BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **222,** 64–70 (1996)
ARTICLE NO. 0698

Reduced Growth Capacity of Hepatocytes from c-myc and c-myc/TGF-α Transgenic Mice in Primary Culture

Chien-Yuan Kao, Valentina M. Factor, and Snorri S. Thorgeirsson[1]

Laboratory of Experimental Carcinogenesis, Division of Basic Sciences, National Cancer Institute, Bethesda, Maryland 20892

Received March 29, 1996

We have previously shown that coexpression of c-myc and TGF-α in the liver results in accelerated replicative senescence and promotes tumor development in young adult transgenic mice. Here we describe the characteristics of hepatocyte proliferation in primary cultures established from 10-week-old control, c-myc and c-myc/TGF-α transgenic mice. A variety of cellular and functional changes occurred in the transgenic livers at this age including enhanced polypoidization and impairment of hepatic functions. Control mouse hepatocytes demonstrated a high level of DNA synthesis in serum-free medium with a maximum at day three in culture at which time 70% of the cells were in S phase. In contrast, DNA synthesis peaked one day later and was reduced by 50% in the cultured c-myc and c-myc/TGF-α hepatocytes. Also, higher frequency of apoptosis was observed in the transgenic hepatocytes. However, in hepatocytes isolated from c-myc/TGF-α mice, which show early appearance of preneoplastic lesions *in vivo,* the DNA synthesis continued for 6 days in culture in contrast to a sharp decrease in the labeling index of control and c-myc hepatocytes after 3–4 days in culture. The results suggest that proliferative features of the transgenic hepatocytes *in vitro* reflect the general properties of these cells *in vivo* and thus may provide a model for studies on senescence and transformation of hepatocytes.

The steady loss of a replicative capacity in cultured human and rodent cells is a well established phenomenon generally termed cellular senescence or the finite lifespan phenotype (1–3). There is a growing body of evidence that the replicative lifespan of cells in culture is inversely proportional to the age of tissue donor (4,5). However, the relationship between reduced growth properties in culture and aging *in vitro* is still unclear. An important approach to study cellular aging is a modulation of organismal or tissue growth and determination of senescence associated alterations *in vitro* (6–8).

Recently a c-myc/TGF-α double transgenic mouse model has been established in our laboratory in which a continuous cell proliferation induced by liver specific expression of c-myc and transforming growth factor-alpha (TGF-α) results in premature aging of differentiated hepatocytes and dramatically accelerates hepatic tumor development (9,10).[2,3] The most obvious signs of aging were attenuated growth response following two-thirds partial hepatectomy and the reduction in a fraction of cells involved in the regenerating response in young adult mice.[2]

To extend these observations the present study was aimed at characterizing the proliferative potential of hepatocytes isolated from c-myc and c-myc/TGF-α transgenic mice in primary culture. For this purpose we employed tissue culture conditions that resulted in a rapid down regulation of the c-myc transgene expression and therefore allowed an appropriate comparison of the intrinsic proliferative properties of transgenic and control hepatocytes. The results suggest that the reduced

[1] Fax: (301) 496-0734, E-mail: snorri_thorgeirsson@nih.gov.

Abbreviations: TGF-α: transforming growth factor-alpha; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; GGT: γ-glutamyl transferase.

[2] Factor, V.M., Jensen, M.R. and Thorgeirsson, S.S. Constitutive expression of c-myc and TGF-α in livers of transgenic mice results in replicative senescence of hepatocytes. Submitted for publication.

[3] Santoni-Rugiu, E., Nagy, P., Jensen, M.R., Factor, V.M. and Thorgeirsson, S.S. Evolution of neoplastic development in the liver of transgenic mice coexpressing c-myc and transforming growth factor-α. Submitted for publication.

0006-291X/96 $18.00

growth properties of adult transgenic hepatocytes *in vitro* might reflect a replicative senescence caused by enhanced cell proliferation driven by transgenes *in vivo* and emphasize the usefulness of the c-myc/TGF-α double transgenic model to study the relationship between aging and hepatocarcinogenesis.

