STEREOLOGICAL ESTIMATION OF ITO CELLS FROM RAT LIVER USING THE OPTICAL FRACTIONATOR - A PRELIMINARY REPORT

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ABSTRACT

In the last two decades, much light has been shed on hepatic fibrosis, and the activation / proliferation of Ito cells (IC) emerged to play a central role. Therefore, it is essential to have solid quantitative data in nonpathological statuses; yet, this data is scarce and confined to "number per area" or semiquantitative information. Moreover, the supposed heterogeneous distribution of IC in the hepatic lobule was never analysed with design-based (unbiased) stereology. In the present study, the total number (N) of IC in rat liver was estimated for the first time, by combining immunocytochemistry with the optical fractionator. Quantification was extended to the hepatocytes, to disclose the IC index, an often-used ratio in hepatology. Systematic uniform random liver sections were obtained from male Wistar rats (n = 3), and immunostained against glial fibrillary acidic protein (GFAP), a known specific marker for hepatic IC. For the first time, these were marked against GFAP in thick (30 µm) paraffin sections. The estimated N of IC was 224E⁰⁶; with a coefficient of error of 0.04 or 0.06, depending on the particular equation used (based on the so-called "quadratic approximation"). The IC index was 91 IC/1000 hepatocytes. Concerning the lobular heterogeneity, it was proved the liver harbours a larger total number of periportal IC and hepatocytes.

Keywords: disector, hepatocytes, Ito cells, liver, optical fractionator.

INTRODUCTION

Ito cells (IC) are perisinusoidal liver cells, located in the space of Disse and between the hepatocytes. These cells, also termed interstitial cells, hepatic stellate cells, fat-storing cells or lipocytes exert four major functions: (1) storage and release of retinoids; (2) production and turnover of extracellular matrix; (3) regulation of blood-flow in the sinusoids; (4) production of mediators (Kawada, 1997). IC are normally in a quiescent, nonproliferative state, but these cells multiply when activated. This happens after partial hepatectomy, focal hepatic lesions, and in different conditions that lead to fibrosis (Geerts et al., 1994). In the last two decades, much light has been put into hepatic fibrosis, and IC clearly emerge as the principal fibrogenic cell in the liver (Kawada, 1997). To better evaluate both the activation and proliferation of IC, it is essential to have solid quantitative data in nonpathological conditions. In this vein, quantitative data has been gathered both in human and rat livers. However, this data is scarce in literature and confined to either "number per area" or to semiquantitative information. Moreover, the heterogeneous distribution of IC within the liver (Jungermann and Kietzmann, 1996) was never study with "design-based" stereology.

It is now recognised that the best strategy for estimating total cell numbers in microscopy is the optical fractionator (West et al., 1991). This technique combines the optical disector (Gundersen, 1986), a 3D probe that samples cells in proportion to their number, with the fractionator, in which a known fraction of the organ is sampled (systematically random). The optical fractionator does not rely on shape, size, or orientation assumptions, and is unaffected by shrinkage, thus making it ideal to use in material embedded in paraffin. However, the thick sections required by the optical disector rise problems for immunocytochemistry, often used to accurately identify the cells to be counted.

The aim of the present study was to estimate the total number (N) of GFAP-positive IC. To disclose the IC index (an often used ratio in hepatology), quantification was extended to the nucleus of hepatocytes (HEP).
MATERIALS AND METHODS

We used four-month old male Wistar rats (n = 3). The animals were deeply anaesthetised with chloral hydrate and cardiacaclly perfused for 15 min, using a peristaltic pump (Gilson). The fixative used was 10% buffered formaline. Then, the liver was removed, weighted, and sliced in 4 mm thick slices (firstly cut at a random distance from the edge). After performing the so-called smooth fractionator (Nyengaard, 1999), a mean of 10 liver fragments was obtained per animal. These were further immersed in the same fixative used for perfusion, for 16 hours, and routinely processed for paraffin embedding. Half of the fragments were sampled, and exhaustively sectioned (30 µm thick), according to fractionator rules (Gundersen, 1986). Every 20th section was then systematically sampled, and, to assure reliable adhesion, mounted on precleaned slides primed with 3-aminopropiltriethoxysilane (Marcos et al., 2001).

IC marking was achieved by immunocytochemistry against glial fibrillary acidic protein (GFAP). After deparaffinization, microwave treatment was carried out for antigen retrieval (600 W, 4 plus 4 min). After rinsing in phosphate-buffered saline (PBS), the endogenous peroxidase was blocked using 0.3% H2O2, for 30 min. A streptavidin-biotin protocol then followed (Histostain Plus, Zymed). Briefly, after rinsing in PBS, a 10% nonimmune goat serum was applied over the thick sections for 90 min, followed by an incubation with 1:3000 rabbit polyclonal antibody against GFAP (Dako), for 4 days at 4ºC. The primary antibody was either omitted or replaced by rabbit nonimmune serum (Dako).

