

## Morphology, Proliferation and Apoptosis of Mouse Liver Epithelial Cells Cultured as Spheroids

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The MLEC10 is an epithelial cell line derived from an untreated, normal C3H/HeN mouse liver. We previously demonstrated that tumorigenic variants from this cell line produced moderately differentiated hepatocellular carcinomas in nude mice. However, it has remained unclear whether the parental MLEC10 cells represent immortalized hepatocytes or so-called oval cells, both of which may serve as precursors for hepatocellular neoplasms. In this study, we performed 3-dimensional, spheroid culture of the MLEC10 cells in order to facilitate histological assessment of their lineage. Spheroidal aggregates were formalin-fixed and embedded in paraffin for routine light-microscopic observation of hematoxylin and eosin-stained sections. Histopathologically, the MLEC10 cells were indistinguishable from immature hepatocytes and distinct from oval cells. At the electron-microscopic level, their hepatocytic nature was evidenced by bile canaliculus structures and glycogen storage. Intriguingly, the spheroids contained fragmentary material reminiscent of Councilman bodies, implying apoptosis of the hepatocytes. Although the cells significantly proliferated during the first three days of culture, apoptotic death then resulted in a 75% decrease in viable cell number. Thereafter, both apoptosis and cell division appeared silent, the numbers being unchanged. Expression of the *p53* tumor suppressor gene became gradually elevated, correlating positively with growth arrest, but negatively with apoptosis, suggesting that the cell death occurred independently of *p53*. Our results indicate that at least some liver epithelial cell lines derived from untreated murine livers exhibit a hepatocytic morphology in spheroid culture. Also, the present culture system provides a useful tool for investigating biological phenomena, e.g. apoptosis, specifically involving liver cells, under 3-dimensional conditions.

Key words: Liver epithelial cell — Spheroid culture — Morphology — Apoptosis — Mouse

Long-term culture of cells from untreated, grossly normal murine livers gives rise to permanent epithelial-like cell lines known as liver epithelial cell lines.<sup>1,2)</sup> Those cell lines occasionally exhibit hepatocytic functions such as production of albumin. Furthermore, examples transformed either spontaneously or by carcinogenic agents have been reported to produce hepatocellular carcinomas in syngeneic or immuno-deficient animals.<sup>3,4)</sup> Although these findings suggest that at least a portion of liver epithelial cell lines originate from hepatocytes, there is still controversy regarding this issue.

In the normal liver, only two types of epithelial cells, hepatocytes and cholangiocytes, are histologically discernible. However, there is a third type of hepatic epithelial cells designated "oval cells," which emerge when livers are treated with highly cytotoxic chemicals or diets.<sup>5)</sup> The oval cells appear around periportal areas and can be morphologically distinguished from hepatocytes because of

their scanty cytoplasm, small size and oval- to spindle-shaped nuclei. In rat livers, the oval cells show a biochemical profile intermediate between those of hepatocytes and cholangiocytes. Based on this, some investigators have considered them to be derived from facultative stem cells, which are capable of differentiating into both cell lineages.<sup>6,7)</sup>

A number of hepatocarcinogens are known to cause oval cell proliferation in rat livers before induction of hepatocellular neoplasms. Since neoplastic hepatocytes exhibit biochemical properties resembling those of oval cells, it has been proposed that a substantial proportion of hepatocellular tumors are of oval cell origin.<sup>6,7)</sup> Significantly, a rat liver epithelial cell line established by Tsao *et al.* was also found to mimic the biochemical and morphological features of oval cells rather than mature hepatocytes or cholangiocytes.<sup>8)</sup> Subsequently, the same group demonstrated that chemically induced transformants from the cell line gave rise not only to hepatocellular carcinomas, but also cholangiocellular carcinomas and hepatoblastomas in syngeneic animals.<sup>4)</sup> Such findings, together with the fact that primary hepatocytes have very limited growth ability in culture, have led to the prevailing

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hypothesis that most liver epithelial cell lines share the same origin as the oval cells *in vivo*, i.e. the putative stem cells.<sup>6, 7, 8-10</sup>

