—RAPID COMMUNICATION—

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ACTIVE PROLIFERATION OF MOUSE HEPATOCYTES IN PRIMARY CULTURE UNDER DEFINED CONDITIONS AS COMPARED TO RAT HEPATOCYTES

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Mouse and rat hepatocytes were investigated for replicative potential in primary culture under defined conditions. Mouse hepatocytes replicated at least twice or three times by the 7th day of culture in response to EGF, while only a half of rat hepatocytes replicated once. Thus a far greater potential for proliferation was demonstrated by mouse than rat cells, although the mouse cells ceased dividing by the 10th day of culture. Immunocytochemical examination revealed that the cells produced both albumin and α-fetoprotein. Ultrastructural investigation revealed that these cells were immature hepatocytes in nature, based on the presence of microbodies with nucleoids. Thus, primary cultures of mouse hepatocytes seem to be a useful system for studying proliferation and transformation of hepatocytes *in vitro.*

Key words: Mouse hepatocytes — Primary culture — Cell proliferation

Rat hepatocytes in primary culture have been widely employed to investigate hepatic functions, and their regulation by various hormones. Recently, several investigators have succeeded in inducing hepatocyte DNA synthesis *in vitro* by using EGF.[1] However, after one replication, very few of the cultured rat hepatocytes reenter the cell cycle under conditions of low Ca^{2+} concentration.[2] Since transformation requires several cell divisions,[3] this very limited potential for replication seems to have been the bottleneck in applying the primary culture system of rat hepatocytes for the *in vitro* study of hepatocarcinogenesis.

Although the mouse liver offers an equally good system for elucidation of hepatocarcinogenesis, in comparison with rat hepatocytes, very little effort[4, 5] has been made to study the mechanisms involved using primary cultures of mouse parenchymal liver cells. This is surprising given the fact that the mouse has distinct advantages for analysis of carcinogenesis,[5, 6] because the genetics of mice have been studied to a greater extent than those of the rat.[7]

We show in this communication that proliferation of cells derived from mouse liver parenchyma did indeed occur at a far higher rate than that characteristic of rat liver cells. Thus, this system might offer us an opportunity to transform hepatocytes *in vitro.*

Rat hepatocytes were isolated by collagenase perfusion[8] from female Sprague-Dawley rats (5–6 weeks old; Charles River Japan, Inc., Atsugi). Mouse hepatocytes were similarly isolated by *in situ* collagenase perfusion via the portal vein from female C3H/N mice (5–6 weeks old; MTV-free; Shizuoka Laboratory Animal Center, Hamamatsu) at a flow rate of 8 ml first perfusate[8]/min for 8–10 min and at the same rate of collagenase-containing perfusate for 12–15 min. After obtaining a crude suspension by centrifugation at 50*g* for 1 min twice, viable hepatocytes were selected by an isodensity Percoll method.[9] Hepatocytes in L-15 supplemented with 0.5 μg of insulin/ml, 18m*M* HEPES, and antibiotics[9] were plated onto 60 mm collagen-coated dishes[9] at a cell density of 10^5 viable hepatocytes/dish. After a 2 hr cultivation, the cells were re-fed DMEM/F-12 (1:1) mixture supplemented with insulin, transferrin, selenium (ITS; Collaborative Research, Inc., USA), dexamethasone and antibiotics, with

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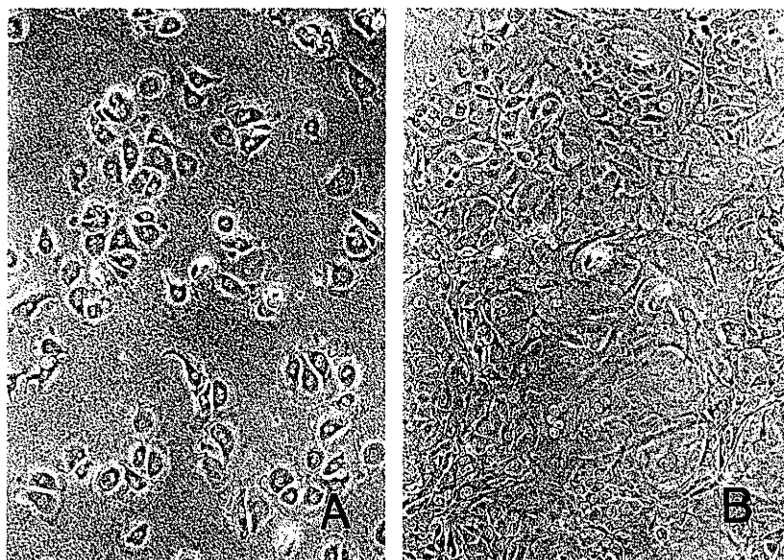