MATERIALS AND METHODS

Transgenic mice. Ten-week-old control B6CBAF1, Alb/c-myc single transgenic and Alb/c-myc/MT-1/TGF-α double transgenic mice were used in this study. The generation of the transgenic mice was as previously described (9,11). All animal study protocols conformed to NIH guidelines for animal care.

Liver functional assay. Blood was obtained from retroorbital puncture, and serum concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), γ-glutamyl transferase (GGT), triglycerides, cholesterol and albumin was measured by an automated multichannel analyzer (Analytics Incorporated, Gaithersburg, MD).

Hepatocytes isolation and proliferation assay. Hepatocytes were isolated by a two-step collagenase perfusion of the liver through the inferior vena cava via the right atrium (12). Liver was perfused 5 min with Hank' balanced solution w/o calcium and magnesium containing 10 mM HEPES (Quality Biological, Inc., Gaithersburg, MD) and 0.2 mM EGTA (Sigma) followed by 10–15 min perfusion with William's medium E (Biofluids, Inc.) containing 10 mM HEPES and 0.03% collagenase H (0.19 U/mg) (Boehringer Mannheim Biochemica). Viable hepatocytes ($\sim$95%) were selected by isodensity centrifugation in percoll (13). Hepatocytes were plated at a density of 0.3×10^6 cells per 35 mm dish in 1:1 mixture (v/v) of DMEM (BioWhittaker, Walkersville, MD) and a HAM's F-12 medium (Biofluids, Inc., Rockville, MD) supplemented with ITS$^+$ (Collaborative Research) (insulin 6.25 μg/ml, transferrin 6.25 μg/ml, selenious acid 6.25 ng/ml, bovine serum albumin 1.25 mg/ml, linoleic acid 5.35 μg/ml), 2mM glutamine (BioWhittaker), 30μg/ml proline (Gibco), 1 mg/ml galactose (Gibco), 18 mM HEPES (14), 1 mM sodium pyruvate (Gibco), 1.4×10^{-2} M sodium bicarbonate (Gibco) and 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). After 4 h attachment, media was changed to serum-free media as described above. Medium was changed every day. 25ng/ml EGF (Upstate Biotechnology Incorporated, NY), and 5×10^{-8} M dexamethasone (Sigma) were added when indicated. DNA synthesis in primary hepatocytes culture was measured after incubation of cells with 5 μCi of [methyl-^{3}H]thymidine (85Ci/mmol) (Amersham Life Sciences) for 24 h followed by TCA precipitation of cell extracts as previously described (15). ^{3}H-thymidine incorporation was measured in a liquid scintillation counter and normalized for DNA content.

Autoradiography. Cells labeled for 24 h with 5 μCi of ^{3}H-thymidine were fixed in cold ethanol and covered with Kodak NTB-2 emulsion. After a 2 week exposure at 4°C, autoradiography was developed and counterstained with Giemsa.

Measurements of DNA content. Freshly isolated hepatocytes after percoll purification were stained with propidium iodide (PI) using Cycle TEST PLUS DNA reagent Kit (Becton Dickinson). The intensity of PI in the isolated hepatocyte nuclei was measured using Epics Profile Flow Cytometry (Coulter Electronic, Inc., FL).

Isolation of RNA and Northern blot hybridization. Total RNA was isolated from cells by RNA STAT 60 (TEL-TEST "B", Inc., Friendswood, TX). 10 μg of each RNA sample was separated by electrophoresis in 1% agarose/0.75% formaldehyde gels. The separated RNAs were transferred to Nytran (a nylon membrane) (Schleicher & Schuell, Keene, NH) and hybridized with [^{32}P]-labeled probes using the QuikHyb hybridization solution (Stratagene, La Jolla, CA). The probes utilized were: a 316-base pair BamHI/EcoRV rat TGF-α cDNA fragment, a 1.4-kb BamHI fragment of mouse c-myc cDNA and a 0.51-kb fragment of rat albumin gene. After washing off the unspecific binding, the membrane was exposed to a Kodak XAR film (Eastman Kodak Co., Rochester, NY).