On the other hand, oval cell proliferation is much less common in mouse hepatocarcinogenesis. We therefore believe that most hepatocellular neoplasms in mice develop through initiation and promotion of hepatocytes.<sup>11, 12</sup> Previously, we established multiple liver epithelial cell lines from untreated, young C3H/HeN mice, approximately half of which expressed albumin.<sup>13</sup> Although most of the cell lines did not form tumors when back-transplanted into nude mice, they acquired tumorigenicity after transfection with an activated *H-ras* oncogene<sup>14</sup> or a hepatocyte growth factor gene.<sup>15</sup> The tumors developing in nude mice were histologically diagnosed as moderately differentiated hepatocellular carcinomas.<sup>13-15</sup> Since spontaneous hepatocellular tumors are frequently seen in old C3H/HeN mice,<sup>16</sup> we suggested that the cell lines from C3H/HeN mice may represent spontaneously initiated hepatocytes developing phenotypic immaturity due to long-term culture.<sup>13</sup> Consistent with this idea, liver epithelial cell lines were far more difficult to establish from C57BL/6N mice, which have a low incidence of spontaneous tumors.<sup>13, 16</sup>

From the standpoint of the stem cell theory, however, our mouse cell lines are also likely to be derived from the putative hepatic stem cells or oval cells. Whereas no biochemical markers can unequivocally distinguish between immature hepatocytes and oval cells at present, the definitions of those cell types basically depend on their morphologies in histological sections. In this study, to extend our knowledge of the nature of liver epithelial cell lines, we performed 3-dimensional spheroid culture of our mouse cell line, MLEC10, because multicellular spheroids can be morphologically analyzed by routine histological methods. It has been shown that primary rodent liver cells well reconstitute their *in vivo* functions and morphologies under spheroid culture conditions.<sup>17, 18</sup> We found MLEC10 cells comprising spheroids to be indistinguishable from immature hepatocytes, and they can undergo apoptosis.

## MATERIALS AND METHODS

**Cell line** The MLEC10 cell line used in this study is a clonal epithelial cell line obtained by culture of primary hepatocytes from a 6-week-old, male untreated C3H/HeN mouse as previously described.<sup>13</sup> The cells express albumin, possess electron-microscopic characteristics of epithelial cells and are non-tumorigenic in nude mice.<sup>13-15</sup> The Ras-1 cell line is a clonal tumorigenic variant of MLEC10 obtained by transfection with an activated human *H-ras* oncogene.<sup>14</sup> The Ras-1 cells produce moderately differentiated hepatocellular carcinomas when transplanted into nude mice.<sup>14</sup> MLEC10 and Ras-1 cells were

routinely maintained as monolayers in an incubator set at 37°C with 5% CO<sub>2</sub> and 100% humidity. The basal medium employed throughout the study was Waymouth's MB 752/1 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), and penicillin and streptomycin (Gibco BRL) as antibiotics.

**Spheroid culture** To avoid cellular attachment, each plastic culture dish, 9 cm in diameter, was coated with 5 ml of 1% melted agar (Agar Noble, Difco, Detroit, MI) in water. The solidified agar coating was overlaid with an equal volume of 2× basal medium and equilibrated overnight. Monolayer cultures of MLEC10 cells were detached with 0.25% trypsin solution in 1 mM EDTA and aliquots of 10<sup>6</sup> cells suspended in 10 ml of the basal medium were added to the coated dishes. Each dish was horizontally rotated several times to help spheroidal aggregation of the cells in the center and then placed in the incubator. This starting date was defined as day 0. Spheroid cultures were continued up to day 7 without changing the medium.

**Histological and immunohistochemical analyses** Just before harvest, the spheroids were labeled for 1 h with 10 μM bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) added to the medium. They were washed twice with Hanks' solution (Gibco BRL), fixed with 10% buffered formalin for 30 min, embedded in 1% agar, further treated with formalin overnight and routinely processed for paraffin embedding. Finally, histological sections 4 μm in thickness were cut and stained with hematoxylin and eosin (H&E) for observation by light-microscopy. For determination of BrdU-labeling indices, the paraffin sections were immunohistochemically stained with mouse monoclonal anti-BrdU antibodies (Becton Dickinson, San Jose, CA) using the avidin-biotin complex method as recommended by the manufacturer. Percentages of cells showing nuclear staining were calculated after counting at least 200 cells.

**Electron-microscopic analysis** The harvested spheroids were fixed with 2.5% glutaraldehyde, embedded in 1% agar in water, and again fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. They were processed for embedding in Spurr resin and ultra-thin sections were made, followed by staining with uranium acetate and lead nitrate. Finally, the sections were observed under an Akashi LEM 2000 electron-microscope.