Fig. 1. Mouse hepatocytes in the same field in primary culture, 1 (A) and 5 (B) days after plating. A needle mark was made in the center of the field to facilitate identification on subsequent days for counting cell numbers. ×100.

or without 20 ng of EGF/ml.[10] The medium was thereafter renewed at the 5th and 7th days after plating. Cell number was estimated by counting cells in the same field under 100 times magnification by phase-contrast microscopy (Nikon Diaphot with Microflex UFX).[10] Five different fields were followed up until the 10th day of the culture. Glucose-6-phosphatase activity of the cultured cells was demonstrated by the method of Wachstein and Meisel[11] after drying the cells *in situ.* To identify peroxisomes, the cells in the dishes were fixed in 10% buffered formalin and incubated in alkaline 3,3′-diaminobenzidine medium[12] for demonstration of peroxisomal catalase activity, and then counterstained with hematoxylin. The cells were fixed in chilled absolute ethanol (−20°) and permeabilized with 0.2% Triton X-100 in PBS. Immunostaining of albumin and α-fetoprotein was carried out using a Vectastain™ ABC kit (Vector Laboratories, Inc., CA). Primary antibodies used were sheep antimouse albumin (Inter-Cell Technologies Inc., NJ) and goat antirat α-fetoprotein (Nihon Biotest Laboratory, Inc., Tokyo). Control experiments were performed using non-immunized serum and Swiss 3T3 cells negative for albumin and α-fetoprotein production. After addition of 5 μCi of [methyl-^{3}H]thymidine (20 Ci/mmol, NEN Research Products, USA), cultures were incubated for 24 hr, and then fixed in chilled absolute ethanol and covered with photographic emulsion. After a 1-week exposure in a cold room, the autoradiographs were developed.[10] The cells were fixed in 2.5% glutaraldehyde in 0.1*M* cacodylate buffer, pH 7.4 at 4°, postfixed in 2% osmium tetroxide in the buffer, dehydrated and embedded *in situ* in Epon 812.[13]

In 2-hr or 1-day cultures of both rat and mouse hepatocytes, more than 98% of the attached cells were identified as hepatocytes with the aid of glucose-6-phosphatase and catalase staining. Regarding primary cultures of mouse hepatocytes, more than 80% of the cells were binucleate, as shown in Fig. 1A. As described by Enat *et al.*[14] hepatocytes seemed to be selectively stimulated to replicate under serum-free conditions during the 10 days of cultivation. In agreement with the results of Klaunig *et al.*,[15] mouse hepatocytes were observed to survive much longer than rat hepatocytes in primary culture. In addition, a

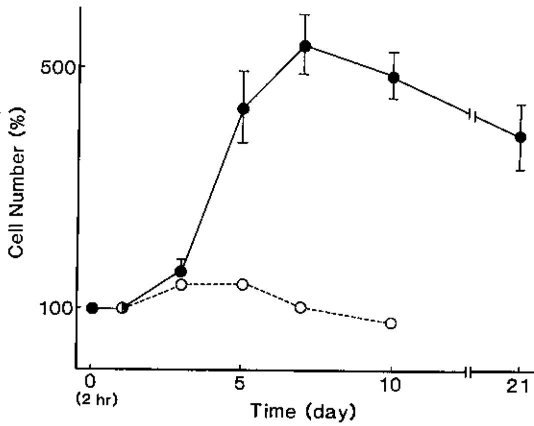


Fig. 2. Time course of change in cell number of rat (○) and mouse (●) hepatocytes in primary culture.

small number of mouse hepatocytes replicated *in vitro* without EGF (data not shown).

