JOURNAL OF VIROLOGY, Sept. 1996, p. 6260–6268 Vol. 70, No. 9
0022-538X/96/$04.00+0

Insulin-Like Growth Factor II Blocks Apoptosis of N-myc2-Expressing Woodchuck Liver Epithelial Cells

DEYUN YANG,[1] RON FARIS,[2] DOUGLAS HIXSON,[2] SUZANNE AFFIGNE,[2] AND CHARLES E. ROGLER[1]*

Marion Bessin Liver Research Center, Departments of Medicine and Microbiology & Immunology, Jack & Pearl Resnick Campus, Albert Einstein College of Medicine, Bronx, New York 10461,[1] and Department of Medical Oncology, Rhode Island Hospital/Brown University, Providence, Rhode Island 02903[2]

Received 26 February 1996/Accepted 5 June 1996

N-myc2 and insulin-like growth factor II (IGF-II) are coordinately overexpressed in the great majority of altered hepatic foci, which are the earliest precancerous lesions observed in the liver of woodchuck hepatitis virus carrier woodchucks, and these genes continue to be overexpressed in hepatocellular carcinomas (HCCs). We have investigated the function of these genes in woodchuck hepatocarcinogenesis by using a woodchuck liver epithelial cell line (WC-3). WC-3 cells react positively with a monoclonal antibody (12.8.5) against woodchuck oval cells, suggesting a lineage relationship with oval cells. Overexpression of N-myc2 in three WC-3 cell lines caused their morphological transformation and increased their growth rate and saturation density in medium containing 10% serum. Removal of serum from the medium increased cell death of the N-myc2-expressing lines, whereas cell death in control lines was minimal. The death of N-myc2-expressing WC-3 cells was accompanied by nucleosomal fragmentation of cellular DNA, and DAPI (4′,6-diamidino-2-phenylindole) staining revealed condensation and fragmentation of the nuclei, suggesting that N-myc2-expressing WC-3 cells undergo apoptosis in the absence of serum. In colony regression assays, conducted in the absence of serum, control colonies were stable, while N-myc2-expressing colonies regressed to various degrees. Addition of recombinant human IGF-II to the serum-free medium blocked both cell death and colony regression in all the N-myc2-expressing lines. Therefore, coordinate overexpression of N-myc2 and IGF-II in woodchuck altered hepatic foci may allow cells which otherwise might die to survive and progress to hepatocellular carcinoma.

The three mammalian hepadnaviruses, hepatitis B virus, woodchuck hepatitis virus (WHV), and ground squirrel hepatitis virus, can establish long-term persistent infection in their hosts. Persistent infections of the liver cause a progressive liver disease which frequently culminates in primary hepatocellular carcinoma (HCC) (21). In the case of WHV persistent infection, HCC has been reported in nearly 100% of infected woodchucks (18, 23, 26). Progressive liver disease in WHV carrier woodchucks is associated with the proliferation of oval cells (10), which have been identified as hepatocyte progenitor cells in other experimental systems (9). Precancerous lesions in the liver, described as altered hepatic foci (AHFs), are also present in the liver of long-term WHV carrier woodchucks (1, 29). Studies of cell lineages during chemically induced hepatocarcinogenesis in rats have demonstrated a precursor-product relationship between oval cells and hepatocytes in precancerous lesions (9). Another line of investigation has used monoclonal antibodies to demonstrate lineage relationships between oval cells and hepatocytes in the liver (5, 6, 12). One such monoclonal antibody, which reacts with both woodchuck oval cells and hepatocytes, is described in this report.

One of our goals has been to characterize the changes in gene expression which occur in AHFs in vivo in order to design in vitro experiments to test whether these changes cause malignant progression of hepatocytes. Our initial approach was to utilize in situ hybridization to identify growth control genes which were deregulated in AHFs. These studies have led to the identification of two genes which are overexpressed in almost all the AHFs in WHV-infected woodchucks. These genes are the fetal growth factor, insulin-like growth factor II (IGF-II), and the nuclear proto-oncogene, N-myc2 (29, 30).

The predominant pattern of expression of N-myc2 and IGF-II in AHFs is a slightly elevated steady-state level of transcripts throughout the AHFs (29, 30). Superimposed upon this slightly elevated uniform pattern is a highly elevated expression pattern in limited groups of cells which have been termed "foci within foci" (29, 30). These small foci of high expression are rare compared with the common pattern of slightly elevated expression. This dual pattern of IGF-II expression is not unique for the woodchuck model, since a second cancer model, which overexpresses simian virus 40-Tag in the pancreas of transgenic mice, shows a similar expression pattern (3). In that model, high levels of IGF-II expression have been specifically linked to progression to pancreatic cancer (3). In WHV carrier woodchucks, high levels of both IGF-II and N-myc2 are very common in fully malignant HCCs (29, 30). Overexpression of IGF-II has also been observed in a wide variety of other malignancies (4).

Programmed cell death, termed apoptosis, has emerged as an important process in tumorigenesis (16). When apoptosis is blocked, tumor progression is enhanced, presumably because of the survival of mutagenized cells that would normally be eliminated from the tissue. The molecular genetic controls of apoptosis are complex and involve both proto-oncogenes and tumor suppressor genes (19, 28). One common finding is that overexpression of myc family proto-oncogenes can have dramatically different effects on cell growth depending upon the

* Corresponding author. Mailing address: Marion Bessin Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2607. Fax: (718) 430-8975.

