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Technical note

Tracking the kinetics of intrahepatic immune responses by repeated fine needle aspiration of the liver

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ABSTRACT

Liver disease is an increasing global health burden. The final sequalae of cirrhosis, liver failure and hepatocellular carcinoma are often the result of inflammation driven by intrahepatic lymphocytes. Accurate assessment of organ-specific diseases ideally employs tissue sampling though this is rarely performed. Here we report our experiences of utilising repeated fine needle aspirations (FNAs) to assess liver-derived leukocytes. In 88 patient samples, we obtained a mean of 36,959 lymphocytes from each FNA-derived biopsy (SD 22,319 cells, range 5034–91,242 cells) measured by flow cytometry. This quick technique required minimal analgesia compared to liver biopsy ($p = 0.03$); was well tolerated and safe, and hence repeated sampling up to 3 times within a week was feasible. We detail the technique to rapidly derive a single cell suspension suitable for multiparameter flow cytometry analysis. Finally we illustrate the importance of organ-derived sampling by showing that natural killer (NK) cells from FNA samples have a markedly altered phenotype compared to those assessed in peripheral blood. In combination these data validate FNA as a powerful and well-tolerated method of sampling intrahepatic lymphocytes to study the immunology of acute and chronic liver diseases.

1. Introduction

Inflammatory liver diseases, including chronic viral hepatitis, autoimmune hepatitis and steatohepatitis, represent a major global health burden. Lymphocytes are often stated to be the drivers of these clinical disorders, but direct evidence is often lacking. It is well recognised that, although easy to sample, peripheral blood lymphocytes may not reflect the function and phenotype of cells within the liver. Sampling cells from within the liver has remained problematic. Liver biopsy, using 16–18 gauge sheathed (Tru Cut) needles or suction (Menghini) needles, remains the clinical gold standard to assess liver fibrosis and inflammation and can provide valuable diagnostic information. However, the invasive nature of the liver biopsy, coupled with significant morbidity (and very rarely mortality) has driven interest in development of biochemical and non-invasive markers (i.e. liver elastography) to assess chronic liver disease (Castera, 2012). Percutaneous liver biopsy has a mortality rate of 1 in 10,000 and the most common complication is abdominal or right shoulder pain in up to 25% of patients (Bravo et al., 2001). Whilst the parenchyma of the liver has a relatively paucity of nerve fibres the liver capsule is well innervated and together with the skin requires infiltration with local anaesthetic; in spite of this some patients require additional analgesia. Other rare (<1%) risks associated with liver biopsy include bleeding, pneumothorax, perforation and peritonitis (Bravo et al., 2001).

To gain a sufficient number of lymphocytes for analysis, it has been necessary to obtain a separate biopsy core designated for research purposes. The risk of complications from liver biopsy obviously increases with the number of passes of the needle into the liver. This separate core requires physical and enzymatic degradation to isolate lymphocytes. The clinical risks associated with liver biopsy prevent the use of this technique to monitor lymphocytes at frequent intervals within the same individual (Ahlenstiel et al., 2011). During interferon-based treatment of viral hepatitis, we and others have found that the largest reductions in viral load take place during the first week, thus it would be useful to closely monitor intrahepatic changes during this period of marked perturbation (Davis et al., 2003; Pembroke et al., 2012).

These limitations to the use of standard liver biopsy techniques led us to explore the use of Fine Needle Aspiration (FNA) to obtain samples. FNA needles rely on aspiration of a clinical sample via a small-bore needle (usually 23 gauge). This is a clinical technique that provides single cell cytology samples and as such is unable to provide histological information. However, it is reliable, relatively safe and often provides the required diagnostic information. FNA has been used to obtain lymphocytes from organs to monitor disease (Oliveira et al., 1997; Claassen et al., 2011).

Aspiration of tissue from within a solid organ, including from within the liver capsule, will inevitably contain a mixture of blood cells and cellular debris from the stroma. A hurdle to the use of FNA as a technique to sample intrahepatic lymphocytes is the need to remove

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sampling debris to enable effective labelling of lymphocytes and analysis by flow cytometry. In this report we discuss our experiences of performing 88 liver FNAs to sample intrahepatic lymphocytes, including repeat FNA sampling on a subset of 9 patients. We discuss the practicality of this approach with respect to patients' tolerance, the procedure itself and sample processing. We also highlight comparative datasets obtained from intrahepatic and blood lymphocytes derived from the same individuals.

2. Methods

2.1. Ethics statement

South East Wales Local Research Ethics Committee reviewed this project (10/WSE02/45). Patients who participated were given a study information sheet and provided informed written consent.

2.2. Study subjects

Fifty-three consecutive patients attending the hepatology department for outpatient liver biopsy or IFNα treatment of HCV were recruited. Liver biopsy was performed on 49 individuals as part of their routine workup whereas FNA alone was conducted on 4. Nine patients undergoing IFNα treatment were sampled by FNA a combined total of 35 times at days 1, 3, 7, and 14 & months 1, 3 and 6 following the initiation of treatment (Table 1).

