A New Approach to an Unbiased Estimate of the Hepatic Stellate Cell Index in the Rat Liver: An Example in Healthy Conditions

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SUMMARY  Evaluation of activation and proliferation of hepatic stellate cells (HSCs) must be grounded on solid quantitative data under normal conditions. The HSC index (HSCI), number of HSCs per 1000 hepatocytes (HEP), is often used in hepatology but has been never determined using stereology. Systematically sampled sections were immunostained against glial fibrillary acidic protein and carcinoembryonic antigen, allowing unequivocal distinction of HSC and mononuclear/binuclear HEP. With the optical disector the HSCI was estimated as 109 (coefficient of error = 0.04). This work provides a sound technical basis for experiments in which the estimation of HSCI and/or simultaneous quantification of HSC and HEP are relevant. (J Histochem Cytochem 51:1101–1104, 2003)

KEY WORDS  hepatic stellate cell index  double immunohistochemistry  disector

When hepatic stellate cells (HSCs) were first described 125 years ago, no one could imagine that this research would be pivotal for understanding liver fibrosis. HSCs produce extracellular matrix and cell mediators, store vitamin A, and regulate sinusoidal blood flow (Geerts 2001). The HSC location between endothelial cells and hepatocytes (HEPs) soon suggested that their regulation depended on cell-to-cell interactions. These were quantitatively expressed by the “hepatic stellate cell index” (HSCI), i.e., the number of HSCs per 1000 HEPs (Horwath et al. 1973). This index changes in liver fibrosis (Ballardini et al. 1983) as the number of HEPs becomes altered. Moreover, in various conditions leading to fibrosis, such as dimethylnitrosamine or CCl₄ liver injury, HSCs become activated and proliferate (Geerts 2001). This may also change the HSCI. To further evaluate HSCs activation, it is important to estimate their index in non-pathological conditions. However, data in such conditions are scarce, and the previous estimations did not consider binuclear HEPs, which represent around 30% of hepatocyte pool in rats (Jack et al. 1990). Furthermore, modern “design-based” stereology was never used to determine the HSCI.

Currently, the unbiased disector principle is the most adequate baseline procedure to estimate cell number in microscopy (Howard and Reed 1998). This modern stereological technique depends neither on shape, size, nor orientation assumptions. It uses either thin sections (physical disector) or thick sections (optical disector). The optical disector is usually preferred because it is more practical and faster than the physical counterpart. In optical disector, a continuous scan is performed through the thick sections (>20 μm) by confocal or light microscopy. In the latter, the microscope needs a micrometer, to measure stage displacements in the z-axis, and a high numerical aperture immersion lens, with a matched condenser, to obtain the smallest possible focal depth. The only prerequisite for this technique is that the cells to be counted are unambiguously identified. To achieve this, immunohistochemistry (IHC) can be applied. However, thick sections raise problems of achieving good immunostaining throughout the section depth.

We aimed to obtain a precise and unbiased estimation of the HSCI in healthy conditions. For this, we combined the optical disector with a double IHC
against (a) glial fibrillary acidic protein (GFAP), a specific marker for quiescent HSCs in the rat liver (Neubauer et al. 1996; Cassiman and Roskams 2002), and (b) carcinoembryonic antigen (CEA) which, by marking biliary canaliculi, circumscribes hepatocyte cell limits and allows a clear distinction of mononuclear from binuclear cells.

Five male Wistar rats (Gulbenkian Institute of Science; Oeiras, Portugal), 3 months old and weighing about 336 g (CV = 0.12) were used. Liver fixation was achieved by controlled flux perfusion of 10% formalin with a peristaltic pump. The liver was removed, weighed, and a fractionator sampling scheme was applied (Howard and Reed 1998). First, each liver was sliced into 4-mm-thick slabs. Half of these were sampled (systematically, with a random start), and cut into 4-mm-thick bars. Again, half of these bars were sampled (systematically, with a random start) and a mean of 10 fragments per animal was obtained. These were postfixed in 10% buffered formalin for 16 hr and processed for paraffin embedding. Half of the fragments were sampled and exhaustively sectioned (30 μm thick). Every twentieth section was systematically sampled and mounted on precleaned slides primed with aminopropyltrithoxysilane.