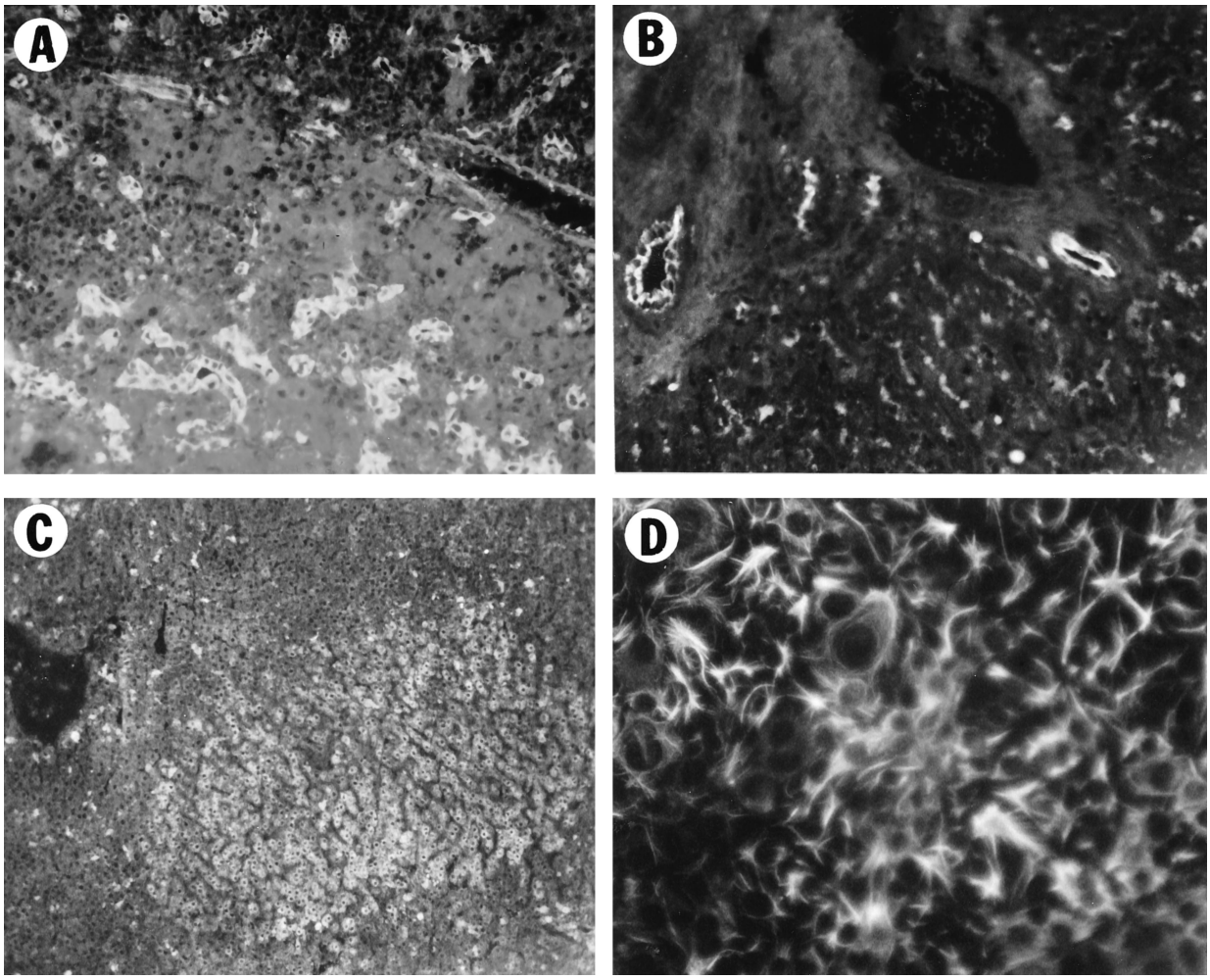


FIG. 1. Identification of cells which react with monoclonal antibody (MAb) 12.8.5 in the liver of woodchucks. (A) Micrograph showing 12.8.5-positive oval cells in the liver of a WHV carrier woodchuck. Magnification of immunofluorescent staining, ×200. (B) Micrograph of a normal woodchuck liver in which only the bile ducts were stained positively by MAb 12.8.5. Magnification of immunofluorescent staining, ×200. (C) Altered hepatic focus stained positively by MAb 12.8.5. Magnification of immunofluorescent staining, ×80. (D) Positive staining of WC-3 cells by MAb 12.8.5. Magnification of immunofluorescent staining, ×320.

expression pattern of other genes in the cell (8). In the presence of the correct growth factors, or with the expression of a set of cell survival genes, myc overexpression can lead to mitogenesis and promote malignant progression. However, in the absence of factors which enable the cell to progress through the cell cycle in response to myc overexpression, cells are often driven into an apoptotic pathway (8).

Previous surveys of growth factor overexpression in woodchuck liver have revealed overexpression of IGF-II, while overexpression of several other growth factors was not observed (10, 29, 30). This has also been the case in other animal models for hepatocarcinogenesis (24). In contrast, in humans, transforming growth factor α (TGF-α) overexpression has been reported (13, 22). Both IGF-I and IGF-II can promote mitogenesis as opposed to apoptosis in cells which overexpress myc proto-oncogenes (11, 27).

In this study we have tested the effects of N-myc2 and IGF-II on growth and morphological transformation of immortalized woodchuck liver epithelial cells, since these genes are overexpressed in naturally occurring HCCs. The woodchuck liver epithelial cell line which we have used, WC-3, has a hepatocytic phenotype and it expresses albumin and glucose-6-phosphatase (14). The WC-3 cell line also reacts with a monoclonal antibody which marks woodchuck bile ducts, oval cells, and transitional hepatocytes in vivo. We demonstrate that overexpression of N-myc2 in WC-3 cells causes morphological and growth changes characteristic of a partially transformed phenotype. Removal of serum from the medium of these cells transiently increases cell death compared with control cells. Treatment of the N-myc2-expressing cells with IGF-II blocks cell death induced by serum removal in several assays. These data suggest that in vivo overexpression of IGF-II in the liver may promote survival of N-myc2-expressing cells, which in turn may promote their propensity to progress to fully malignant HCC.

(Data in this paper are from a thesis to be submitted by Deyun Yang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.)

MATERIALS AND METHODS

Plasmid construction. The N-myc2 expression vector used in this study is schematically depicted in the top panel of Fig. 2. The pRc/RSV vector (Invitro-

gen) contains a Rous sarcoma virus long terminal repeat upstream from the multiple cloning site and a neomycin-resistant gene for selection. Plasmid Ph58-2b, containing a dimer of wild-type N-myc2 allele, was a kind gift of G. Fourel and M. Buendia. An *Avr*II-*Ssp*I fragment of plasmid Ph58-2b was inserted into the *Spe*I site of the pRc/RSV vector by blunt-end ligation to construct the N-myc2 vector.

Isolation of the 12.8.5 monoclonal antibody. The 12.8.5 monoclonal antibody was developed as previously reported (12). Initially, woodchuck oval cells were isolated from a WHV carrier woodchuck by a previously reported method (12). These cells were injected into a recipient mouse for the production of cells to be used for monoclonal antibody production as described earlier (12).

Cell culture and transfection. WC-3 cells were the kind gift of Tomo Kitagawa, Cancer Institute, Tokyo, Japan. The cells were routinely cultured in Dulbecco's minimal essential medium/F12 (GIBCO BRL) containing 1× antibiotics and 10% fetal bovine serum. Transfection was performed with Lipoamine (GIBCO BRL). WC-3 cells were transfected with either pRc/RSV/N-myc2 or pRc/RSV vector. Briefly, 2×10^5 WC-3 cells were plated into 35-mm plates and allowed to grow to 80% confluence. Lipofectin suspension containing 2 μg of plasmid DNA, prepared according to the manufacturer's instructions, was added per plate, and 15 h later medium containing 20% serum was added. Seventy-two hours after transfection, cells were split into 10 100-mm plates, and selection medium containing 400 μg of G418 per ml was added. Single colonies were selected and amplified into cell lines.

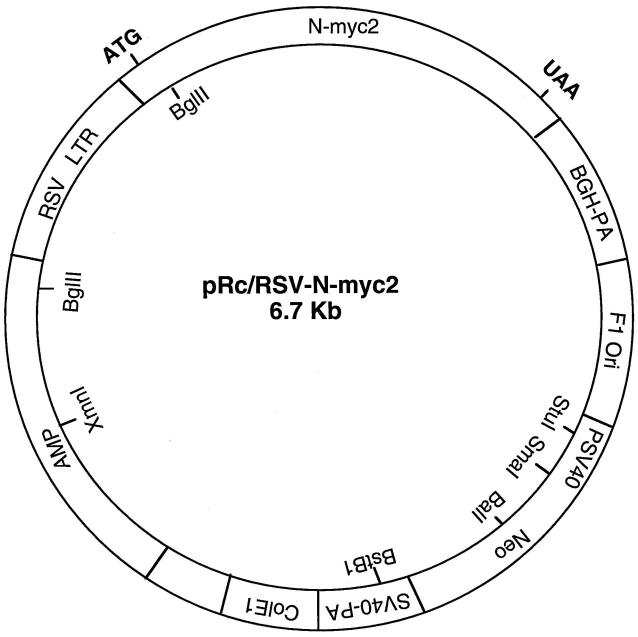
RNA isolation and Northern (RNA) blot hybridization. Total RNA was isolated with TRIZOL reagent (GIBCO BRL). Total RNA (20 μg per lane) was separated by 1.2% denaturing agarose gel electrophoresis and transferred to a nylon membrane (Genescreen; Dupont); this was followed by baking in an 80°C vacuum oven for 2 h. The blots were prehybridized in 10× Denhardt's solution at 42°C for 2 h. Hybridization was performed at 42°C overnight with ^{32}P-labeled DNA probes (random priming) in hybridization buffer which contained 2.5× Denhardt's solution, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 50% formamide. The blots were washed twice in 2× SSC plus 0.1% sodium dodecyl sulfate (SDS) for 15 min each time at room temperature; this was followed by two 30-min washes in 0.1× SSC plus 0.1% SDS at 65°C. After being washed, the blots were exposed to X-ray film and stored at −70°C for 1 to 3 days.

