Cells were cryopreserved in suspension medium containing 10% DMSO in 5-mL tubes by freezing to -80°C in 1°C decrements per minute and then transferred to liquid nitrogen after 24 h.

**Hepatocyte spiking & purging**

*In vitro* and *in vivo* studies were performed using suspensions of isolated human hepatocytes spiked with various numbers of HT-29 human colorectal cell-line tumor cells and then treated or not with immunomagnetic beads (CELLlection™ Epithelial Enrich; Dynal AS, Oslo, Norway), in the method described by Kielhorn *et al.*[18] and Flatmark *et al.*[9] Specifically, we used 4.5 µm magnetic beads coated with mouse IgG1 Ber-Ep4 antibody against Ep-CAM, an antigen highly expressed on colorectal cancer (CRC) cells (including HT-29 tumor cells), but not on mature hepatocytes.[20]

*In vitro hepatocyte and HT-29 cell-line preparation*

Cryopreserved human hepatocytes were thawed in a 37°C water bath, diluted in suspension medium, pelleted by centrifuging at 50 g for 3 min at 4°C and re-suspended in suspension medium. Density gradient centrifugation was performed to purify the thawed hepatocytes. The suspension was then mixed with a Percoll™ (Amersham Biosciences, Uppsala, Sweden) density gradient (25% final concentration) medium and centrifuged at 75 g for 5 min at 4°C to separate viable from dead hepatocytes. Pellets were re-suspended in PBS with 0.1% BSA and placed on ice. Cells were counted in quadruplicate, using a hemocytometer (Neubauer, Germany), and their viability assessed by Trypan blue exclusion. HT-29 cells grown in 75 cm² culture flasks (Greiner Bio-One, Germany) in 95% O₂ and 5% CO₂ at 37°C were trypsinised, washed in PBS (Invitrogen, Auckland, New Zealand), pelleted by centrifugation at 75 g for 5 min, re-suspended in PBS and counted as above.

**Determination of RT-PCR sensitivity for tumor cell detection**

One, 10, 50, 100, 1000, 5000 or 10 000 HT-29 cells were added to suspensions of 1 million human hepatocytes to establish the lower limit of detection of tumor cells by RT-PCR. Suspensions of 1 million HT-29 cells alone and 1 million hepatocytes alone served as positive and negative controls respectively.

Total RNA was extracted using TRIzol® reagent (15596026, Invitrogen) as per the manufacturer’s instructions. Following DNase I treatment (18068-015, Invitrogen), the total RNA concentration and quantity was assessed by spectrophotometry at 260 nm and the RNA stored at -80°C.

**RT-PCR**

cDNA was synthesized from total RNA using One-Step SuperScript III™ system (12574-026, Invitrogen) with target specific primers as per the manufacturer’s instructions. EpCAM primers were as published by Sakaguchi *et al.*[21] with actin housekeeping primers as follows; antisense 5’-GGAGCAATGATCTTGACCTT -3’; sense 5’-CTTCTGGGGATGAGTCCT-3’. The RT-PCR program was as follows; cycle one, 56°C, 30 min, 94°C for 3 min, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, with a final extension for 10 min at 72°C. The cDNA products were visualized on a 1% agarose gel, and sequenced to confirm product identity.

**Immunomagnetic bead treatment of spiked hepatocytes**

Five mL suspensions of 1 × 10⁶ hepatocytes spiked with 1000, 10 000 and 50 000 HT-29 cells per mL were prepared in duplicate. Immunomagnetic beads (CELLlection™ Epithelial Enrich; Dynal AS; 4 × 10⁶ beads/mL), were washed in PBS + 0.1% (w/v) BSA, and added to half the tubes in the ratio of 20 beads to 1 HT-29 cell. The remaining preparations constituted controls and contained no immunomagnetic beads. All tubes were placed in a rotator (15 r/min) at 4°C for 30 min, to allow maximal tumor cell-bead contact and capture. Treated tubes were placed in the magnetic particle concentrator provided by the manufacturer for 2 min, the supernatant transferred to new tubes and the process repeated. One mL from each sample was collected after treatment for RT-PCR analysis to assess the efficacy of immunomagnetic bead-mediated tumor-purging.

**In vivo study**

Male Balb/C athymic nude mice (Animal Resource Centre, Perth, WA, Australia) were housed and fed under specific pathogen-free conditions according to study protocols approved by the Animal Care & Ethics Committee of UNSW (approval No. 02/103). The athymic mouse was chosen due to its minimal cellular immunity, so as to minimize risk of rejection of human hepatocytes and facilitate tumor engraftment. The main aims were to study (a) the tumor load required for tumorigenesis following intraperitoneal transplantation, (b) the effect of co-transplantation of human hepatocytes on tumorigenesis and (c) the effect of our immunomagnetic purging protocol on tumorigenesis. The experimental protocol is described below in Table 2.