Determination of apoptosis. The apoptotic death was determined in a series of 3-day cultures each established from 3 transgenic and control animals. Hepatocytes were plated in chamber slides with the same cell density as for DNA synthesis analysis. Cells were fixed in 70% ethanol for 30 min at 4°C, and stained with propidium iodide (5 μg/ml) in the presence of RNase A (25 U/ml) for 1 hr at 37°C. The apoptotic bodies were visualized by fluorescent microscopy. Their frequency was determined after counting of 1000 nuclei.

RESULTS

Liver functions. To monitor the transgene-induced hepatocyte alterations we measured the activity of circulating levels of aspartate aminotransferase and alanine aminotransferase (Table 1). The levels of both enzymes were 9–11 fold higher in c-myc and c-myc/TGF-α mice than in age-matched controls indicating the injury of liver parenchyma in young adult transgenic animals (Table 1). The activity of alkaline phosphatase was about twofold higher in c-myc single transgenic than in c-myc/TGF-α double transgenic mice when compared with that in controls (Table 1). c-myc/TGF-α transgenic mice demonstrated 2–2.5 fold increase in serum levels of triglycerides and cholesterol indicating changes in lipid metabolism. The serum levels of albumin were similar in control and transgenic mice.

TABLE 1
Liver Function Assays in 10-Week-Old Transgenic and Control Mice

Group of mice	No. of animals	Alanine amino-transferase (u/l)	Aspartate amino-transferase (u/l)	Alkaline phosphatase (u/l)	γ-Glutamyl transferase (u/l)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	Albumin (g/dl)
Control*	4	35.0 ± 4.7	38.5 ± 9.4	124.3 ± 8.6	3.5 ± 0.3	91.0 ± 2.8	101.3 ± 2.7	3.6 ± 0.1
c-myc	7	290.1 ± 99.6	380.6 ± 194	505.4 ± 91.8	5.6 ± 2.0	102.1 ± 6.5	185.1 ± 11.6	3.4 ± 0.1
c-myc/TGF-α	10	351.4 ± 86.5	451.3 ± 147.0	264.0 ± 2.6	2.1 ± 0.9	194.5 ± 26.2	251.5 ± 28.2	4.7 ± 0.4

* Data from (B6 × CBA)F1.
Note. Data represent the mean ± standard error.

Changes in ploidy levels. In 10 week old control liver 55% of nuclei were diploid and 42% were tetraploid as measured by flow cytometry of nuclear DNA content after percoll purification (Table 2). On average, the hepatocytes isolated from c-myc and c-myc/TGF-α mice were more polyploid indicating a higher rate of nuclear polyploidization in transgenic livers. However, the ploidy profile of isolated nuclei was different in c-myc and c-myc/TGF-α transgenic mice. c-Myc mice exhibited a significant shift of DNA distribution to polyploid (4n and 8n) nuclei with a sharp decrease in a fraction of diploid cells as occurs during the normal process of liver aging. In c-myc/TGF-α mice, despite a 4–5 fold increase in the proportion of 8n nuclei, the frequency of diploid cells was significantly higher. Taking into account a larger liver size in c-myc/TGF-α mice the difference in absolute number of 2n and 8n nuclei was even more pronounced.