Growth curve assays. WC-3 cells were plated at a density of 1.5×10^5 per 60-mm plate or 10^5 per 35-mm plate and allowed to attach overnight in complete medium. Then, the serum containing medium was removed and the cells were washed twice in phosphate-buffered saline (PBS) before serum-free medium, serum-free medium plus recombinant IGF-II (R&D Systems), or complete medium was added to the culture. The number of cells was determined by using either Trypan blue exclusion or Coulter Counter at different time intervals. Three plates were counted for each time point. For the ratios of dead to living cells, the numbers of dead and living cells were determined at each time point by using Trypan blue exclusion. Growth curves and ratios of dead to living cells were plotted by using the StatView 4.01 program.

Colony regression assays. Cells were plated at a density of 10^4 per 100-mm plate and between 47 and 72 colonies of 100 to 500 cells were identified for each experimental group and counted at the beginning of the experiment. The plates were washed twice in PBS before serum-free medium or serum-free medium plus recombinant IGF-II (R&D Systems) was added to the culture. The morphology of colonies was observed by phase-contrast microscopy at different times after the onset of serum starvation. Colonies containing a clearly increased number of dead cells or which had completely detached from the plate were categorized as regressing. The total number of regressing colonies was determined at 24 h and used as the numerator. The total number of colonies at 0 h served as the denominator. The results are shown as percentages of colonies showing regression.

Immunofluorescence. Five-micrometer cryostat sections of liver tissue, or cultured cells on chamber slides, were fixed in ice-cooled acetone for 10 min; this was followed by washing twice in PBS. Nonspecific staining was blocked with 3% normal goat serum in PBS for 1 h at 37°C. Supernatant of monoclonal antibody 12.8.5 was applied to the slides and incubated for 1 h at 37°C in a humidified chamber. Supernatant of the P3 mouse myoloma cell line was used as a negative control. After being washed in PBS three times for 5 min each at room temperature, the slides were incubated with fluorescein-conjugated goat anti-mouse immunoglobulin G (1:40 dilution) for 30 min at 4°C. After three 5-min washes in PBS, the slides were mounted with buffered glycerol and examined under a fluorescent microscope. Occasionally, the slides were counter-stained with Ewins blue before mounting.

DNA fragmentation assay. N-myc2-expressing cells and control cells were plated at a density of 10^6 per 100-mm plate and allowed to attach overnight in the complete medium. After removal of complete medium, cells were grown in serum-free medium, and at the indicated times, cells were harvested from the dishes and medium and total DNA was extracted from the pooled cells by lysis with 0.5% SDS buffer plus proteinase K (400 μg/ml) at 55°C overnight; this was followed by phenol extraction. The samples were treated with RNase A (100 ng/ml) for 1 h at 37°C; this was followed by phenol-chloroform extraction and ethanol precipitation. Ten micrograms of total DNA was electrophoresed through 1.7% agarose gels containing 0.5 μg of ethidium bromide per ml and examined on a UV transilluminator.



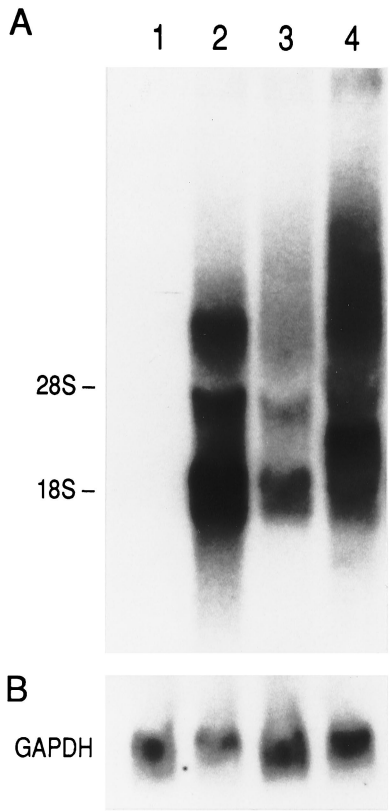


FIG. 2. Top panel depicts the N-myc2 expression vector used in this study. (A) Expression of N-myc2 in WC-3 cells and three N-myc2 transfectant lines as detected by Northern blot hybridization. Lanes: 1, WC-3 cells; 2, N-myc2-expressing cell line 10; 3, N-myc2-expressing cell line 40; 4, N-myc2-expressing cell line 36. (B) The same blot was eluted and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as loading control.

Tunel assay. N-myc2-expressing cells and control cells were plated at a density of 5×10^4 per well on four-chamber slides and allowed to attach overnight in complete medium. After removal of complete medium, the cells were maintained in serum-free medium plus or minus 100 nM recombinant IGF-II for 24 h. The cells were fixed in 4% paraformaldehyde in PBS for 30 min at room

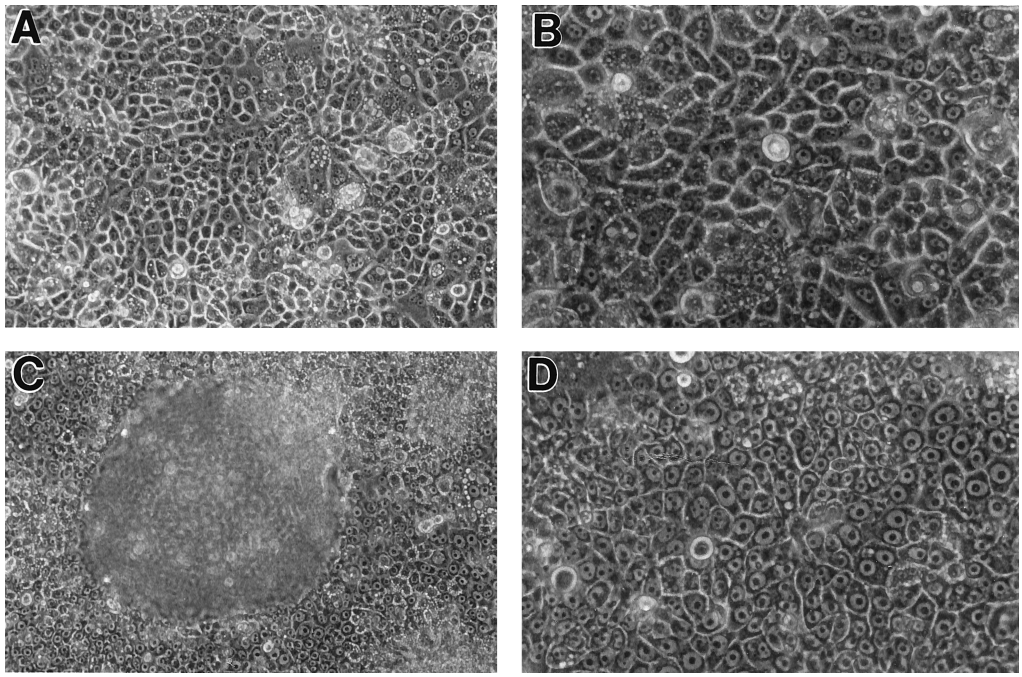


FIG. 3. Morphological changes of N-myc2-expressing and control WC-3 cells. (A and B) Micrographs of control WC-3 cells transfected with pRc/RSV vector. Magnification: panel A, ×100; panel B, ×400. (C and D) Micrographs of N-myc-expressing cell line 10. Magnification: panel C, ×100; panel D, ×400.

temperature. Endogenous peroxidase activity was blocked by incubating cells with 0.5% H_2O_2 in methanol (vol/vol) for 30 min at room temperature. After treatment with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, the cells were incubated with Tunel reaction mixture according to the manufacturer's manuals (in situ Cell Death Detection Kit; Boehringer Mannheim) at 37°C for 1 h. After being washed in PBS, the cells were incubated with converter-peroxidase solution for 30 min at 37°C; this was followed by washing in PBS. The slides were reacted with diaminobenzidine (DAB) solution (1.0 mg of DAB and 60 ng of H_2O_2 per ml of PBS) for 1 min; this was followed by several washes in H_2O. Then the cells were counter-stained with 0.5% methyl green in 0.1 M sodium acetate, pH 4.0, for 10 min, dehydrated in *n*-butanol, and cleared in xylene. After mounting in premount, the slides were observed under a light microscope.