Hepatocytes and HT-29 cells were prepared as per the *in vitro* arm, separately and in combination to produce suspensions containing the cell numbers required per mL PBS (cf Table 2). Two hundred µL samples, containing 20% of the cell number in each 1 mL inoculation, were collected for negative control,
positive control and immunomagnetic bead groups, before and after purging for RT-PCR analysis.

Mice were monitored for 28 d post-transplantation after which they were sacrificed with a lethal 6 mg dose of pentobarbital sodium. The abdomen, pelvis and thorax were examined visually for the presence of tumor.

RESULTS

Isolation of hepatocytes

The total viable hepatocyte yield averaged at $9.3 \times 10^8$ cells per isolation (range $2.0 \times 10^{2-36.5} \times 10^8$; mean ± SD viable hepatocyte yield $9.33 \times 10^8 \pm 6.0 \times 10^6$ cells/g digested liver tissue), with the five most recent isolations each yielding over $1.0 \times 10^9$ cells. The mean viability of freshly isolated hepatocytes was $70.5\% \pm 8.1\%$. Mean warm ischemic time was $31 \pm 19$ min (range, 25-60 min); mean cold ischemic time was $1.5 \pm 0.6$ h (range, 0.5-16 h).

Tumorigenesis of transplanted hepatocyte and tumor cell suspensions

RT-PCR analysis demonstrated clear single bands at the 515 bp position, indicating Ep-CAM detection, in representative samples of all hepatocyte/HT-29 cell suspensions transplanted into mice belonging to Positive Control 2 and pre-treatment Bead groups. No detectable bands were seen in the samples transplanted into the Negative Control and post-treatment Bead groups (gel images not shown), indicating in the latter case the removal of Ep-CAM positive cells (including HT-29 cells) to below the detection limit of 1 tumor cell in 1 million hepatocytes (Figure 1) and thus, a maximum tumor purging efficacy of immunomagnetic bead treatment of at least 400,000 fold (Figure 2).

In the control groups, all mice injected with 100,000 HT-29 cells and below showed no tumor, except for one animal inoculated with 100,000 HT-29 cells only. The mouse developed a small (0.1 g) skin nodule at the injection site and had no evidence of intra-abdominal tumor on detailed examination. This is probably due to an inadvertent subcutaneous rather than intraperitoneal injection of cells, and could thus be excluded on the basis of technical error. All except two mice inoculated with at least 500,000 HT-29 cells (83%) developed tumor. There was no significant difference in tumor expression between mice injected with or without hepatocytes (Tables 2 and 3). These results would suggest that the minimum tumor load required for engraftment and growth was between 100,000 and 500,000.

There was a complete absence of tumor development in any mouse injected with HT-29 cell-spiked hepatocyte

### Table 2 Mice & cell transplantation details

| Treatment groups                  | Mouse group | IP injection             | No. of mice |
|-----------------------------------|-------------|--------------------------|-------------|
| Negative control (Hepatocyte only)| 1           | 5 x 10^6 hepatocytes     | 3           |
| Positive control 1 (HT-29 only)   | 2           | 5000 HT-29 cells         | 3           |
|                                   | 3           | 100,000 HT-29 cells      | 3           |
|                                   | 4           | 500,000 HT-29 cells      | 3           |
|                                   | 5           | 2 million HT-29 cells    | 3           |
| Positive control 2 (Hepatocytes + HT-29) | 6     | 5 million hepatocytes + 5000 HT-29 | 3       |
|                                   | 7           | 5 million hepatocytes + 100,000 HT-29 | 3       |
|                                   | 8           | 5 million hepatocytes + 500,000 HT-29 | 3       |
|                                   | 9           | 5 million hepatocytes + 2 million HT-29 | 3     |
| Bead (Hepatocyte + HT-29)         | 10          | 5 million hepatocytes + 5000 HT-29 | 3       |
|                                   | 11          | 5 million hepatocytes + 100,000 HT-29 | 3       |
|                                   | 12          | 5 million hepatocytes + 500,000 HT-29 | 3       |
|                                   | 13          | 5 million hepatocytes + 2 million HT-29 | 3     |