Expression levels of endogenous and exogenous c-myc and TGF-α mRNAs. Expression of the c-myc transgene was detectable in freshly isolated hepatocytes only in the presence of dexamethasone (Fig. 1A,B). The expression of human TGF-α transgene did not depend on the presence of dexamethasone and despite a tendency to decline with time was relatively high throughout the period of study (Fig. 1). Because a stable expression of c-myc confers a strong proliferative advantage to cultured cells (16), we selected a serum free medium without dexamethasone as the most appropriate condition for comparison of the intrinsic proliferative capacity of control and transgenic hepatocytes. Under this tissue culture condition, both control and transgenic hepatocytes showed comparable levels of induction of endogenous c-myc and TGF-α mRNAs which correlated with the rate of hepatocyte proliferation (Fig. 1 and 2A). The c-myc/TGF-α primary hepatocyte cultures demonstrated a continuous expression of endogenous c-myc and TGF-α transcripts which did not decline during 6 days in culture.

DNA synthesis in primary culture of hepatocytes. Hepatocytes from the c-myc and c-myc/TGF-α showed a dramatic loss of viability during the perfusion procedure. Before percoll purification, the average percent of viable cells was ~30% in both transgenic mice compared with ~80% in control animals. However, more than 90% viability was achieved after percoll purification in all strains of mice. The percoll-purified hepatocytes showed the same plating efficiency as control cells (~70%) and formed monolayer in 24 h.

Control mouse hepatocytes cultured in serum-free medium had a high level of DNA synthesis which reached a sharp peak by the third day in culture when about 70% of nontransgenic cells

TABLE 2
Distribution of Nuclear Ploidy of Hepatocytes Isolated from 10-Week-Old Transgenic and Control Mice

Group of mice	2N	4N	8N	≥16N	Average ploidy (N)
Control	55.1 ± 0.35	42.1 ± 1.2	2.8 ± 0.6	0	2.99 ± 0.03
c-myc	28.2 ± 3.2	60.0 ± 1.4	11.1 ± 2.1	0.7 ± 0.3	3.96 ± 0.21
c-myc/TGF-α	39.7 ± 8.2	45.5 ± 5.3	13.9 ± 3.6	0.9 ± 0.5	3.91 ± 0.32

Note. Data represent the mean ± standard error of 3 mice (%).