For quantitation, 6 to 8 fields of ×200 magnification from each slide were randomly selected and photographed. The numbers of positive cells and total cells per field were determined for each slide. To avoid subjective bias, the quantitation was performed before decoding the micrographs. The data were entered into a computer by using the StatView 4.01 program. A histogram was generated and an unpaired *t* test was performed for statistical analysis.

DAPI staining. N-myc2-expressing cells and control cells were plated at a density of 2×10^5 per 35-mm plate. At 0, 3, 6, 12, and 24 h of serum starvation, the cells were fixed with 10% buffered formalin plus 0.01% Triton X-100 for 30 min, stained with 50 μg of DAPI (4′,6-diamiidno-2-phenylindole) per ml for 10 min, and then examined microscopically under an UV illuminator.

RESULTS

Oval cells and selected hepatocytes in woodchuck liver share a common marker with WC-3 liver epithelial cells. Woodchuck oval cells were isolated from the liver of a WHV carrier woodchuck by a method which employed both collagenase and pronase digestion of the liver, as reported for the isolation of rat liver oval cells (12). When aliquots of woodchuck oval cell preparations were cultured in vitro, we observed groups of liver epithelial cells with the appearance of hepatocytes (data not shown). A cell suspension of the woodchuck oval cells was used to immunize BALB/c 3 mice for the preparation of monoclonal antibodies as described earlier (12). We screened the supernatants of approximately 450 hybridoma cell lines for reaction with specific cell types in WHV-infected woodchuck livers. We obtained a series of monoclonal antibodies which reacted with various components of woodchuck liver, including sinusoidal lining cells, hepatocytes, oval cells, and bile ducts (data not shown). We selected a monoclonal antibody, designated 12.8.5, which reacted with woodchuck liver cells in the pattern expected for oval cells (Fig. 1).

This oval cell antibody reacted with cells in WHV carrier woodchuck liver which extended out from the portal tracts and were present as either single cells or groups of cells which appeared as pseudoduct-like structures (Fig. 1A). The monoclonal antibody also reacted with bile ducts (Fig. 1B), which is consistent with the proposed origin of oval cells from stem cells in the canals of herring (25). Using the 12.8.5 antibody, we were not able to detect any oval cells in normal uninfected woodchuck liver (Fig. 1B). The degree of oval cell proliferation detected by the 12.8.5 antibody varied greatly among woodchucks according to the severity of pathological changes associated with persistent WHV infection.

We also identified small groups of hepatocytes which reacted positively with the 12.8.5 antibody in chronically WHV-infected livers (Fig. 1C). We did not detect any positively reacting hepatocytes in uninfected woodchuck liver (Fig. 1B). The pattern of immunofluorescence in 12.8.5-positive hepatocytes suggested that the antigen identified by the 12.8.5 antibody was present in the cytoskeleton of hepatocytes. The WC-3 woodchuck liver epithelial cell line also reacted positively with the 12.8.5 antibody, and the pattern of immunofluorescence in WC-3 cells also suggested that the antibody was reacting with a component of the cytoskeleton (Fig. 1D). These data are consistent with an oval cell origin for the WC-3 cell line.

Overexpression of N-myc2 causes a partially transformed phenotype of WC-3 cells. WC-3 cells were transfected with the N-myc2 expression vector, pRc/RSV/N-myc2, and three stably transfected lines were selected which expressed N-myc2 in high abundance (Fig. 2A). Two lines which were stably transfected with the pRc/RSV vector were also established as controls. Morphologically, the N-myc2-expressing cells exhibited an in-

TABLE 1. Characterization of N-myc2 transfectant lines

Cell line	Doubling time (h)	Saturation density/ 100-mm plate	Foci formation/cm^2	Soft agar growth
WC-3 RSV9	25.0	3.6×10^6		None
WC-3 MYC10	21.5	7.0×10^6	57	None
WC-3 MYC36	18.0	9.4×10^6	118	None
WC-3 MYC40	18.0	1.0×10^7	ND[a]	None

[a] ND, not done.

creased nuclear/cytoplasmic ratio in comparison to control cells and contained prominent nucleoli as shown in Fig. 3D. The N-myc2-expressing cells formed foci when grown on plastic, suggesting a loss of contact inhibition, while the control WC-3 cells remained as a monolayer (Fig. 3A and C; Table 1). However, the N-myc2-expressing cells failed to grow in soft agar (Table 1). In comparison to the control cell lines, all three N-myc2-expressing lines exhibited moderately increased growth rates and increased saturation density when maintained in complete medium (Table 1).

IGF-II blocks cell death in WC-3 cells which overexpress N-myc2. We next determined whether overexpression of N-myc2 was associated with increased growth or cell death upon removal of serum from cell culture media. N-myc2-expressing lines and control lines were plated at 10^5 per 60-mm plate in complete medium; this was followed by removal of serum from the medium. Twenty-four hours after serum removal, survival of N-myc2-expressing cells was reduced to a greater extent than it was in control cells (Fig. 4A). However, between 24 and 48 h of serum starvation, N-myc2-expressing cells did not continue to die in the absence of serum; instead, they resumed growth as did all the other cell lines whether in complete or serum-free medium (Fig. 4A).

Twenty-four hours after serum removal, the ratio of dead to living cells in cultures was threefold higher in N-myc2-expressing cultures compared with controls, whereas by 48 h the ratio of dead to living cells returned to normal (Fig. 4B). These data suggest that after 24 h WC-3 cells secrete autocrine growth factors which support their growth in the absence of serum.

Next we tested whether addition of recombinant IGF-II to the serum-free medium increased the survival of N-myc2-expressing cells during the first 24 h after serum removal. The results are shown in Fig. 5. The base level of cell death was greater in the N-myc2-expressing lines compared with controls in the presence of 10% serum, suggesting that growth factors were limiting in the serum. Both control and N-myc2-expressing cells exhibited an increase in the ratio of dead to living cells after 24 h of serum starvation. However, addition of IGF-II in the serum-free medium had no effect on the survival of the control cells, whereas addition of 100 nM recombinant IGF-II to the serum-free medium increased the survival of each of the N-myc2-expressing cell lines (Fig. 5).

Finally, we conducted a colony regression assay to study the effect of N-myc2 overexpression on colony survival in the presence or absence of serum or added recombinant IGF-II. This assay would determine both whether variability existed among the responses of subclones of cells within each cell line to the removal of serum and the effect of IGF-II on restoration of cell survival of specific subclones. We tested N-myc2-expressing lines and colony regression was judged by the integrity of the colony (Fig. 6).