**Detection of apoptosis** Fragmented genomic DNA specific for apoptotic cells was isolated from fresh spheroids according to a method that preferentially extracts low-molecular-weight DNA as previously described.<sup>19</sup> Electrophoresis of the DNA samples was performed on 2% agarose gels, and ladder formation due to fragmentation was visualized with ethidium bromide under ultraviolet illumination.

**Quantification of cell number** Fresh spheroids were washed twice with phosphate-buffered saline (PBS) con-

taining 1 mM EDTA and then treated with 0.25% trypsin solution at 37°C for 20 min. They were again washed with PBS and gently pipetted to make single cell suspensions. The numbers of viable cells were determined by the trypan blue exclusion test.

**Northern blot analysis** Northern blot studies were performed with established procedures.<sup>19,20</sup> Total RNA from fresh spheroids was extracted by the guanidinium thiocyanate method,<sup>21</sup> and 20 µg aliquots were electrophoresed in 1.2% agarose gels containing formaldehyde and stained with ethidium bromide to check equal loading and the intactness of 18S and 28S ribosomal RNAs. The RNA was then transferred onto a nylon filter and hybridized with rat *p53*,<sup>22</sup> human *c-myc*,<sup>19</sup> rat albumin,<sup>14</sup> or mouse β-actin (Wako, Osaka) probe cDNA <sup>32</sup>P-labeled by the random prime method. After stringent washing, hybridization signals on the filters were visualized by autoradiography with X-ray film overnight.

## RESULTS

### Morphology of MLEC10 cells comprising spheroids

Non-adherent cultures of MLEC10 cells resulted in the formation of multicellular spheroids, typically 5 to 10 cells in thickness, within 24 h. Histological examination of one-day-old spheroids revealed that the cells had fairly eosinophilic, abundant cytoplasm with round to ovoid nuclei carrying one or two prominent nucleoli (Fig. 1A). Binuclear cells were occasionally noticed. Considering those features, we histopathologically diagnosed the MLEC10 cells to be of hepatocytic lineage, although the cells appeared immature in terms of nuclear atypia and resembled hepatocellular carcinoma cells rather than normal hepatocytes (Fig. 1A). Their nuclei tended to become smaller with culture time. Thus, the spheroidal MLEC10 cells on day 7 exhibited a more benign morphology (Fig. 1B). Another notable finding was that the spheroids contained numerous eosinophilic apoptotic bodies resembling the Councilman bodies described in human livers with acute hepatitis (Fig. 1A). Intra-spheroidal apoptotic bodies were located within either intercellular spaces or the cytoplasm of viable cells. Extra-spheroidal apoptotic bodies floating in the medium were also encountered.

The Ras-1 cell line showed a morphological appearance similar to that of the parental cell line. However, the nuclear atypism on day 1 was more pronounced (Fig. 1C). Time-dependent reduction in nuclear size was also evident for the Ras-1 cells on day 7 (Fig. 1D).

Electron-microscopic examination was performed on spheroids of MLEC10 on day 1. Most cells possessed numerous swollen mitochondria, well-developed microvilli and dense cytoplasmic deposits, possibly resulting from phagocytosis of apoptotic bodies (Fig. 2A). Bile canaliculi sealed with junction complexes were occasionally formed