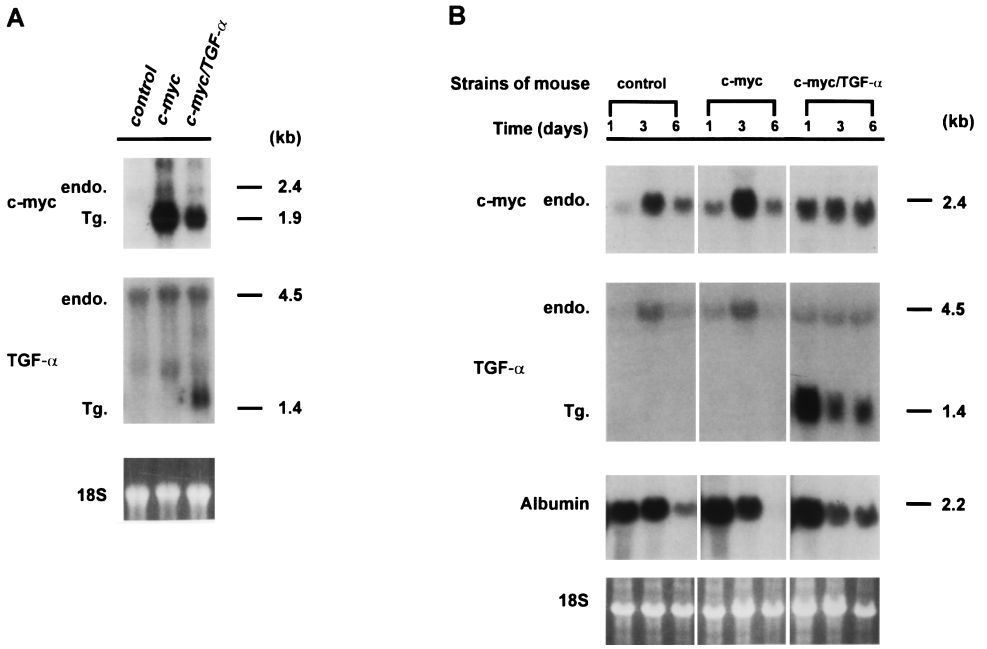

FIG. 1. The expression patterns of c-myc and TGF-α mRNAs in primary culture of hepatocytes isolated from control, c-myc, and c-myc/TGF-α transgenic mice in a serum-free medium with (A) or without (B) dexamethasone (5×10^{-8}M). (A) Total RNA was obtained from freshly isolated hepatocytes which were kept in the medium with dexamethasone. (B) Total RNA was obtained from hepatocytes cultured in serum free medium for 1, 3, and 6 days. In both (A) and (B), total RNA (10 μg/lane) were electrophrosed, blotted, and probed with c-myc, TGF-α, and albumin cDNA. Endo. and Tg. refer to endogeneous and transgene transcripts, respectively. Equal loading was confirmed by ethidium bromide stained 18sr-RNA.

incorporated ^{3}H-thymidine (Fig. 2A and 3). The proliferation of control hepatocytes resulted in 1.5 fold increase in DNA content per dish by the third day in culture (Fig. 2B).

The ability of transgenic hepatocytes to proliferate was significantly decreased under the same culture conditions (Fig. 2A). The rate of DNA synthesis peaked one day later and intensity of ^{3}H-thymidine incorporation was twofold less as estimated by both liquid scintillation counter and autoradiography. In contrast to control cells, the amount of DNA was gradually decreased in c-myc and c-myc/TGF-α transgenic hepatocyte cultures (Fig. 2B). These results were corroborated by data showing high frequency of apoptotic death, 6.9 ± 0.8% and 3.7 ± 0.8% in c-myc and c-myc/TGF-α transgenic hepatocytes, respectively, compared with 1.4 ± 0.2% in nontransgenic cells.

In contrast to both control and c-myc hepatocytes, the rate of DNA synthesis in the c-myc/TGF-α hepatocytes during the culture period (Fig. 2A). In addition, a significant variability was observed in DNA synthesis of c-myc/TGF-α hepatocytes among individual animals, unlike the uniform proliferative response in cultures established from nontransgenic as well as c-myc transgenic mice (Fig. 2C).

Supplementation of EGF did not further stimulate DNA synthesis in the hepatocytes from all strains of mice (data not shown). The addition of dexamethasone also did not influence the magnitude of proliferative response in control and transgenic hepatocytes but reduced and delayed the peak of DNA synthesis to the 6th day of culture (data not shown).

DISCUSSION

The present results show that the proliferative potential of young adult c-myc and c-myc/TGF-α transgenic hepatocytes is far less in culture when compared with age-matched nontransgenic cells.