As shown in Figs. 1 and 2, the cell number of mouse hepatocytes increased with culture time. In particular, an increase of three-fold was observed between the 3rd and 5th days, and small mononucleate cells became dominant. During this period, 80–90% of the cells were autoradiographically labeled in any 24 hr period (Fig. 3A), showing that the majority of the cultured cells had entered the S phase and completed cell division. At the 8th day after plating, however, the percentage of labeled cells was about 10%. Light microscopic cytochemistry of peroxisomes, specific for the parenchymal component of livers,[12] revealed that, on the 4th day, all of the cells in mitosis and more than 99% of the cultured

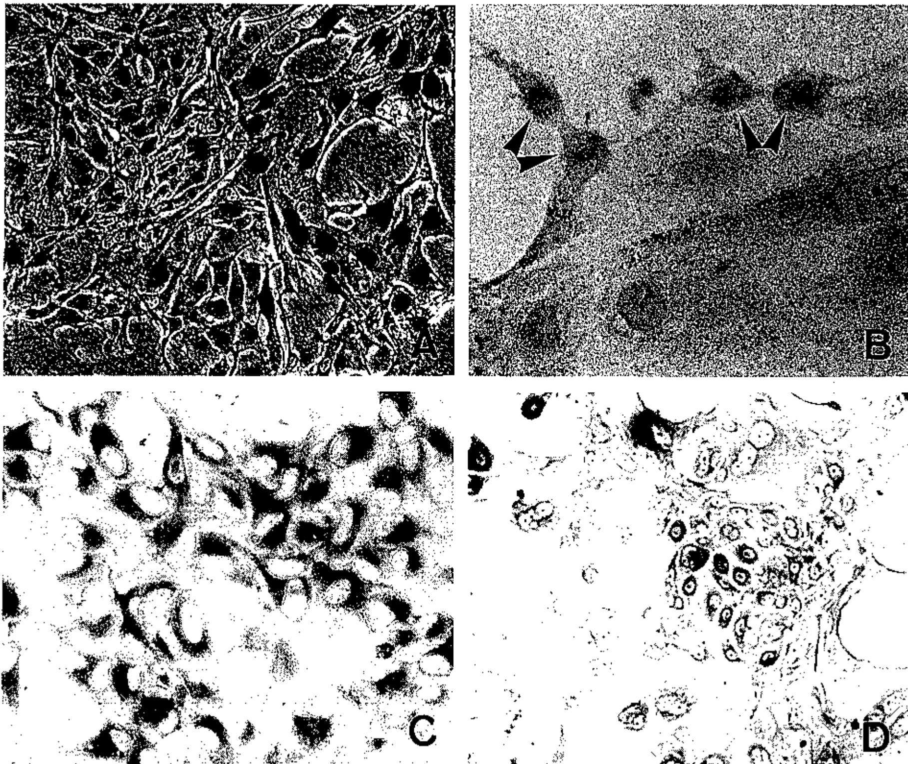
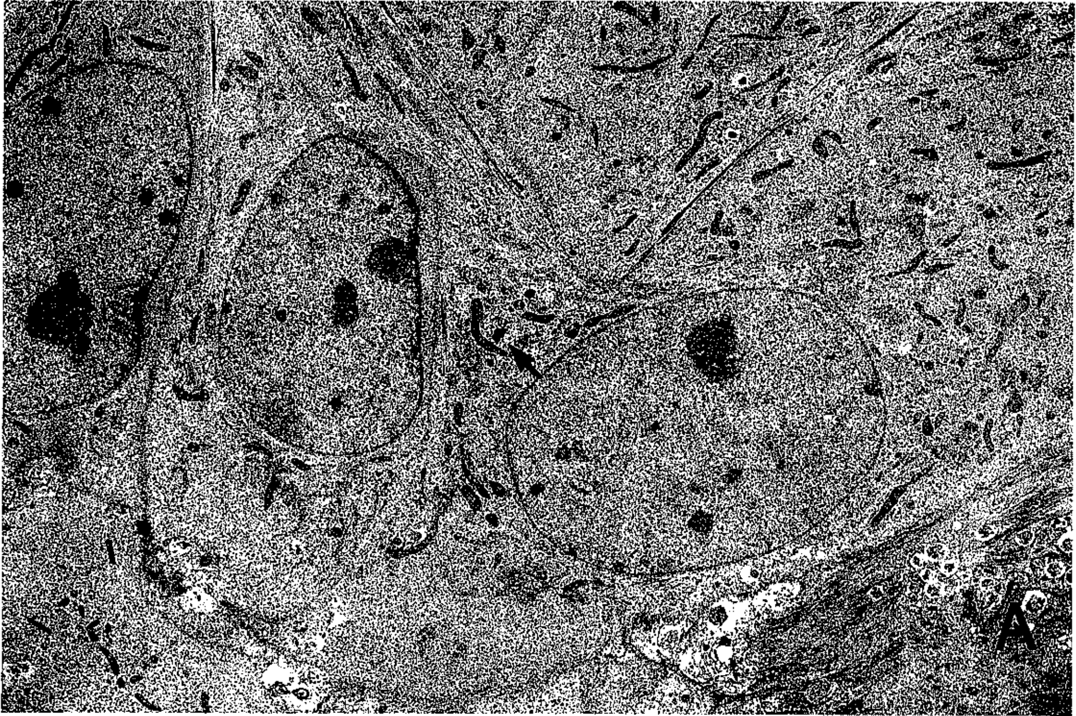