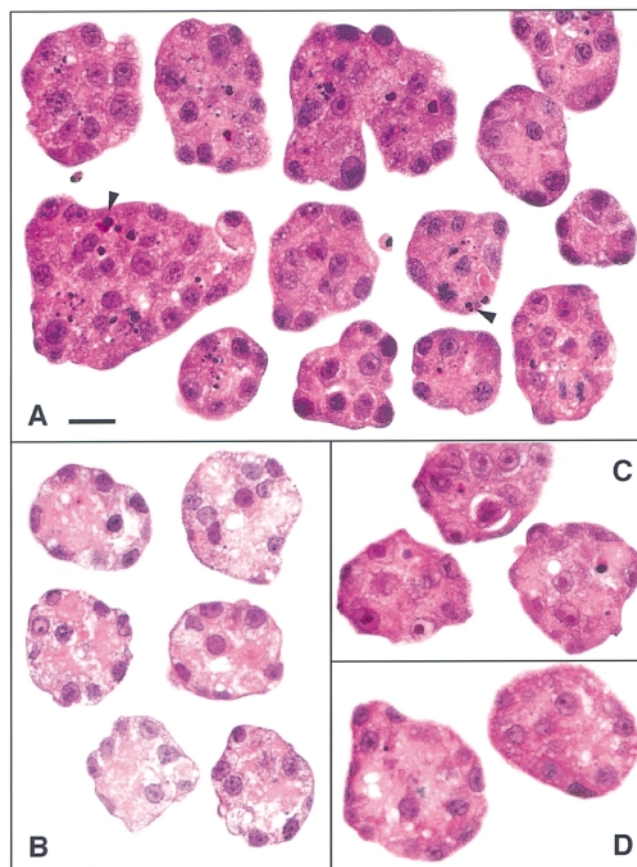


Fig. 1. Histological appearance of mouse liver epithelial cells in spheroidal aggregate culture (H&E staining. Bar=20 µm). (A) MLEC10 cells on day 1. (B) MLEC10 cells on day 7. (C) Ras-1 cells on day 1. (D) Ras-1 cells on day 7. Arrowheads indicate representative apoptotic bodies. For A and C, only relatively intact spheroids were selected for presentation in order to show the morphology of viable spheroidal cells.

between adjacent cells (Fig. 2B). In addition, some cells were found to accumulate glycogen particles (Fig. 2B). These findings are consistent with a hepatocytic nature.

### Characterization of apoptosis and cellular proliferation

Fragmented genomic DNAs were extracted from spheroids and apoptosis was visualized in terms of ladder formation on gel electrophoresis. For both MLEC10 and Ras-1, DNA ladders indicating apoptosis were observed until day 3 (Fig. 3). Ladders then became undetectable, although smears of degraded DNAs were seen in the very low molecular weight range.

Viable cell number and BrdU-labeling of the MLEC10 and Ras-1 cells were sequentially quantified (Fig. 4). Although the number of viable MLEC10 cells slightly increased during the first 24 h, it rapidly decreased due to

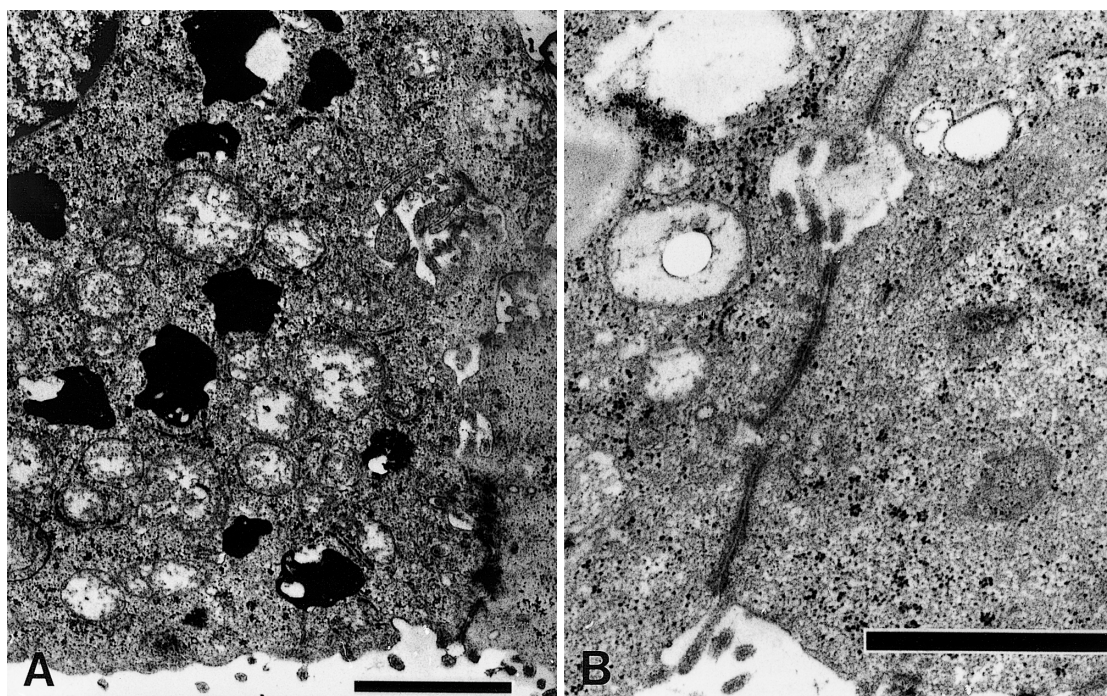


Fig. 2. Electron-microscopic appearance of MLEC10 cells on day 1 of spheroid culture (Bars=2  $\mu$ m). (A) Relatively intact cells with numerous swollen mitochondria, dense deposits in lysosomes, microvillous projections and junction complexes. (B) A representative bile canaliculi intercellular space sealed with junction complexes. Note that the cells contain glycogen particles.