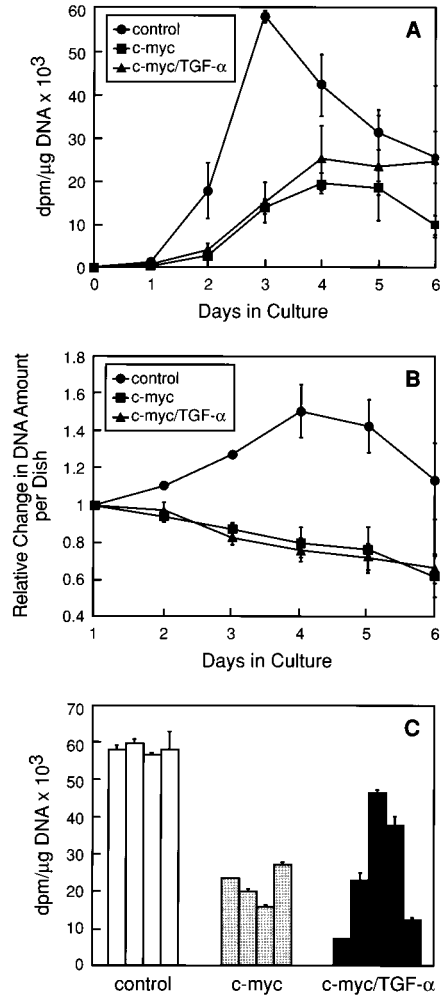

FIG. 2. Growth kinetics of hepatocytes derived from control, c-myc, and c-myc/TGF-α transgenic mice in primary cultures. Hepatocytes were isolated from 10-week-old transgenic and nontransgenic mice and cultured in serum-free medium as described. Medium was changed every day, and hepatocytes were labeled with ^{3}H-thymidine for 24 h prior to trichloroacetic acid precipitation. (A) The time course of ^{3}H-thymidine incorporation in primary cultures. Data represent the mean ± standard error from 4–5 experiments carried out on triplicate plates. (B) Changes in the relative amount of DNA per dish, as compared to that at the 0 time point, during tissue culture. Data represent the mean ± standard error from 4–5 experiments carried out on triplicate plates. (C) Variability in DNA synthesis in the primary cultures established from different control, c-myc, and c-myc/TGF-α mice at the 3rd day of culture. Data represent the mean ± standard error. Bar, one experiment carried out on triplicate plates.

Under conventional serum-free conditions which favor primary hepatocyte proliferation and turn off c-myc transgene expression, the entry into S phase was twofold lower in transgenic hepatocytes in spite of similar levels of induction of endogenous c-myc and TGF-α mRNAs.

Inability to initiate DNA synthesis in response to growth stimulation is the most consistent feature of senescent cells (1–5). It therefore appears that reduced growth properties of cultured transgenic hepatocytes can reflect the exhaustion of the proliferative function caused by excessive cell proliferation during preceding liver growth *in vivo.* Similar conclusions were drawn from the studies involving suckling versus adult rat hepatocytes (17), and different types of cells from a variety of tissues derived from transgenic mice harboring the growth hormone gene (8) or rats fed on a caloric restriction diet (6,7).

