Note

Staurosporine Promotion of Formation of Continuous Monolayers of Primary Rat Hepatocytes by Improving Attachment and Spreading

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Primary rat hepatocytes form discontinuous monolayers even at their maximum density. Here, we show that staurosporine promotes attachment and spreading of hepatocytes onto culture substrates, so that hepatocytes form a close, continuous monolayer. This treatment did not attenuate major hepatic functions. Therefore, this technique is promising for making seamless cell-sheet structures, which will be applicable for cell-polarity experiments or artificial liver construction.

Key words: staurosporine; primary hepatocyte; monolayer

Primary cultured hepatocytes, like most other cells, form a monolayer within 24 hours after cell seeding. However, due to some characteristics of primary hepatocytes, such as their poor proliferation in vitro, it is difficult to make a close, continuous (called ‘confluent’) cell layer, even if the cells are seeded at their maximum density. Here, we report a marked ability of staurosporine, a potent protein-kinase inhibitor,1,2 to make primary rat hepatocytes form a close monolayer, which will be applicable for in vitro cell-polarity experiments or construction of artificial liver modules.

Hepatocytes were prepared from Wistar rats (female, 7–9 weeks old, Nippon Clea) by an in situ collagenase perfusion method3 and four subsequent low-speed centrifugations. Cell viability was assessed by the trypan blue exclusion method, and was always higher than 90%. The isolated hepatocytes were resuspended in Williams’ medium E (ICN Biomedicals) with 10% fetal bovine serum (Filtron) and 10−7 M dexamethasone (Sigma), and were seeded at a density of 10×10^5 cells/cm^2 into 6-well or 24-well culture plates with type I collagen-coat (Sumitomo Bakelite) or into 3-micron pore size polytetrafluoroethylene transwell filters with type I collagen-coat (Corning). After 4 h of incubation at 37°C in a 5% (v/v) CO2 atmosphere, the medium was replaced with fresh Williams’ medium E with 10−7 M insulin (Sigma), 10−7 M dexamethasone, and 0.7 μg/ml aprotinin (Bayer). After a further 20 h of culture, the cells were analyzed by the methods described in the figure legends or in other papers.4,5 Staurosporine (Wako) was dissolved in dimethylsulfoxide (infinity-pure grade, Wako), and was added to the culture medium at both 0 h and 4 h.

By starting the cultures of primary rat hepatocytes in the presence of staurosporine, we found a marked effect of staurosporine on the morphology and adhesion of hepatocytes within 24 h. Staurosporine is known to inhibit a wide spectrum of intracellular protein kinases, including serine/threonine kinases and tyrosine kinases.1,2 A preliminary test showed that none of several other protein-kinase inhibitors that were tested, e.g. H-7, KN-62, genistein,3 etc., showed such an effect (data not shown), suggesting that this effect is staurosporine-specific. Another preliminary test showed that staurosporine concentrations around 50 nM appeared to be strong enough to have the desired morphological effect but not strong enough to be toxic (data not shown). Therefore, we used 50 nM staurosporine in all the following experiments.

Figure 1 shows the morphologies of primary rat hepatocytes in the absence or presence of staurosporine. After 24 h of culture, control cells formed hundreds of patch-like layers with much blank space (Fig. 1-A), while staurosporine-treated cells formed an almost continuous, close layer with little space (Fig. 1-B), and appeared to attach and spread better than control cells. MTT assays4 showed that staurosporine treatment tends to improve the viability of hepatocytes slightly but reproducibly, suggesting an increase in adherent cell numbers in the staurosporine-treated culture. As a typical example, the relative viability* of a staurosporine-treated sample at 24 h was 1.09±0.03 (a mean ± a standard deviation of quadruplicate measurements), compared

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; EHS, Engelbreth/Holm-Swarth sarcoma

* Relative viability was calculated relative to the OD value of the control, which was defined as 1.

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with the control (1.00±0.02). Moreover, a dye-permeation assay using transwell filters demonstrated that the staurosporine-treated cell layer passed much less dye than the control cell layer (Fig. 2), indicating the close structure of the staurosporine-treated monolayer.

To find whether staurosporine causes hepatocytes to lose their hepatic functions, we analyzed two major indices of hepatic function: the productions of albumin and bile acids. Albumin concentration was analyzed by a sandwich ELISA method and total bile acids was analyzed with a Wako Total Bile Acids Test (Wako) as described previously. In a typical experiment, in the absence of staurosporine the concentrations of albumin and total bile acids in the culture medium at 24 h were 8.44±0.36 μg/ml and 16.06±0.30 pmol/ml (means ± standard deviations of quadruplicate measurements), respectively. On the other hand, in the presence of staurosporine, their concentrations were 8.14±0.46 μg/ml and 15.74±0.49 pmol/ml, respectively. Thus, staurosporine did not significantly alter the abilities of hepatocytes to produce albumin and bile acids, suggesting that it has minimal adverse effects on hepatic functions despite its marked effect on morphology.

Taken together, these results reveal that the staurosporine treatment of primary rat hepatocytes at the beginning of culture is useful for forming close, continuous monolayers that preserve hepatic functions. Although in these data the monolayers appear to be not completely seamless, adjustment of culture conditions, including the manner of staurosporine administration, the initial cell density, etc., should make it possible to form practically seamless cell-sheet structures.

Similar effects of staurosporine on cell morphology and adhesion were reported in other cells: e.g. a human colon cancer cell, and human megakaryoblas-
tic leukemia cells. Analyses of their mechanisms showed that in the staurosporine-treated cells there occurred several changes in the cytoskeleton and focal adhesion complex as well as changes in the phosphorylation states of specific proteins such as Src and tensin. The interaction of the cells with specific ECMs was also suggested to be an important factor regulating the morphological changes. However, the overall mechanisms including the primary target(s) of staurosporine have not yet been identified. Considering the morphological changes in the staurosporine-treated hepatocytes, it is likely that similar molecular processes may occur in the hepatocytes. Further molecular analyses will be required to understand the phenomenon shown in this report as well as the similar phenomena observed in other cell systems.

As an application of the close monolayers of primary hepatocytes, they can be used for in vitro cell polarity experiments. Promotion of physical contacts between adjacent hepatocytes may help to reconstitute the functional interaction between the cells, resulting in the formation of the lateral and the apical (bile canalicular) domains in the plasma membrane. Combining the use of a differentiation-inducing ECM, such as the EHS gel and the type I collagen-sandwich gel, should facilitate the formation of the polarized structure.

Another possible application of the seamless hepatocyte layer is that it can be used as a component of an artificial liver module. Since the inherent polarity of hepatocytes in vivo is distinctive (they have polygonal surfaces, and their apical surfaces face bile canaliculi between adjacent cells), it is usually impossible to form the typical epithelial-cell-like polarity on a culture substratum in vitro. Nonetheless, if some techniques make it possible to artificially control the polarity of the hepatocyte layer on a permeable culture membrane, we could obtain a unique hepatocyte-culture system, in which the secretion of bile acids is physically separated from the secretion and metabolism of blood components by the intervening seamless hepatocyte layer. Such an architecture would be suitable for artificial liver modules, where the leakage of bile acids into the bloodstream should be avoided. We are presently attempting to develop new methods for forming such an artificial polarity.

References