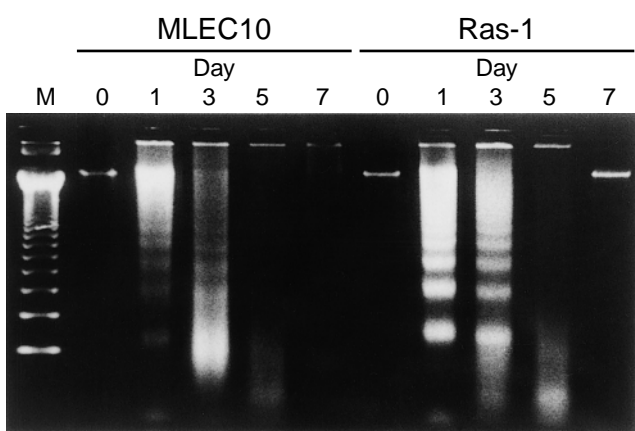


Fig. 3. Fragmentation of genomic DNAs due to apoptosis of spheroidal MLEC10 and Ras-1 cells visualized by ethidium bromide staining of an agarose gel after electrophoresis. Ladder formation indicates apoptosis. For day 0, DNA was extracted from monolayer cells. M=123 bp ladders as DNA size markers.

apoptotic death until day 3, when the cell number was only 25% of the initial figure (Fig. 4A). The BrdU-labeling index of MLEC10 cells was as high as 14% on day 1.

On day 2 and later, however, the cells were virtually unlabeled (Fig. 4B). The Ras-1 cells showed similar courses of changes in both the cell number and the labeling index. However, the onset of decrease in cell number was delayed by one day (Fig. 4A). In addition, their labeling index was always higher than that of the parental cells and, a low steady level of labeling (4%) remained even after day 3 (Fig. 4B).

**Expressions of the *p53*, *c-myc* and albumin genes**  
Expression of *p53* and *c-myc*, known to be involved in apoptosis and proliferation of many cell types,<sup>23)</sup> was studied by northern blot analysis, along with the albumin gene as an indicator of hepatocytic maturation.

When the MLEC10 cells were maintained as monolayers, expression from the *c-myc* and albumin genes was much higher in the logarithmic growth phase with 70% confluence than in the growth-restricted phase with 100% confluence (Fig. 5). A converse tendency was observed for expression of the *p53*. On the other hand, in spheroid culture, expression of all three genes was sequentially elevated, associated with growth arrest and cessation of apoptosis (Fig. 5).

As for the Ras-1 cells in monolayers, growth suppression due to 100% confluence caused higher expression of *c-myc* and albumin than with active growth under 70%