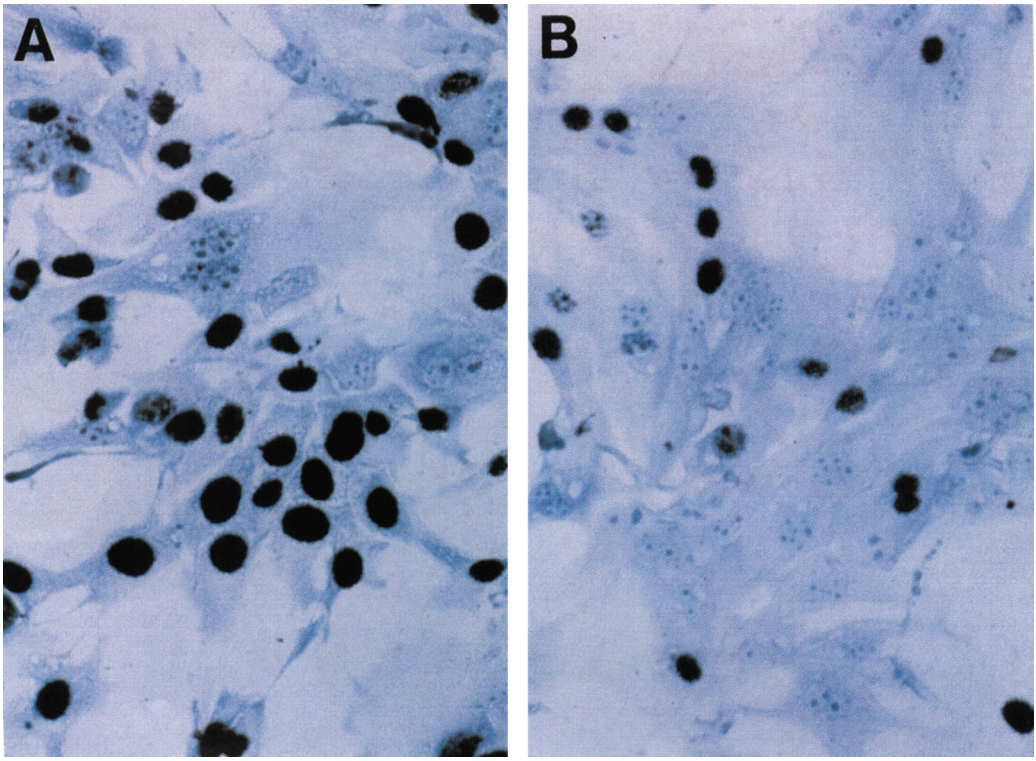

FIG. 3. Representative autoradiography of 3 days primary hepatocyte cultures. Control (A) and transgenic (B) cells were labeled with ^{3}H-thymidine for 24 h before fixation (200×, original magnification).

The reduced growth capacity of the cultured transgenic hepatocytes can be partly due to an enhanced polyploidization. A steady decrease of proliferative potential with an increase in ploidy levels is considered to be a part of a normal differentiation program of hepatocytes (18). With age, an increasing proportion of high ploidy hepatocytes fail to enter into S phase after mitogenic stimulation with partial hepatectomy (18). The reduced growth potential of polyploid hepatocytes versus diploid ones has also been demonstrated in primary culture of hepatocytes (19–21).

The hepatic cells proliferation was more vigorous and prolonged in the c-myc/TGF-α double transgenic mice than in c-myc montransgenic mice and resulted in twofold increase in liver mass between 1–2 months of age despite the substantial apoptotic death indicating both higher rate of cell turnover and cell injury.[2,3] The present results, taken together with our previous data (9,10),[2,3] show that hepatocytes in young c-myc/TGF-α transgenic mice display a number of cellular, functional and genetic changes which may be linked to the aging process. These include: (a) pronounced dysplasia defined by accumulation of giant cells with abnormal chromatin structure, (b) progressive polyploidization, (c) impairment of liver functions, (d) high frequency of chromosomal aberrations, (e) attenuated growth response following partial hepatectomy, and (f) reduced capacity for DNA synthesis *in vitro.*

In addition to extensive dysplasia, the most important feature that distinguishes young adult c-myc/TGF-α transgenic livers from age-matched c-myc tissue is an early emergence of preneoplastic cells.[3] Recently we have shown that transplantation of hepatic tissue from 10-week-old c-myc/TGF-α transgenic mice into nude mice gives rise to hepatocellular carcinomas composed of small diploid cells.[3] These data strongly suggest the presence of initiated cells in the early dysplastic livers. Our data on ploidy distributions show that despite a clear tendency towards increased

polyploidization, c-myc/TGF-α transgenic mice exhibit a higher frequency of diploid nuclei than c-myc monotransgenic mice. The appearance and selective growth of mononucleated diploid cell populations is a general feature of preneoplastic growth in the liver (22,23). The evidence supports the notion that these diploid cell populations most likely contain initiated cells capable of progression to malignant phenotypes (24).

The data presented in this report show that the rate of DNA synthesis did not decline in primary hepatocyte cultures established from c-myc/TGF-α double transgenic mice during 6 days in culture, in contrast to a sharp decrease in labeling indices in control and c-myc cultured hepatocytes. Furthermore, the extended proliferative capacity correlated with a constitutive level of expression of endogenous c-myc and TGF-α mRNAs, a stable expression of TGF-α transgene mRNA and a decrease in frequency of cells undergoing apoptosis. These data suggest that among c-myc/TGF-α transgenic hepatocytes there may exist cells with a potential for multiple replication in culture and that TGF-α may be capable of enhancing the survival of initiated hepatocytes (25).

In conclusion, we have described a culture system which might be useful for a selection of initiated hepatocytes versus terminally differentiated aging cells and may provide a relevant model to study multistep hepatocarcinogenesis *in vitro.*

ACKNOWLEDGMENT

The assistance of David Stephany (Flow Cytometry Section, LMS, NIAID, NIH) in flow cytometry analysis is gratefully acknowledged.