N-myc2-expressing cell lines and control cell lines were plated at low density and colonies were grown in the presence of serum. Recombinant IGF-II was added at the time of serum removal, and 24 h later, survival or regression of a large number of premarked colonies was determined. This analysis showed that colonies of the control cell line showed no regression (Fig. 6A and B), while many N-myc2-expressing colonies exhibited regression (Fig. 6C and D). Addition of 100 nM recombinant IGF-II to the serum-free medium blocked colony regression of N-myc2-expressing colonies (Fig. 6E and F). A quantitative analysis of colony regression in N-myc2-expressing lines 36 and 40 is shown in Fig. 7. Colonies from both of these lines exhibited dramatic colony regression, and addition of 100 nM recombinant IGF-II to the medium immediately after serum removal almost completely blocked colony regression.

From these data we deduced that there were three different responses to N-myc2 and IGF-II among the colonies in each cell line. The responses included (i) no colony regression (about 60 and 40% of the colonies for line 40 and line 36, respectively), (ii) colony regression which could be blocked by the addition of recombinant IGF-II (approximately 35 and 55% of the colonies for lines 40 and 36, respectively), and (iii)

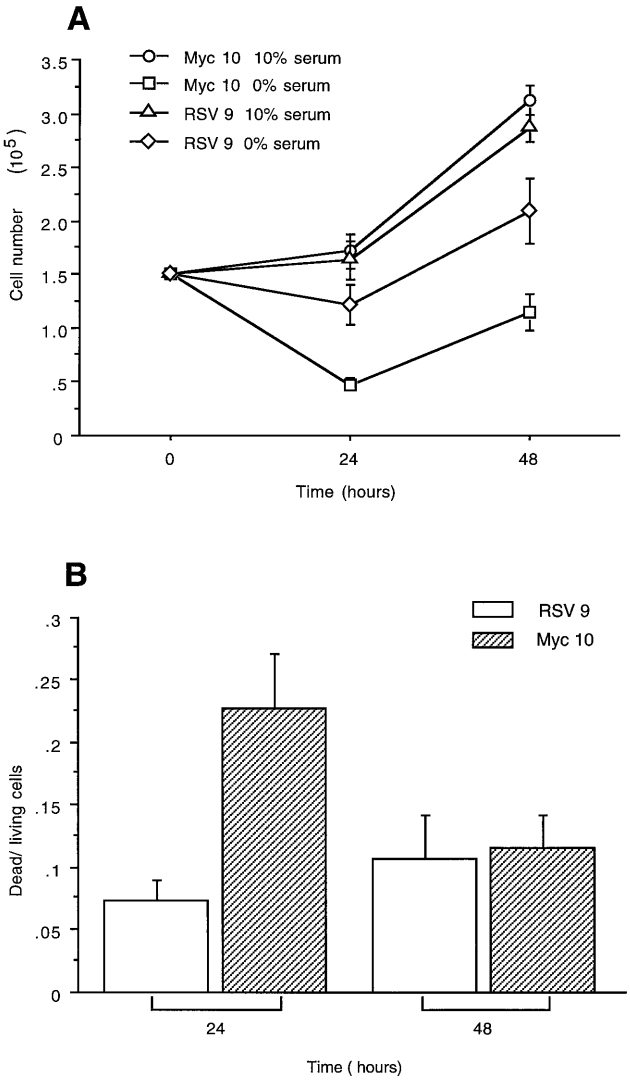


FIG. 4. Cell death induced by N-myc2 overexpression. (A) Growth curves of a N-myc2-expressing cell line (Myc10) and a control cell line (RSV9) in the presence or absence of 10% serum. (B) A histogram depicting the ratios of dead to living cells in the same growth curve assays as in panel A.

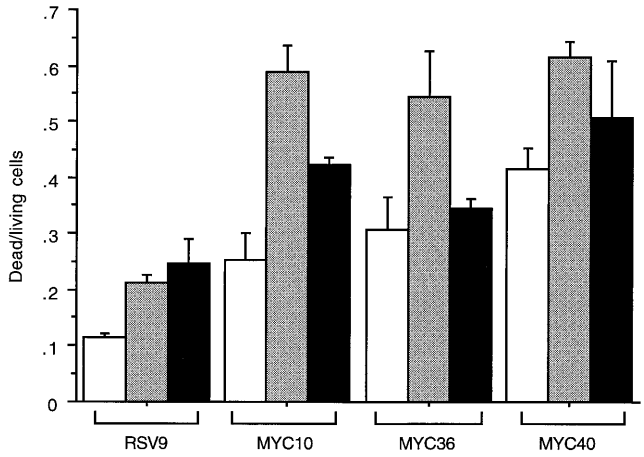


FIG. 5. Effects of recombinant IGF-II on cell death of N-myc2-expressing and control cell lines. The numbers of dead and living cells were determined 24 h after serum removal by using the trypan blue exclusion assay. The data are averages of three determinations. The ratios of dead to living cells were calculated and plotted as a histogram by using the Macintosh StatView 4.01 program. Open bar, 10% serum; shaded bar, 0% serum; solid bar, 0% serum and IGF-II.

colony regression not blocked by the addition of recombinant IGF-II (approximately 5% of the colonies in each cell line). Possible reasons for this variability are under investigation and will be discussed. However, a predominant response in both cell lines was a dramatic reduction in colony regression in response to the addition of recombinant IGF-II.

Apoptosis occurs in N-myc2-expressing WC-3 cells. Having demonstrated that overexpression of N-myc2 can cause cell death in WC-3 cells, we next investigated the nature of cell death by conducting three tests for apoptosis. These tests included (i) a DNA fragmentation assay, (ii) DAPI staining, and (iii) a Tunel assay.

Nucleosomal fragmentation of DNA has been described as a hallmark of apoptosis, although such DNA fragmentation does not occur in all the apoptotic processes (17). To determine whether the N-myc2-induced cell death is accompanied by nucleosomal fragmentation, we isolated total DNA from an N-myc2-expressing line and a control cell line at different times after initiation of serum starvation. Typical DNA laddering was observed in the N-myc2-expressing line at 24 h of serum starvation, while the control cells did not show such nucleosomal DNA fragmentation (Fig. 8). These results suggest that apoptosis occurred in N-myc2-expressing WC-3 cells.

We next performed DAPI staining, which reveals nuclear fragmentation, on the N-myc2-expressing cells and control cells. While we observed very few cells with nuclear condensation and fragmentation in control cells, nuclear condensation could be observed as early as 3 h, and nuclear fragmentation became evident at 24 h of serum starvation as shown in Fig. 9A. These changes in nuclei were also consistent with an apoptosis mode of cell death.

Finally, we conducted a Tunel assay to quantitate the effects of N-myc2 overexpression and recombinant IGF-II on the survival of WC-3 cells. Figure 9B represents an example of a Tunel assay in which two apoptotic cells were positively la-