### Table 3 Tumor growth

| Treatment groups                  | IP injection             | Mouse with tumor | Percentage expression (%) |
|-----------------------------------|--------------------------|------------------|---------------------------|
| Negative control (Hepatocyte only)| 5 x 10^6 hepatocytes     | 0/3              | 0                         |
| Positive control 1 (HT-29 only)   | 5000 HT-29 cells         | 0/3              | 0                         |
|                                   | 100,000 HT-29 cells      | 1/3              | 0°                        |
|                                   | 500,000 HT-29 cells      | 2/3              | 67                        |
| Positive control 2 (Hepatocytes + HT-29) | 2 million HT-29 cells    | 3/3              | 100                       |
|                                   | 5 million hepatocytes + 5000 HT-29 | 0/3              | 0                         |
|                                   | 5 million hepatocytes + 100,000 HT-29 | 0/3              | 0                         |
|                                   | 5 million hepatocytes + 500,000 HT-29 | 3/3              | 100                       |
|                                   | 5 million hepatocytes + 2 million HT-29 | 2/3              | 67                        |
| Bead (Hepatocyte + HT-29)         | 5 million hepatocytes + 5000 HT-29 | 0/3              | 0                         |
|                                   | 5 million hepatocytes + 100,000 HT-29 | 0/3              | 0                         |
|                                   | 5 million hepatocytes + 500,000 HT-29 | 0/3              | 0                         |
|                                   | 5 million hepatocytes + 2 million HT-29 | 0/3              | 0                         |

1Injection-site tumor in skin only; In italics: Groups in which tumor was found; In standard form: Tumor-free.
To our knowledge, our centre is the first to propose immunomagnetic purging of hepatocytes isolated from liver resections performed annually in our unit, of which approximately half will be suitable for hepatocyte isolation. Thus, a minimum of forty hepatocyte transplants could potentially be performed annually.

Our mean viable yield of $9.33 \times 10^6 / g$ is a significant improvement from our preliminary study$^{[22]}$ and marginally exceeds that reported by Richert et al$^{[23]}$. To achieve this, the original isolation protocol was altered to include sealing of cannula points and cut surfaces prior to perfusion and ensure a cold ischemic time of less than 5 h; both of these changes have been independently identified to positively influence viable yield$^{[22,23]}$.

The number of hepatocytes transplanted in clinical experiences to date has ranged between $2 \times 10^5$ to $4 \times 10^5$ cells$^{[24]}$. Given a mean viable yield of $9.3 \times 10^6$ cells per isolation, our technique offers the possibility of one hepatocyte transplant for every hepatocyte isolation. In the order of 80-100 liver resections are performed annually in our unit, of which approximately half will be suitable for hepatocyte isolation. Further, a minimum of forty hepatocyte transplants could potentially be performed annually.

A comparison of viable hepatocytes yield from resection specimens versus cells obtained from explanted organs rejected for OLT shows that the resected specimens have a consistently higher viable yield$^{[22]}$. Further, recovery from cryopreservation of hepatocytes derived from normal resected liver is significantly higher compared to that of cells obtained from organs rejected for liver transplantation$^{[25]}$, thus improving the potential quality and quantity of bankable cells for use on-demand. Such factors, combined with the opportunity to utilize a hitherto untapped hepatocyte source, further enhance the potential application of hepatocyte transplantation as a clinically relevant treatment modality.

An important concern regarding the use of hepatocytes isolated from liver resections performed for malignancy has been the possibility of co-transplanting contaminating tumor cells. Although immunomagnetic cell separation has been widely utilized to enhance detection of tumor cells in different body compartments, purging has been mainly limited to ex vivo removal of tumor cells from autologous stem cell transplants$^{[18,19,26,27]}$. To our knowledge, our centre is the first to propose immunomagnetic purging of any residual, contaminating tumor cells from isolated hepatocyte suspensions.

The Ep-CAM cell-surface antigen is consistently present on both HT-29 colorectal cancer cells and most colorectal metastases$^{[28]}$, the pathology in the majority of our liver resection specimens in this study. The antigen is not expressed by mature hepatocytes$^{[29]}$ making differential separation of Ep-CAM-expressing tumor cells by immunomagnetic beads (coated with Ber-EP4) possible. Various other carcinomas, including all the types from our patient cohort, also express Ep-CAM$^{[29]}$. As Ep-CAM has been shown consistently to be absent in hepatocellular carcinoma, patients with such tumors, along with other Ep-CAM-negative lesions, were excluded from our study to avoid undetectable tumor contamination. Whilst hepatocytes were harvested only from patients whose tumors expressed Ep-CAM in this situation the viable hepatocyte yield would be less.

FIGURE 1
 Detection sensitivity. RT-PCR detection of HT-29 cells in $1 \times 10^6$ hepatocytes. Lane M: 100 bp ladder (100 bp to 1 kb); Lane β: β-actin; Lane 1: 100 HT-29 cells; Lane 2: 10 HT-29 cells; Lane 3: 5 HT-29 cells; Lane 4: 1 HT-29 cell; Lane 5: Hepatocytes only; Lane 6: HT29 only.