REFERENCES

1. Hayflick, L., and Moorhead, P. S. (1961) *Exp. Cell Res.* **25,** 585–621.
2. Peacocke, M., and Campisi, J. (1991) *J. Cell. Biochem.* **45,** 147–155.
3. Goldstein, S. (1990) *Science* **249,** 1129–1133.
4. Dice, J. F. (1993) *Physiol. Rev.* **73**(1), 149–159.
5. Cristofalo, V. J., and Pignolo, R. J. (1993) *Physiol. Rev.* **73,** 617–638.
6. Pendergrass, W. R., Li, Y., Jiang, D., Fei, R. G., and Wolf, N. S. (1995) *Exp. Cell Res.* **217,** 309–316.
7. Wolf, N. S., Penn, P. E., Jiang, D., Fei, R. G., and Pendergrass, W. R. (1995) *Exp. Cell Res.* **217,** 317–323.
8. Pendergrass, W. R., Li, Y., Jiang, D., and Wolf, N. S. (1993) *J. Cell. Physiol.* **156,** 96–103.
9. Murakami, H., Sanderson, N. D., Nagy, P., Marino, P. A., Merlino, G., and Thorgeirsson, S. S. (1993) *Cancer Res.* **53,** 1719–1723.
10. Sargent, L. M., Sanderson, N. D., and Thorgeirsson, S. S. (1996) *Cancer Res.* **56,** (in press).
11. Jhappan, C., Stahle, C., Harkins, R. N., Fausto, N., Smith, G. H., and Merlino, G. T. (1990) *Cell* **61,** 1137–1146.
12. Benveniste, R., Danoff, T. M., Ilekis, J., and Craig, H. R. (1988) *Cell Biochem. Funct.* **6,** 231–235.
13. Kreamer, B., Staecker, J. L., Sawada, N., Sattler, G. L., Hsia, M. T. S., and Pitot, H. C. (1986) *In Vitro Cell. Dev. Biol.* **22,** 201–211.
14. Li, Y., Sattler, G. L., and Pitot, H. C. (1993) *Biochem. Biophy. Res. Commun.* **193,** 1339–1346.
15. Michalopoulos, G., Houck, K. A., Dolan, M. L., and Luetteke, N. C. (1984) *Cancer Res.* **44,** 4414–4419.
16. Marcu, K. B., Bossone, S. A., and Patel, A. J. (1992) *Annu. Rev. Biochem.* **61,** 809–860.
17. Baribault, H., Leroux-Nicollet, I., and Marceau, N. (1985) *J. Cell. Physiol.* **122,** 105–112.
18. Brodsky, W. YA., and Uryvaeva, I. V. (1977) *Int. Rev. Cytol.* **50,** 275–332.
19. Sawada, N., and Ishikawa, T. (1988) *Cancer Res.* **48,** 1618–1622.
20. Gerlyng, P., Grotmol, T., Erikstein, B., Stokke, T., and Seglen, P. O. (1992) *Carcinogenesis* **13,** 1795–1801.
21. Mossin, L., Blankson, H., Huitfeldt, H., and Seglen, P. O. (1994) *Exp. Cell Res.* **214,** 551–560.
22. Saeter, G., Schwarze, E., and Seglen, P. O. (1988) *J. Natl. Cancer Inst.* **80,** 950–958.
23. Saeter, G., Schwarze, P. E., Nesland, J. M., Juul, N., Petterson, E. O., and Seglen, P. O. (1988) *Carcinogenesis* **9,** 939–945.
24. Hohne, M. W., Zieroth, S., Veser, U., Kahl, G. F., and Schwarz, L. R. (1993) *Mol. Carcinogen.* **7,** 180–189.
25. Nagy, P., Bisgaard, H. C., Santoni-Rugiu, E., and Thorgeirsson, S. S. (1996) *Hepatology* **23,** 71–79.