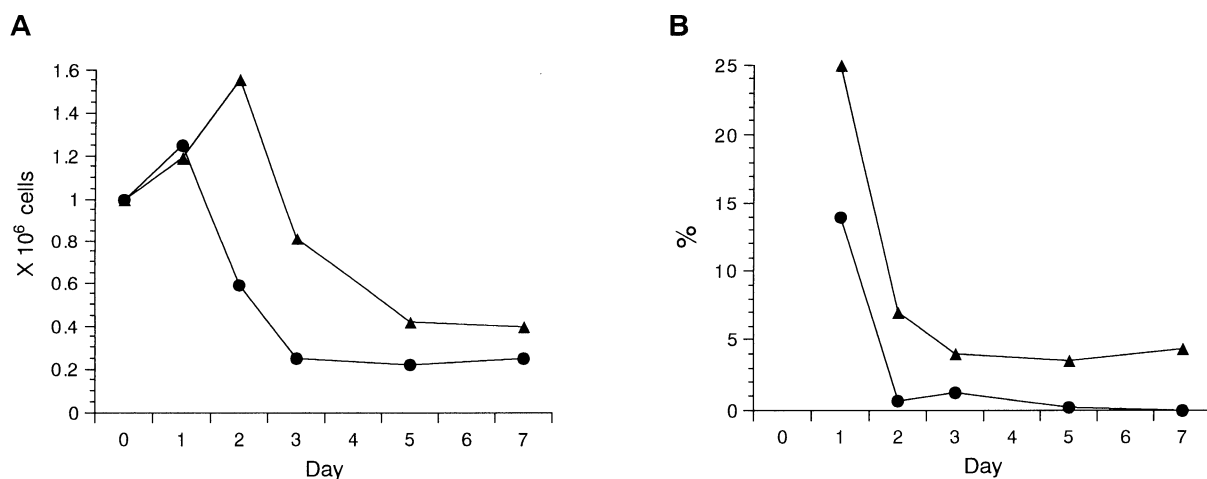


Fig. 4. Sequential quantification of viable cell numbers (A) and BrdU-labeling indices (B) for spheroidal MLEC10 and Ras-1 cells. Each value indicates the mean of data from three independent experiments. ● MLEC10, ▲ Ras-1.

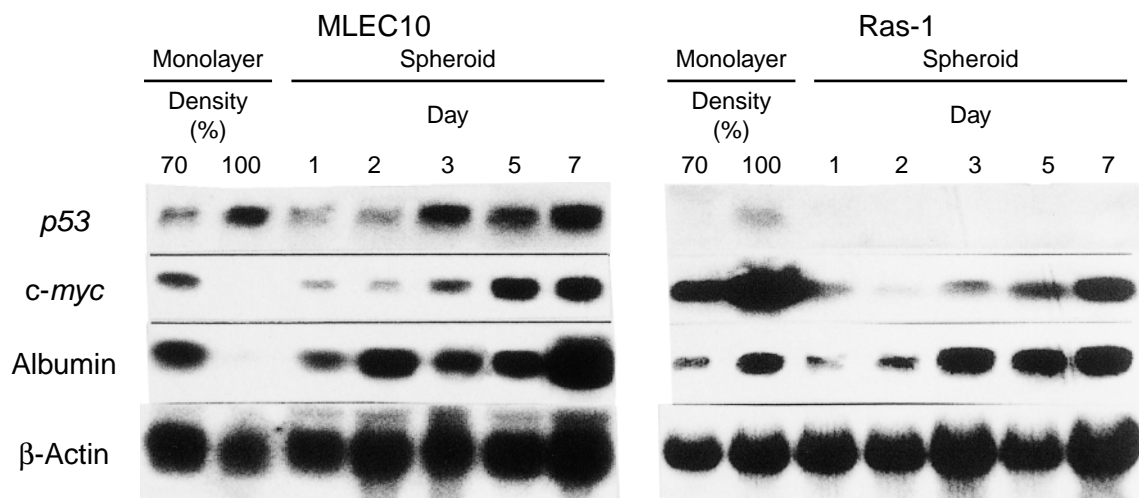


Fig. 5. Northern blot analysis of *p53*, *c-myc* and albumin gene expression in spheroids composed of MLEC10 or Ras-1 cells. Expression of  $\beta$ -actin was also analyzed to confirm equal loading of the samples.

confluence, in contrast to the parental cells (Fig. 5). With spheroid culture, on the other hand, expression patterns of both genes were similar to those of the parental cells (Fig. 5). In the Ras-1 cells, a trace level of *p53* mRNA was detected when they were cultured as 100% confluent monolayers (Fig. 5). Otherwise, the *p53* expression was undetectable at any time under any condition (Fig. 5). This was confirmed by long-term exposure northern blotting (data not shown).

## DISCUSSION

The present study was conducted to assess the histological cell type of MLEC10 cells, known to serve as precursors for hepatocellular carcinomas,<sup>13-15</sup> placed in a 3-dimensional environment. In order to determine whether they correspond to altered hepatocytes<sup>11, 12</sup> or oval cells<sup>6, 7</sup> *in vivo*, two options were initially considered. One was to back-transplant the cells into particular *in vivo* sites such

as the liver, spleen and dorsal fascia, because those organs are known to accept untransformed liver cells.<sup>24-26</sup> The other was to produce non-adherent, spheroidal aggregates in 3-dimensional culture, which can be then analyzed by routine histological methods.<sup>17, 18</sup> We selected the latter option because of its simplicity.