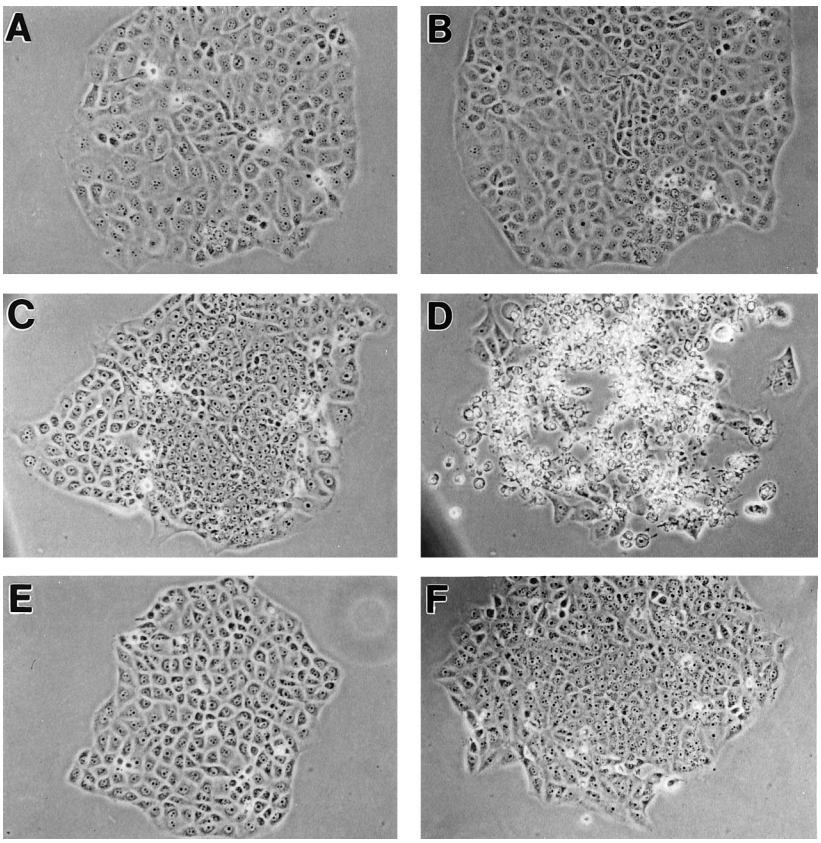


FIG. 6. Colony regression induced by N-myc2 overexpression is blocked by recombinant IGF-II. (A and B) A colony of control cell line (RSV9) at 0 and 24 h of serum starvation, respectively. (C and D) A colony of N-myc2-expressing cell line (Myc10) at 0 and 24 h of serum starvation, respectively. (E and F) A colony of Myc10 cells at 0 and 24 h of serum starvation to which 100 nM recombinant IGF-II was added to the medium at 0 h.

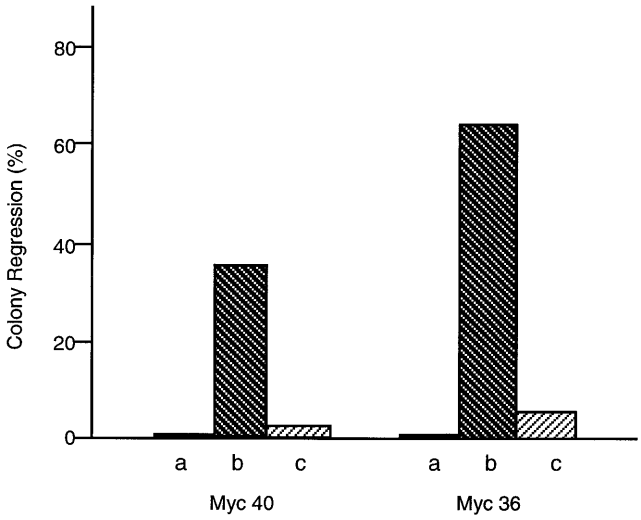


FIG. 7. Quantitative analysis of colony regression induced by N-myc2 overexpression after serum starvation and reversal by IGF-II. Lanes: a, regression of colonies maintained in 10% serum (complete medium); b, regression of colonies in which serum was removed 24 h earlier; c, regression of colonies in which 100 nM recombinant IGF-II was added to the medium at the time of serum removal. Myc 40, N-myc2-expressing cell line 40; Myc 36, N-myc2-expressing cell line 36. Colony regression is expressed as a percentage of the total number of colonies observed at the beginning of the experiment. The number of colonies present in the Myc 40 cell line at the beginning of the experiment were as follows: 10% serum, 68; 0% serum, 72; and 0% serum plus 100 nM IGF-II, 52. The respective numbers of colonies for the Myc 36 cell line were 62, 47, and 59.

beled. The results of a quantitative Tunel assay are shown in Fig. 9C. At 24 h of serum starvation, only a small number of control WC-3 cells were positive, while nearly 50% of N-myc2-expressing cells were positively labeled by the Tunel assay ($P < 0.001$). The number of positive cells was reduced dramatically for N-myc2-expressing cells in the presence of 100 nM recombinant IGF-II ($P < 0.01$).

From these data we conclude that the N-myc2-induced cell death is by apoptosis and that this mode of cell death can be blocked by IGF-II.

DISCUSSION

The WC-3 cell line was the first liver epithelial cell line established from a woodchuck liver (14). In this report we have shown that WC-3 cells share a common antigen, recognized by monoclonal antibody 12.8.5, with oval cells in the liver of WHV-infected woodchucks. The antigen is not present in hepatocytes from uninfected woodchucks, but it is present in isolated foci of hepatocytes in persistently infected woodchuck liver. These data are consistent with a possible cellular lineage between 12.8.5-positive oval cells and 12.8.5-positive hepatocytes in woodchuck liver.

The WC-3 cell line also expresses the 12.8.5 antigen. WC-3 cells also express γ-glutamyl transpeptidase, which is a fetal hepatocyte marker expressed in oval cells and hepatocytes in AHFs in woodchuck liver (14, 30). WC-3 cells also express glucose-6-phosphatase and albumin, which attest to their hepatocytic differentiation. Therefore, we conclude that WC-3 cells are woodchuck liver epithelial cells which share a common lineage with oval cells and transitional hepatocytes in WHV carriers.

In the light of the work which demonstrated that deregulated expression of c-myc could cause apoptosis of rat fibroblasts in the absence of serum, and that a number of growth factors, including IGF-II, could block c-myc-induced apoptosis (8, 11), we hypothesized that IGF-II might function in a similar manner in woodchuck liver cells which overexpress N-myc2.

Overexpression of N-myc2 in WC-3 cells caused the cells to undergo a morphological transformation and increased the growth rate of cells maintained in complete medium. While the cells lost contact inhibition, as judged by increased foci formation, they were not capable of forming colonies in soft agar. Thus, while N-myc2 expression alters cell growth properties, the cells were not completely transformed to the fully malignant phenotype. This suggests that additional mutational events required for cooperation together with myc genes, such as point mutations in ras, are not preexisting in WC-3 cells.

By several criteria we have demonstrated that overexpression of N-myc2 increased cell death of WC-3 cells when they were maintained in serum-free medium. Further analysis revealed a typical apoptosis as the mode of cell death in N-myc2-expressing WC-3 cells. However, in our cell culture system, the N-myc2-induced apoptosis is only transient, and after 24 h of serum starvation, WC-3 cells resume growth. Northern blot analysis of poly(A)$^+$ mRNA did not detect endogenous expression of IGF-II in WC-3 cells grown in the presence of serum (data not shown). Endogenous IGF-II expression was also not detected in the N-myc2-expressing WC-3 cell lines (data not shown). Therefore, N-myc2 expression does not induce IGF-II expression in WC-3 cells.

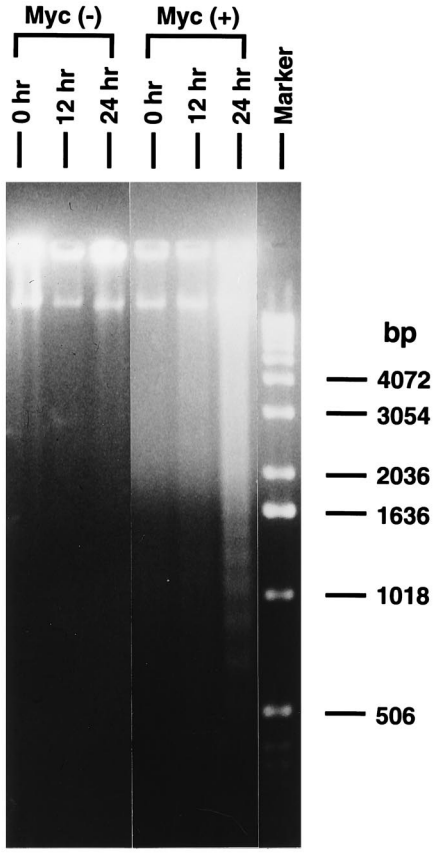


FIG. 8. DNA fragmentation assay of N-myc2-expressing WC-3 cells. Total DNA from cells was isolated from N-myc2-expressing [Myc (+)] and control [Myc (−)] WC-3 cells at the indicated times after the initiation of serum starvation and then examined by agarose gel electrophoresis as described in Materials and Methods. Note the DNA ladders at 24 h of serum starvation in the N-myc2-expressing WC-3 cells (lane 6). Molecular size markers (in base pairs) are on the right.