Fig. 3. (A) Autoradiograph of 6-day-cultured hepatocytes labeled with [^{3}H]thymidine, ×100. (B) Histochemical demonstration of peroxisomes in replicating cells on the 4th day of culture, ×300. Arrowheads indicate dividing small cells with peroxisomes. (C) Immunostaining of albumin in 7-day-cultured hepatocytes, ×200. (D) Immunostaining of α-fetoprotein in 7-day-cultured hepatocytes, ×100.



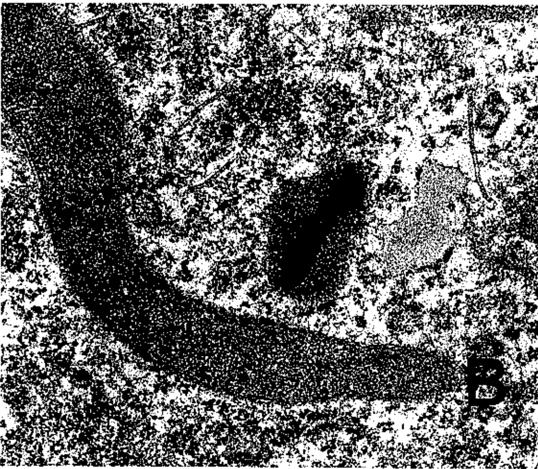


Fig. 4. (A) Electron micrograph of actively proliferating cells on the 7th day of culture, ×4000. (B) A higher magnification of the portion indicated by an arrow, showing a peroxisome with nucleoid, ×30,000.

cells were of hepatocyte nature (Fig. 3B). Thus, at the 7th day, the cell number had increased 5 times as compared to the 1st-day cultures. Ultrastructural examinations on 7-day-cultured cells demonstrated that these small and mononucleate cells had plenty of mitochondria, desmosomes and bile-canaliculus-like structures. Moreover, these cells had a few peroxisomes with a band-shaped nucleoid characteristic of hepatocytes (Fig. 4), strongly suggesting that these cells were derived from hepatocytes. Immunocytochemical examination of the cultured cells revealed production of both albumin and α-fetoprotein in primary culture (Figs. 3C and 3D), showing that the mononucleate cells appearing after frequent cell division were indeed hepatocytes or immature hepatocytes. Production

of α-fetoprotein in primary cultures of adult rat hepatocytes was reported by Sirica *et al.*[16] and *in vivo* production of α-fetoprotein was demonstrated in adult mouse hepatocytes after partial hepatectomy.[17] Thus, one can conclude that the increase in cell number during the cultivation period resulted from two or three cell divisions and thereafter the cell proliferation ceased. The use of this system in future investigation of the regulatory mechanisms of hepatocytes would appear warranted.

Although several investigators have demonstrated a high frequency of cell division of rat hepatocytes in primary culture,[10, 18-20] only a half of the rat cells in the present experiments replicated once. Very few cells replicate twice under low Ca^{2+} conditions,[2] and most investigators have described an increase in cell number of rat hepatocytes in primary culture limited to 50%. This compares unfavorably with the 500% increase observed for mouse hepatocytes. While Enat *et al.* did show that the presence of liver biomatrix was associated with promotion of rat hepatocyte proliferation *in vitro*,[14] their system did not give readily reproducible results.[21] Thus, there seems to be a species difference in proliferation potential between the rat and mouse.

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