A double IHC procedure against GFAP and CEA followed. After deparaffinization, slides were placed in buffered citrate (pH 6.0) and microwave treatment was carried out for antigen retrieval (600 W, 12 min). After rinsing in PBS, the endogenous peroxidase was blocked with 0.3% H2O2 for 30 min. The first streptavidin–biotin protocol then followed (Histostain Plus; Zymed, San Francisco, CA). After rinsing in PBS, 10% non-immune goat serum was applied for 90 minutes, followed by a long incubation with 1:3000 rabbit non-immune serum showed no immunomarking (negative controls).

The HSCI was estimated with the optical disector, using a workstation made of a microscope (Olympus BX-50; Tokyo, Japan), a microcator (Heidenhain MT-12; Traunreut, Germany) to control the movements in the z-axis (accuracy 0.5 μm), a motorized stage (Prior; Fulbourn, UK) for stepwise displacement in the x–y axis (accuracy 1 μm), and a CCD video camera (Sony; Tokyo, Japan) connected to a 17" PC monitor (Sony). The entire system was controlled by the software Olympus CAST-Grid version 1.5 (Albertslund, Denmark). A ×100 oil immersion lens (Olympus Uplan; NA 1.35) provided a high magnification at the monitor (×4750), allowing unambiguous recognition of HSC and HEP borders. The first field was selected randomly. Thereafter, fields were sampled systematically by stage stepwise movements (stepx,y = 1250 μm). Through the disector height (20 μm), software-generated counting frames with defined areas (1673 μm² for HSCs and 418 μm² for HEPs) were superimposed.

The HSCs and HEPs were counted when two conditions simultaneously met: (a) the rim of the nucleus was in focus at a plane below 4 μm and above or equal to 24 μm (the upper and lower surfaces were avoided with guard heights of 4 and 7 μm, respectively); (b) at the plane of focus, the nucleus was within the counting frame or touching the inclusion lines but did not touch the forbidden lines or their extensions. In every disector it was confirmed that the immunomarking extended beyond 24 μm, ensuring that all HSC and HEP borders could be unambiguously recognized. At the same time, in every fifth field, section thickness was measured with the microcator. The same author (R. Marcos) performed the countings.

The HSCI was estimated via the Nv, obtained from the optical disector, by the formula

$$\text{HSCI} = 1000 \times \frac{N_HSC}{N_{HEP}}$$

where Nv (HSC) and Nv (HEP) are the numerical density of HSC and HEP, ΣQ−HSC and ΣQ−HEP are the total number of cells counted, and αHSC and αHEP are the counting frame area for HSCs and HEPs. To determine the coefficient of error (CE) of the index estimate, a two-step procedure was used. The CE of the number of cells counted, HSC and HEP, was estimated in each rat by the formula:

$$\text{CE}(\Sigma Q^-) = 1/\sqrt{\Sigma Q^-}.$$ 

Since the CE of the index represents a ratio, another formula was then used: (Howard and Reed 1998)

$$\text{CE} = \sqrt{[\text{CE}^2(\Sigma Q^-_{HSC}) + \text{CE}^2(\Sigma Q^-_{HEP})]}$$

A consistent and reliable marking of HSCs and biliary canaliculi was achieved with GFAP and CEA. The
Brown staining of HSCs, visualized at high magnification, allowed an accurate identification of these cells at every section depth and in all sections. In contrast, biliary canaliculi were black stained. This outlined HEP borders, permitting clear distinction and counting of mononucleated and binucleated cells (Figure 1).

A mean of 1340 dissectors was analyzed per rat, with an average of 907 HSCs and 2099 HEPs counted (1540 MnHEP and 559 BnHEP). The HSCI was 109 HSCs/1000 HEPs (CV = 0.11), with CE = 0.05 only.