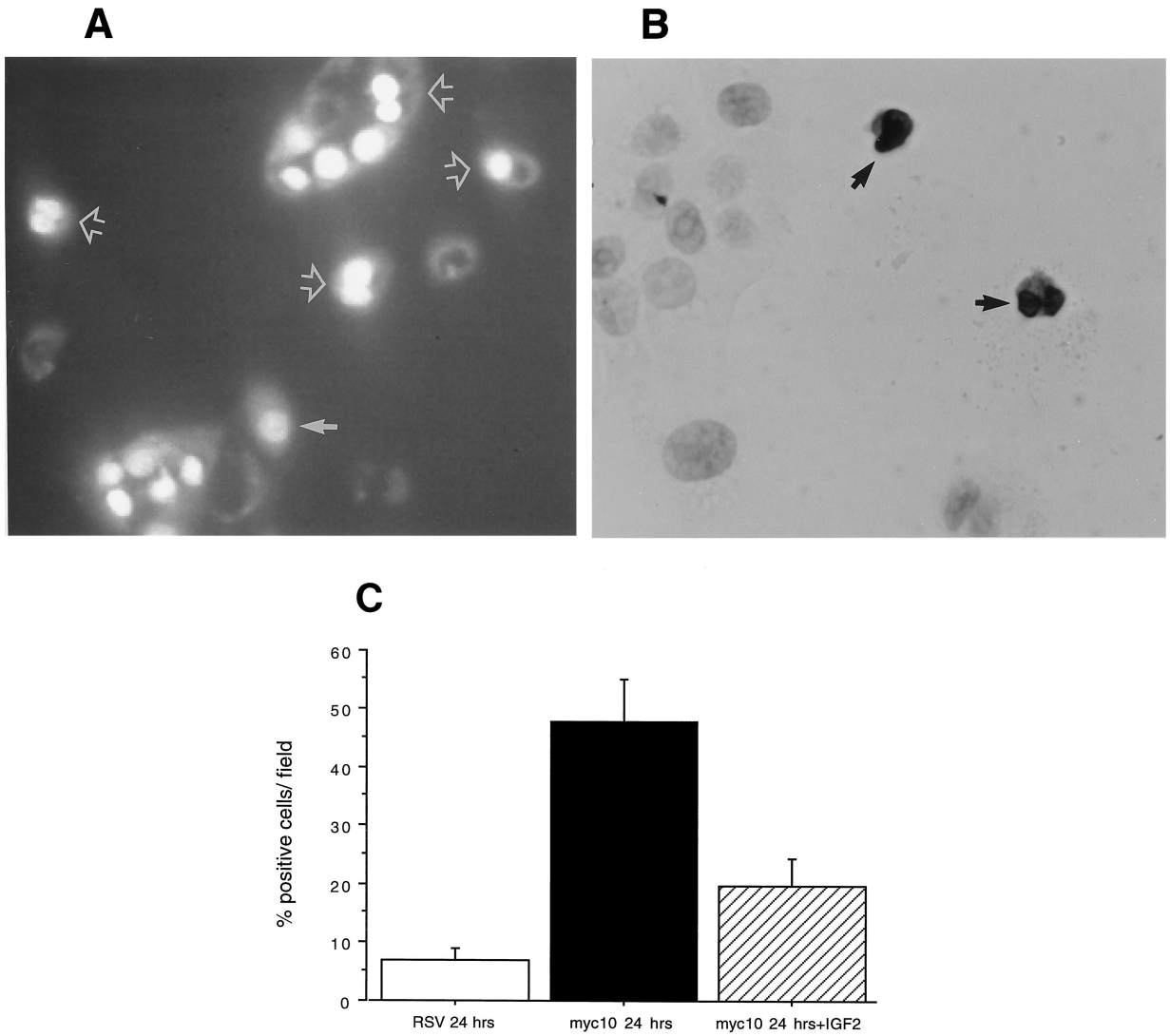


FIG. 9. DAPI staining and a Tunel assay for apoptosis of N-myc2-expressing WC-3 cells. (A) Micrograph of UV fluorescence depicting nuclear condensation and fragmentation of N-myc2-expressing cells 24 h after initiation of serum starvation as determined by DAPI staining. The solid arrow denotes a normal WC-3 cell and the open arrows denote nuclear condensation and fragmentation typical of apoptosis. Magnification, ×180. (B) Light field micrograph showing N-myc2-expressing WC-3 cells in a Tunel assay. Note the two apoptotic cells, which are denoted by arrows. Light brown cells with a normal nucleus are non-apoptotic cells. Magnification, ×360. (C) Histogram of a quantitative Tunel assay in which cells were labeled with a Tunel assay and the percentage of positive cells was determined as described in Materials and Methods. Note the increased frequency of cell death in N-myc2-expressing WC-3 cells (myc10) compared with control WC-3 cells (RSV) ($P = 0.0004$) and the dramatic reduction in apoptosis in the presence of 100 nM recombinant IGF-II (IGF2) ($P = 0.0057$).

The identity of the cell survival factors which allow WC-3 cells to resume growth in serum-free medium after 24 h are unknown. Previously we have not detected epidermal growth factor, TGF-α, or TGF-β expression in woodchuck precancerous livers. While TGF-α may play a role in immortalization of hepatocytes, a recent study demonstrated that TGF-α overexpression was not sufficient to block N-myc2-induced apoptosis of an immortalized mouse hepatocyte cell line (27). IGF-II, on the other hand, was capable of reversing the N-myc2-induced apoptosis in the same TGF-α-expressing hepatocytes (27).

The data from our colony regression assays revealed a heterogeneous response of cells to serum removal. A significant proportion of the WC-3 colonies in our assays remained completely healthy while others regressed very rapidly. These data are consistent with possible epigenetic changes in the control of the apoptosis pathway in a subpopulation of the N-myc2-expressing WC-3 cells. One possibility is that individual subclones of cells may produce autocrine factors which promote their survival and that other subclones may not produce those factors. Alternatively, some subclones may down regulate N-myc2 expression.

Epigenetic changes in gene expression, by their nature, may not be stable. Alterations of DNA methylation patterns are one mechanism for epigenetic control of gene expression which has been linked to carcinogenesis (2, 20). Altered DNA methylation patterns may regulate IGF-II expression and expression of a closely linked candidate tumor suppressor gene, H19 (7). H19 has been implicated in control of IGF-II expression through controlling the expression of the maternally imprinted IGF-II allele (7, 15).

Interestingly, H19 and IGF-II are closely linked in the mouse and they are also oppositely imprinted (7). Studies from

our laboratory (11a) have shown that imprinting of the maternal IGF-II allele is reversed in HCCs which arise in TGF-α transgenic mice. Investigations into the imprinting status and allelic expression of the IGF-II and H19 genes in subclones of WC3 cells, therefore, may provide insights into the mechanisms of cell survival and cell death if variable expression of imprinted alleles is observed.