The following controls were performed: (1) positive control, in which astrocytes were tagged by GFAP in the rat brain; (2) negative controls, in which the first antibody was omitted or replaced by rabbit nonimmune serum (Dako). The N of IC and of HEP, in the whole liver was estimated as:

$$N = \sum Q \cdot (1/ssf) \cdot (1/asf) \cdot (1/hsf)$$

(1)

where $\sum Q$ is the number of IC or HEP counted, $ssf$ is the fraction of sections sampled (in this case $ssf = 1/160$), $asf$ is the sectional area sampled ($asf = 1/935$ for IC, and $1/3730$ for HEP), and $hsf$ is the fraction of the section thickness sampled ($hsf = 0.65$). The number of IC and HEP per gram of liver ($N/g$) was also calculated.

The IC index was also calculated for each rat, by the ratio:

$$\text{IC index} = \frac{N \text{ (IC)}}{N \text{ (HEP)}} \times 1000$$

(2)
The coefficient of error (CE) of N was estimated applying two variants of the "quadratic approximation formula" (West et al., 1996; Gundersen et al., 1999). The CE estimations were compared with the observed total variance (OCV²), according to the formula (West, 1993):

\[ OCV^2 = BCV^2 + CE^2 \]  

where BCV² is the biological variance and CE² is the quadratic mean of the CE for each rat.

RESULTS

All the examined livers showed a normal histology. A consistent and reliable marking of IC was achieved with GFAP (not stained in negative controls). The IC were clearly visualised at every depth in all the sections (Fig. 1), and were distributed throughout the hepatic lobule. Most IC had clear cytoplasmic spots intermingled the GFAP staining ("reticular" pattern).

A mean of 905 disectors per rat was analysed, in which a mean of 633 IC and of 1728 HEP were counted. As to the lobular zones, 231 disectors per rat were rated pericentrally, and 166 IC and 429 HEP were counted. In periportal zones, 318 disectors were examined, thus counting 207 IC and 613 HEP. The global IC index was 91 IC/1000 HEP (CV = 0.14); no significant differences were found among different zones. The N of IC and HEP are presented in Table 1, which also includes the N/g (as it facilitates comparing animals with different liver weights), and the estimates of the CE. The mean section thickness was 31.1 µm (CV = 0.01).

Table 1. Estimations of Ito cell (IC) and hepatocyte nucleus (HEP) number per gram of liver (N/g) and of total number (N), in the whole liver, and in the pericentral and periportal zones. CE is the coefficient of error of N, estimated according to the formula used by West et al. (1996), CE₁, and to its revised version by Gundersen et al. (1999), CE₂. Data are expressed as Mean (Coefficient of Variation).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N/g</th>
<th>N</th>
<th>CE₁</th>
<th>CE₂</th>
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</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>IC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole liver**</td>
<td>15 E⁰⁶ (0.03)</td>
<td>224 E⁰⁶ (0.09)</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Pericentral</td>
<td>4 E⁰⁶ (0.03)</td>
<td>59 E⁰⁶ (0.06)</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>Periportal</td>
<td>5 E⁰⁶ (0.07)*</td>
<td>72 E⁰⁶ (0.09)*</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>HEP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole liver**</td>
<td>169 E⁰⁶ (0.13)</td>
<td>2511 E⁰⁶ (0.24)</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Pericentral</td>
<td>41 E⁰⁶ (0.16)</td>
<td>617 E⁰⁶ (0.27)</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Periportal</td>
<td>59 E⁰⁶ (0.06)*</td>
<td>878 E⁰⁶ (0.16)*</td>
<td>0.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Indicate significant differences between pericentral and periportal zones (t-test, p < 0.05).
** Includes besides the pericentral and periportal, other unclassifiable zones.

Fig. 1. Image from a 30 µm thick section. Two GFAP-marked Ito cells (IC) stand out among hepatocytes and their roundish nuclei. IC display their stellate morphology. The clear vertical slit is a sinusoid. ×1,250.

For estimating the N of IC and of HEP, and according to equation (3), the sampling procedure was responsible for 46% and 4% of total variance, respectively (i.e., the biological variance was more important in both cases). For the IC, in pericentral and periportal zones, the sampling procedure was responsible for 100% and 84% of total variance, respectively. For the HEP, in the same zones, sampling contributed to 5% and 17% of the respective total variance.
DISCUSSION

Ito cells ("Sternzellen") were described over a century ago, but this is the first time that an unbiased stereological tool, combined with immunocytochemistry, is applied to these cells. The immunocytochemical approach here used was mandatory, as IC cannot be distinguished in routine sections (Hall and Rojko, 1996). We tagged IC with GFAP, an intermediate filament (IF) protein recognised as a specific marker for these hepatic cells (Neubauer et al., 1996). GFAP allows a more precise identification/counting of the densely packed hepatocyte nuclei. Moreover, recognition of the irregular IC profiles was facilitated, and counting the densely packed hepatocyte nuclei was made easy. The potential bias from lost caps was avoided by having upper and lower guarding zones within our thick sections, despite their use is still a matter of debate (Hatton and Von Bartheld, 1999).