Based on routine histopathological examination of the spheroids, the MLEC10 cells were indistinguishable from immature hepatocytes, but distinct from oval cells. This does not necessarily preclude the idea that the cells originated from an oval cell as a putative stem cell *in vivo*, since one can still argue that hepatocytic differentiation occurred during establishment of the cell line or spheroid culture. For example, Coleman *et al.* demonstrated that cells of a rat liver epithelial cell line exhibited a typical morphology of mature hepatocytes after intrahepatic transplantation into syngeneic animals, but they interpreted the result as hepatocytic differentiation of cultured stem cells rather than maturation of immortalized hepatocytes.<sup>27, 28</sup> However, our MLEC10 cell line and its transformed sublines invariably exhibited histological features of hepatocytes in 3-dimensional environments, including back-transplantation.<sup>13-15</sup> No histological evidence of an oval cell nature is yet available.

The spheroids produced by MLEC10 cells contained numerous apoptotic bodies, the extent of apoptosis positively correlating with proliferative activity of the cells. Since primary rodent hepatocytes have been shown to exhibit neither proliferation nor apoptosis in spheroid culture,<sup>17, 18</sup> the phenomena observed in this study may be attributed to the immortal phenotype of MLEC10 cells. We expected that the Ras-1 cell line, an *H-ras*-transformed tumorigenic subclone of MLEC10, might exhibit some resistance to apoptosis relative to the parental line, because reduced susceptibility to apoptosis is often associated with cellular transformation.<sup>29, 30</sup> However, they were actually no less sensitive to apoptosis, indicating that the *H-ras* activation is not sufficient to overcome cell death stimuli. Our results are in contrast to a previous report of activated *H-ras*-associated resistance to apoptosis in murine intestinal epithelial cells maintained as spheroids.<sup>31</sup> Acquisition by MLEC10 cells of such resistance would clearly require more genetic events. Elucidation of such events should allow insights into mechanisms of "progression" as the last stage of carcinogenesis.<sup>32</sup>

In the MLEC10 cells, expression of the *p53* tumor suppressor gene increased with growth arrest, consistent with the notion that the *p53* is a negative regulator of cell proliferation.<sup>23</sup> On the other hand, although functional defects of the *p53* gene can cause insensitivity to apoptosis,<sup>23</sup> in the present case the cell death may have been induced through mechanisms independent of *p53* since a negative

association with this tumor suppressor gene expression was found to exist in the MLEC10 cells. Moreover, whereas the Ras-1 cells did not express detectable amounts of *p53* mRNA, probably due to the *H-ras*-induced transformation, they were found to be susceptible to apoptosis under spheroid culture. Further studies are needed to clarify the molecular pathway of the apoptosis observed in this study.

It is of interest that expression patterns of the *c-myc* gene, a positive regulator of proliferation and apoptosis,<sup>23</sup> and the albumin gene, a marker of hepatocytic maturation, in MLEC10 cells were dependent on culture conditions. In monolayer culture, the cells highly expressed both genes during the logarithmic growth phase, relative to the quiescent phase on reaching confluence, consistent with previous reports for rat liver epithelial cell lines.<sup>8, 33</sup> In contrast, increased levels of mRNAs from those two genes were accompanied by growth arrest in spheroid culture. These findings suggest that growth-arresting mechanisms in spheroid cells are qualitatively different from those acting in monolayers. The gradual increase in *c-myc* expression might indicate that the spheroidal cells continuously try to divide even after growth arrest. However, in order to prevent apoptosis due to proliferation, they might be prohibited from growing by means of an emergency mechanism overcoming the *c-myc* expression. It is of interest that, as for Ras-1 cells, higher expression of the genes was always associated with suppressed growth activity irrespective of culture type. This may be relevant to autonomous growth regulation in transformed cells.

Most epithelial cells *in vivo* lie on a basement membrane covering the surface of stroma composed of connective tissue. Thus, where this is the case, monolayer cultures imitate the *in vivo* status. However, both normal and neoplastic hepatocellular plates are surrounded by Disse's space, which contains fluid from the sinusoids, but lacks basement membrane. This particular situation may be better reconstituted by spheroid culture. In conclusion, the present *in vitro* system should be a simple and useful tool for investigating the morphology and biology of liver cells placed into *in vivo*-like conditions. Histological characterization of other liver epithelial cell lines and so-called oval cell lines should provide important information on their origins and lineages.

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