It is generally assumed that IGF-II effects are mediated through the IGF-I receptor (3, 8), and it is also possible that the differential response of WC-3 subclones could reflect an increase in the expression of IGF-I receptors in the cells. Additional factors which may affect the level of IGF-II biological activity are IGF-binding proteins and the IGF-II–mannose-6-phosphate receptor (3, 8). In situ hybridization and immunocytochemistry has revealed that the genes which encode both of these proteins, which would be expected to interfere with IGF-II activity, are down regulated in woodchuck HCCs (30a). The IGF-II–mannose-6-phosphate receptor gene is also imprinted in mice and humans (7). Altered expression of the IGF-II–mannose-6-phosphate receptor or IGF-binding protein in cell culture could also account for variable responses of subclones of N-myc2-expressing cells.

In summary, we have shown that IGF-II can block apoptosis in N-myc2-expressing woodchuck liver epithelial cells. These data suggest that IGF-II expression may promote tumor progression by increasing the survival of hepatocytes which overexpress N-myc2 in precancerous woodchuck liver.

ACKNOWLEDGMENTS

We thank Tomo Kitagawa for his kind gift of the WC-3 cell line.

This work was supported by Public Health Service grants CA37232 and CA56076 and center grants P30CA13330 and 5P30DK41294. C.E.R. is the recepient of an Irma T. Hirschl-Weiler Career Scientist Award.

REFERENCES

1. **Abe, K., T. Kurata, R. Shita, and B. C. Tennant.** 1988. Enzyme altered liver cell foci in woodchucks infected with woodchuck hepatitis virus. Jpn. J. Cancer Res. **79:**466–472.
2. **Barlow, D. P.** 1995. Gametic imprinting in mammals. Science **270:**1610–1613.
3. **Chrislofori, G., P. Nalk, and D. Hanahan.** 1994. A second signal supplied by insulin like growth factor II on oncogene induced tumorigenesis. Nature (London) **369:**414–417.
4. **Cullen, K. J., D. Yee, and N. Rosen.** 1991. Insulinlike growth factors in human malignancy. Cancer Invest. **9:**443–454.
5. **Dunsford, H. A., C. Karnasuta, J. M. Hunt, and S. Sell.** 1989. Different lineages of chemically induced hepatocellular carcinoma in rats defined by monoclonal antibodies. Cancer Res. **49:**4894–4900.
6. **Dunsford, H. A., and S. Sell.** 1989. Production of monoclonal antibodies to preneoplastic liver cell populations induced by chemical carcinogens in rats and to transplantable Morris hepatoma. Cancer Res. **49:**4887–4893.
7. **Efstratiadis, A.** 1994. Parental imprinting of autosomal mammalian genes. Curr. Opin. Genet. Dev. **4:**265–280.
8. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:**119–128.
9. **Evarts, R. P., P. Nagy, E. Marsden, and S. Thorgeirson.** 1987. A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis **8:**1737–1740.
10. **Fu, X. X., C. Y. Su, Y. Lee, R. Hinz, L. Biempica, R. Snyder, and C. E. Rogler.** 1988. Insulin-like growth factor II expression and oval cell proliferation associated with hepatocarcinogenesis in woodchuck hepatitis virus carriers. J. Virol. **62:**3422–3430.
11. **Harrington, E. A., M. R. Bennett, A. Fanidi, and G. I. Evan.** 1994. c-Myc induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J. **13:**3286–3295.

11a. **Harris, T., and C. E. Rogler.** Unpublished data.

12. **Hixson, D. C., and J. P. Allison.** 1985. Monoclonal antibodies recognizing oval cells induced in the liver of rats by N-2-fluorenylacetamide or ethionine in a choline-deficient diet. Cancer Res. **45:**3750–3760.
13. **Hsia, C. C., C. A. Axiotis, A. M. Di Bisceglie, and E. Tabor.** 1992. Transforming growth factor-alpha in human hepatocellular carcinoma and coexpression with hepatitis B surface antigen in adjacent liver. Cancer **70:**1049–1056.
14. **Lee, G. H., T. Kawaguchi, K. Nomura, and T. Kitagawa.** 1987. Establishment and characterization of a diethylnitrosamine-initiated woodchuck hepatocyte cell line. Hepatology **7:**937–940.
15. **Leighton, P. A., R. S. Ingram, J. Eggenschwiler, A. Efstratiadis, and S. M. Tilghman.** 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature (London) **375:**34–39.
16. **Mark, J.** 1993. Cell death studies yield cancer clues. Science **259:**760–761.
17. **Oberhammer, F., W. Bursch, R. Tiefenbacher, G. Froschl, M. Pavelka, T. A. Purchio, and R. Schulte-Hermann.** 1993. Apoptosis is induced by transforming growth factor-β1 within 5 hours in regressing liver without significant fragmentation of the DNA. Hepatology **18:**1238–1246.
18. **Popper, H., J. W. K. Shih, J. L. Gerin, D. C. Wong, B. H. Hoyer, W. T. London, D. L. Sly, and R. H. Purcell.** 1981. Woodchuck hepatitis and hepatocellular carcinoma: correlation of histologic with virologic observations. Hepatology **1:**91–98.
19. **Raff, M. C.** 1992. Social controls on cell survival and cell death. Nature (London) **356:**397–400.
20. **Rainier, S., L. A. Johnson, C. J. Dobry, A. J. Ping, P. E. Grundy, and A. P. Feinberg.** 1993. Relaxation of imprinted genes in human cancer. Nature (London) **362:**747–749.
21. **Rogler, C. E.** 1991. Cellular and molecular mechanisms of hepatocarcinogenesis associated with hepadnavirus infection, p. 103–140. *In* W. S. Mason and C. Seeger (ed.), Hepadnaviruses: molecular biology and pathogenesis. Current topics in microbiology and immunology, vol. 168. Springer-Verlag, Berlin.
22. **Schaff, Z., C. C. Hsia, I. Sarosi, and E. Tabor.** 1994. Overexpression of transforming growth factor-α in hepatocellular carcinoma and focal nodular hyperplasia from European patients. Hum. Pathol. **25:**644–651.
23. **Scherer, E.** 1984. Neoplastic progression and experimental hepatocarcinogenesis. Biochim. Biophys. Acta **738:**219–236.
24. **Schirmacher, P., W. A. Held, D. Yang, R. V. Chisari, Y. Rustum, and C. E. Rogler.** 1992. Reactivation of insulin-like growth factor II during hepatocarcinogenesis in transgenic mice suggests a role in malignant growth. Cancer Res. **52:**2549–2556.
25. **Sell, S., J. M. Hunt, B. J. Knoll, and H. A. Dunsford.** 1987. Cellular events during hepatocarcinogenesis and the question of premalignancy. Adv. Cancer Res. **48:**37–111.
26. **Snyder, R. L., and J. Summers.** 1980. Woodchuck hepatitis virus and hepatocellular carcinoma. Cold Spring Harbor Conf. Cell Proliferation **7:**447–457.
27. **Ueda, K., and D. Ganem.** 1996. Apoptosis is induced by N-myc expression in hepatocytes, a frequent event in hepadnavirus oncogenesis, and is blocked by insulin-like growth factor II. J. Virol. **70:**1375–1383.
28. **Williams, G. T., and C. A. Smith.** 1993. Molecular regulation of apoptosis: genetic controls on cell death. Cell **74:**777–779.
29. **Yang, D., E. Alt, and C. E. Rogler.** 1993. Coordinate expression of N-myc2 and insulin-like growth factor II in precancerous altered hepatic foci in woodchuck hepatitis virus carriers. Cancer Res. **53:**2020–2027.
30. **Yang, D., and C. E. Rogler.** 1991. Analysis of insulin-like growth factor II (IGF-II) expression in neoplastic nodules and hepatocellular carcinomas of woodchucks utilizing "in situ" hybridization and immunocytochemistry. Carcinogenesis (London) **12:**1893–1901.

30a. **Yang, D., L. E. Rogler, and C. E. Rogler.** Unpublished data.