FNA samples were taken in a day case treatment room in the University Hospital of Wales. Prior to the FNA, all patients underwent a recent ultrasound scan and α-fetoprotein (to exclude abnormal anatomy and intrahepatic lesions) and routine clinical bloods (full blood count, liver biochemistry, coagulation screen) were assessed. Patients were considered eligible for the procedure if platelets were >60, 000/μl and prothrombin time <14.5 s in keeping with local liver biopsy guidelines. Patients were seen on an outpatient basis with a view to discharge following the procedure. Clinicians experienced in biopsying the liver undertook this procedure. Written informed consent was obtained—the risks we described relating to the FNA procedure are as follows:

- Common: approximately 10–25% of procedures:
  - Bruising and soreness
- Serious complications: < than 1 in every 10,000 procedures:
  - Bleeding requiring blood transfusion
  - Damage to organs surrounding the liver—including the lung, bowel and gallbladder
  - Peritonitis
  - Pneumothorax

Table 1
FNA donor characteristics.

Characteristics	Chronic liver disease	HCV
Number	29	24
Mean age (range)	50.6 (29–69)	49 (27–65)
Male:female	16:13	17:7
Median viral load (range)	–	1.3×10^6 (1.9×10^3–3×10^7)
Median NI score (range)	1 (0–6)	5 (2–8)
Median fibrosis score (range)	2 (0–6)	2 (0–2)
HCV genotype		
1	–	16
3	–	8

Separate written consent for liver biopsy was obtained in the usual manner for those undergoing both procedures.

2.3. Materials for Fine Needle Aspiration

1. Roswell Park Memorial Institute (RPMI, Gibco, Paisley UK) media supplemented with 10% foetal calf serum, 250 μl heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2 mM sodium pyruvate (R-10), stored on ice.
2. Sterile dressing pack (Rocialle, Mountain Ash UK) including gloves, gauze swabs, gallipot and sterile paper field.
3. 1% tincture of iodine solution (Videne).
4. 5 and 10 ml syringes.
5. Orange (25 gauge), Green (21 gauge) and White (19 gauge) hypodermic needles (BD Oxford UK).
6. 10 ml 2% lignocaine (Braun, Melsugen Germany).
7. 22 gauge spinal needle Quinke type point with an internal trocar (BD, Oxford UK).
8. Sterile dressing for puncture site (Premier, Enfield UK).

2.4. Fine Needle Aspiration technique

We have developed the following protocol for FNA sampling of intrahepatic lymphocytes. Standard aseptic clinical procedure is observed using a plastic disposable apron, sterile gloves and sterile disposable towels.

1. Obtain a heparinised blood sample for density gradient isolation of lymphocytes as previously described (Gallagher et al., 2009).
2. Place the patient in the supine position with the right hand behind the head. Percuss the lower edge of the liver in the right mid-axillary line and mark suitable site 1–2 intercostal spaces above the lower edge; this is usually 2 intercostal spaces above the costal margin. Confirm that the marked position for FNA sampling over the liver is dull on percussion in full expiration.
3. Clean the skin with iodine solution.
4. Draw up 5 ml 2% lignocaine into a 5 ml syringe; inject 1–2 ml into the dermis with a 25-gauge orange needle. Allow the lignocaine to take effect (30–60 s), then replace this needle with a 23-gauge green needle and continue to infiltrate 2% lignocaine through the intercostal muscles to the hepatic capsule. Occasionally a longer 21 gauge white needle is required for larger patients.

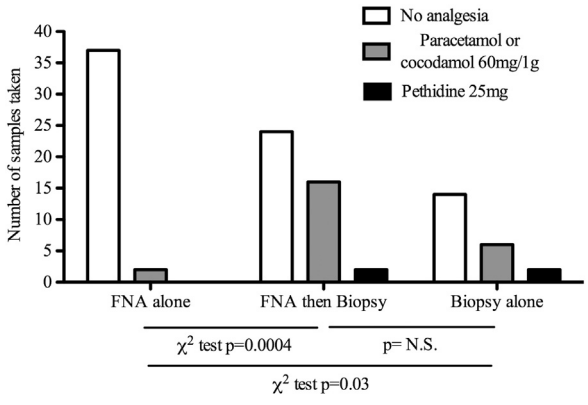

Fig. 1. Post procedure analgesia. Analgesia was offered to patients at regular intervals following FNA and liver biopsy procedures. 2 patients requested simple analgesia after FNA alone; 16 patients who underwent FNA followed by biopsy requested simple analgesia and 2 required pethidine $p = 0.0004$ χ^2 test. Of 22 patients who had biopsy alone 6 required cocodamol and 2 required pethidine (v FNA alone $p = 0.025$ χ^2 test); there was no significant difference in analgesia requirement in biopsy alone v biopsy and FNA ($p =$ ns χ^2 test).

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