FIGURE 2
 Tumor-purging efficacy. RT-PCR detection of EpCAM RNA in HT-29-cell-spiked hepatocytes ($1 \times 10^6$) with and without treatment with Ber-EP4-coated immunomagnetic beads. Lane M: 100 bp ladder (100 bp to 1 kb); Lane β: β-actin; Lane 1: 50,000 HT-29 cells; Lane 2: 50,000 HT-29 cells treated with immunomagnetic beads; Lane 3: 10,000 HT-29 cells; Lane 4: 10,000 HT-29 cells treated with immunomagnetic beads; Lane 5: 10 HT-29 cells treated with immunomagnetic beads; Lane 6: 10 HT-29 cells only.

DISCUSSION

Our results show that clinically relevant numbers of hepatocytes can be recovered from macroscopically normal liver unavoidably removed during hepatic resection for neoplasia. Further, when isolated and subjected to an immunomagnetic cell separation technique, the resulting hepatocytes can be safely transplanted intra-peritoneally in athymic mice without increased risk of development of tumor. Only patients requiring anatomical resection were included in this study. Our technique may be less useful in the case of patients undergoing small, non-anatomical resection; in this situation the viable hepatocyte yield would be less.

Immunomagnetic purging of hepatocytes

Figure 1 Detection sensitivity. RT-PCR detection of HT-29 cells in $1 \times 10^6$ hepatocytes. Lane M: 100 bp ladder (100 bp to 1 kb); Lane β: β-actin; Lane 1: 100 HT-29 cells; Lane 2: 10 HT-29 cells; Lane 3: 5 HT-29 cells; Lane 4: 1 HT-29 cell; Lane 5: Hepatocytes only; Lane 6: HT29 only.

Figure 2 Tumor-purging efficacy. RT-PCR detection of EpCAM RNA in HT-29-cell-spiked hepatocytes ($1 \times 10^6$) with and without treatment with Ber-EP4-coated immunomagnetic beads. Lane M: 100 bp ladder (100 bp to 1 kb); Lane β: β-actin; Lane 1: 50,000 HT-29 cells; Lane 2: 50,000 HT-29 cells treated with immunomagnetic beads; Lane 3: 10,000 HT-29 cells; Lane 4: 10,000 HT-29 cells treated with immunomagnetic beads; Lane 5: 10 HT-29 cells treated with immunomagnetic beads; Lane 6: 10 HT-29 cells only.

suspensions treated with immunomagnetic beads. All mice survived until day 28 without any significant weight loss/gain or signs of malaise or distress. Where intraperitoneal tumors occurred, these were seen only intra-abdominally, expressed as distinct, scirrhous, white nodules adherent to the parietal or visceral peritoneum, without evidence of metastasis.
study, the targeting of additional molecular markers such as CK-20 and CEA enhanced detection of any potential tumour cells without an Ep-CAM phenotype\[^{30,31}\]. Additional surface antigens are currently being studied to potentially increase the tumor-purging efficacy by multiplying the number of target molecules per cell.

In our study, immunomagnetic purging was shown to remove tumor cells by a factor greater than 400,000. This compares favorably with similar large-scale experiments involving breast cancer cells in blood stem cell harvests\[^{32}\]. It also represents a substantial improvement on our preliminary experience\[^{13}\], attributable to optimization of the sample purging treatment and bead to cell ratio employed. The development of an RT-PCR detection assay, in addition to standard immunohistochemical methods, has enabled a more efficient and sensitive detection of tumor contamination (1 tumor cell per 1 million hepatocytes) enabling confidence that in a typical human hepatocyte transplant of 1 billion cells, a maximum tumor load of no more than 1000 cells could be present in the preparation. This is significantly below the 100,000–500,000 cell threshold for tumor engraftment and growth demonstrated in our athymic mouse model in this study. Further, no additional growth potential was conferred to tumor cells by the co-transplantation of hepatocytes, an important observation that indicates that the presence of hepatocytes does not magnify the risk of tumor cell engraftment.

We have demonstrated that with the use of an optimized cell-isolation protocol, liver resection specimens obtained from patients undergoing resection for neoplasia can offer sufficient viable hepatocytes to potentially provide clinically-relevant liver support. We have further shown both in vitro and in a suitable in vivo animal model that immunomagnetic purging can confer safety from the potential of tumor contamination of hepatocyte suspensions. We therefore propose that liver resection specimens, by a simple purging step, may provide a safe, alternative hepatocyte source for clinical transplantation.

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