IHC has been often used in liver research, but this is the first time a double immunotagging is combined with an unbiased stereological tool to determine the HSCI. In recent years, crosstalk between liver cells became a focus of research. Respecting HSCs and HEPs, different modes of communication, either by membrane contact or soluble mediators, have been identified (Gressner and Bachem 1995) and co-culture of these cells has been shown to be beneficial for growth and stability of HSCs. Therefore, the correct assessment of the HSCI in non-pathological conditions stands as fundamental for morphofunctional correlations.

Despite its introduction 30 years ago (Horwath et al. 1973), the HSCI had never been estimated by stereological methods. Examples of its usefulness can be found in the literature (e.g., Ballardiini et al. 1983; Yokoi et al. 1984; Azaïs–Braesco et al. 1997), but these studies contrast with our report in different points. Some researchers used lipid staining, much less accurate than IHC (Azaïs–Braesco et al. 1997), whereas others used desmin immunomarking, shown to be less precise than GFAP (Neubauer et al. 1996). Moreover, semithin sections and “numbers per area” have been used to obtain the HSCI in previous reports. Nowadays, it is recognized that the number of particles can be directly estimated from sections only by the tedious process of 3D reconstruction or by using a 3D probe, such as the disector (Howard and Reed 1998). With this tool, we sampled HSCs and HEPs in proportion to their numbers, irrespective of size, shape, or orientation of cells. It is noteworthy that the amount of our sampling substantially differs from those of previous reports (i.e., we examined at least a 25-fold greater number of fields). In addition, in all previous reports HEPs nuclei were counted directly as “hepatocytes,” thus disregarding the binucleated cells (Jack et al. 1990) and underestimating the HSCI. Indeed, using this approach in our data, an estimate of 86 HSCs/1000 “mononucleated” HEPs would be achieved, instead of the above reported 109 HSCs/1000 HEPs.

In quantitative studies of HSCs involving IHC, routine cryostat and paraffin sections have been used (e.g., Johnson et al. 1992; Azaïs–Braesco et al. 1997), but the thick paraffin sections we applied are still rather unusual in hepatology. As to the thickness, we used 30-μm sections to fulfill the technical requirements of the optical disector. We tried different strategies to accomplish a uniform immunostaining throughout the full section depth, but only long incubation periods with antibodies granted consistently good results. Such an approach was also used for marking astrocytes with GFAP in thick brain sections (Ontoniente et al. 1983). As to the embedding medium, despite the fact that IHC is a priori more straightforward in cryostat sections, we opted for paraffin sections. If immunomarking is achieved, these are advantageous for stereological purposes because they are much easier to handle in systematic sampling schemes, render better morphology, and exhibit less shrinkage from fresh to mounted sections. Shrinkage can be a drawback because it usually leads to an overestimate of N/V. However, respecting the HSCI, and since we determined it as a ratio between N/V computed from the same sections, the amount of shrinkage (about 38%) canceled out.

The marking of HSCs is still controversial. Concerning GFAP, it was established that not all HSCs express this protein (Cassinon and Roskams 2002), and therefore are missed in quantification. In our study, the number of those cells should be low, in view of the observed high numerical density of immunomarked cells (N/V = 20.4 × 10³ HSCs/mm³). It is noteworthy that our study dealt with normal conditions, but the
sampling strategy and stereological procedure appear well suited for the analysis of rat fibrosis and human liver biopsies. In the former, a more appropriate marker, such as α-smooth muscle actin, should be used, whereas in humans the use of a neurotrophin receptor, such as Trk-C, is a better option (Geerts 2001). From liver biopsies, thick sections could be obtained from the same liver pieces used for routine thin sectioning.

In conclusion, the use of stereology and double IHC against GFAP and CEA is presented as an innovative approach to estimating the HSCI and obtaining an unbiased simultaneous quantification of HSCs and HEPs. By using stereology, the CE could be estimated, allowing a critical judgment about the sampling and precision. In view of the high numbers of cells counted and the low CE, we can regard our sampling as efficient and the estimate as precise.

Literature Cited