Although introduced decades ago, the IC index was here estimated for the first time using a stereological approach. Our data (91 IC/1000 HEP) was inferior to previous reports, using 2D biased approaches, namely by Blouin et al. (1977), 130, and by Azaïs Braesco et al. (1997), 109. It is noteworthy that the index was relatively constant in different zones of the lobule, reinforcing the functional and structural relations between IC and hepatocytes, and emphasising the relevance of this index in hepatology.

Concerning the hepatocytes, they were counted in this and in previous studies as if all cells were "mononucleated hepatocytes" (Weibel et al., 1969). However binucleated cells are frequent in rat liver; according to an unbiased study by Jack et al. (1990), 30% of the hepatocytes are binucleated. Taking this into account, the N we estimated (2511E+06 "mononucleated hepatocytes") could correspond to 1930E+06 hepatocytes. Quantification of hepatocytes has been performed, namely by Weibel et al. (1969), who estimated 568E+06 "mononucleated hepatocytes" per gram of liver, and by Carthew et al. (1996), who estimated 1520E+06 hepatocytes per liver (including binucleated cells). These estimations differ 30% and 20%, respectively, from ours. This can be accounted for by the distinct strains, ages of the animals used, and by the methodologies now in disuse employed by both authors, stressing the potential differences between the nowadays called "design-based" stereology we used, and the "assumption-based" techniques of the past.

Quantitative studies of IC have been published (Bronfenmajer et al., 1966; Sztark et al., 1986; Geerts et al., 1991; Niki et al., 1996; Azaïs Braesco et al., 1997), using 3-10 µm sections and obtaining "numbers per area". In the stereology field, it has been often stressed that counting profile number per unit area of a section will not give a meaningful estimate of number. Number estimation of particles in a 2D basis is inevitably affected by the size, form, orientation, and by lost caps (Gundersen, 1986). It has been emphasised that the number of particles, being a zero-dimensional quantity, can only be estimated by a 3D probe, like the disector. The association of the optical disector with the fractionator (i.e., the optical fractionator) here used, proved as efficient as advantageous. The natural 3D alignment of the optical sections made the counting much more straightway than when using the physical disector (Marcos and Rocha, 2001). Moreover, counting of the IC and hepatocytes was made easy. The potential bias from lost caps was avoided by having upper and lower guarding zones within our thick sections, despite their use is still a matter of debate (Hatton and Von Bartheld, 1999).

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It has been known for long that though the liver tissue looks uniform on basic histology, it is heterogeneous at the level of morphometry and histochemistry, both for hepatocytes and sinusoidal cells (Jungermann and Kietzman, 1996). To our knowledge, this is the first study to quantify, using "design-based" stereology, such lobular heterogeneity for IC and hepatocytes. The periportal predominance of IC has been suggested in 2D-analyses, both in the rat (Geerts et al., 1991, Azaïs Braesco et al., 1997) and in the pig (Wake and Sato, 1993). In theory, such predominance could be imputed to a larger reference volume of periportal zones and/or to higher numerical density (Nv). In our case, the first hypothesis seems to prevail, because the Nv did not differ significantly between zones (data not shown). This means that the periportal liver tissue is more abundant and thus it contains more IC (so the N of IC is higher), although...
there is the same IC number per unit volume. This global periportal predominance may explain the higher extracellular matrix deposition and vitamin A storing described in this zone (Geerts et al., 1994). Moreover, our results seem in “physiological” accordance with data suggesting that Kupffer cells (essential for the IC activation) also predominate in periportal zones (Jungermann and Kietzman, 1996).

Based on preliminary tests, all the sampling was designed to result in precise estimations of N, with CE's lower than 10% (West et al., 1996). The estimation of the CE is still a matter of debate among stereologists, and here we used two formulae based on the so-called "quadratic approximation", slightly differing in the weight given to the inter-sectional variance. Because the CE's were much similar and lower than the above cited threshold with both formulae, the conclusions were alike, and so, the sampling procedure could be considered efficient a priori. In all the estimations but two, the CE contributed less than 50% to the total variances (i.e., the biological variance was greater than the variance due to the sampling procedure). Only in N of IC for pericentral and periportal zones, the sampling variance was superior to the biological one. However, we want to emphasise that the OCVs of these estimations were low (<10%); so, even in those cases the sampling effort seems to be well adequate for our present purposes.

REFERENCES


