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# INHIBITION OF PROLIFERATION OF CULTURED RAT LIVER EPITHELIAL CELLS AT SPECIFIC CELL CYCLE STAGES BY TRANSFORMING GROWTH FACTOR-β

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Proliferation of early-passage propagable cultured rat liver epithelial cells derived from normal adult rats is markedly inhibited by transforming growth factor-β (TGF-β). Inhibition, which is completely reversible, is effected at two distinct points of the cell cycle, the $G_1$/S border and the $G_0$ or early $G_1$ phase. With increasing passages in culture, hepatic epithelial cells progressively become less sensitive to the inhibitory effect of TGF-β. 

Transforming growth factor-β (TGF-β) inhibits the proliferation of many cultured cells (Reviewed in 1). Flow cytometric and autoradiographic studies on human prokeratinocytes (2) and rat hepatocytes in primary cultures (3) suggested that TGF-β inhibited the progression of cells from $G_1$ to S phase of the cell cycle. TGF-β also delayed the entrance of aortic endothelial cells into the S phase (4). In this communication we report that the proliferation of early-passage propagable adult rat liver epithelial cells is inhibited by TGF-β in a reversible manner, and that inhibition occurs at two specific points in the cell cycle.

## MATERIALS AND METHODS

### Materials:

The culture medium was Richter's Improved Minimal Essential Medium with zinc salt (IMEMZO) (Irvine Scientific, Irvine, CA). Fetal bovine serum was obtained from Gibco Laboratories (Grand Island, NY) and TGF-β was purchased

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from R and D Systems (Minneapolis, MN). [Methyl-$^3$H]-thymidine (6.7 mCi/mmole) was from ICN Biomedicals (Montreal, Quebec).

Cell lines:

Both WB-F344 and RL-F344-3 cell lines were isolated from the livers of adult male Fischer 344 rats essentially by the method of Williams et al (5). The phenotypic properties of WB-F344 cells has been described previously (6). Cells were passaged regularly in IMEMZO medium containing 10% fetal bovine serum, each passage resulting in approximately 5-6 population doublings. The population doubling times of both cell lines were approximately 21 to 24 hours when these experiments were carried out.

Assay of DNA synthesis:

DNA synthesis was measured by the uptake of $^3$H-thymidine ($^3$H-Tdr) into the acid precipitable cell faction. Cells in 60 mm culture plates were incubated in medium containing 1 µCi/ml of $^3$H-Tdr at 37°C. At 30 to 60 min later the medium was aspirated and the plates were washed three times with ice-cold phosphate-buffered saline (PBS). Subsequently, the cultures were bathed in 5% trichloroacetic acid (TCA) for 30 min at 4°C and then washed twice more with ice-cold TCA. After 3 further rinses in ice-cold 95% ethanol, plates were air-dried. Each plate was incubated with 1.5 ml of 0.3N NaOH in humidified air at 37°C for 6-12 hr, and 0.5 ml of the resulting cell lysate was mixed with 60 µl of 3N HCl and 4.5 ml of Scintiverse solution (Fisher Scientific), and counted in a LKB liquid scintillation counter. All measurements were performed with triplicate plates.

## RESULTS

DNA synthesis and proliferation of early-passage normal rat liver epithelial cell lines were inhibited by TGF-β (Figure 1). With increasing passages in culture, the sensitivity of the cells to the inhibitory effect of TGF-β was diminished (Figure 1, WB-P20 vs WB-P9). In the presence of 1 ng/ml of TGF-β, DNA synthesis in logarithmically growing populations of WB-344 cells at passage 11 was markedly inhibited within the first 24 hr, inhibition persisting as long as TGF-β was present (Figure 2). As soon as TGF-β was removed, cellular proliferation resumed at the rate typical of cell cultures without TGF-β (Figure 3). The reversibility of inhibition of DNA synthesis by TGF-β was complete even in cells which had been cultured in the presence of TGF-β for 4 days.

To study the phase(s) in the cell cycle at which TGF-β inhibited DNA synthesis, cells were incubated in medium containing 10% FBS and 1 ng/ml of TGF-β for 3 days, when it was removed. $^3$H-thymidine uptake was measured during various times following the release of inhibition of DNA synthesis

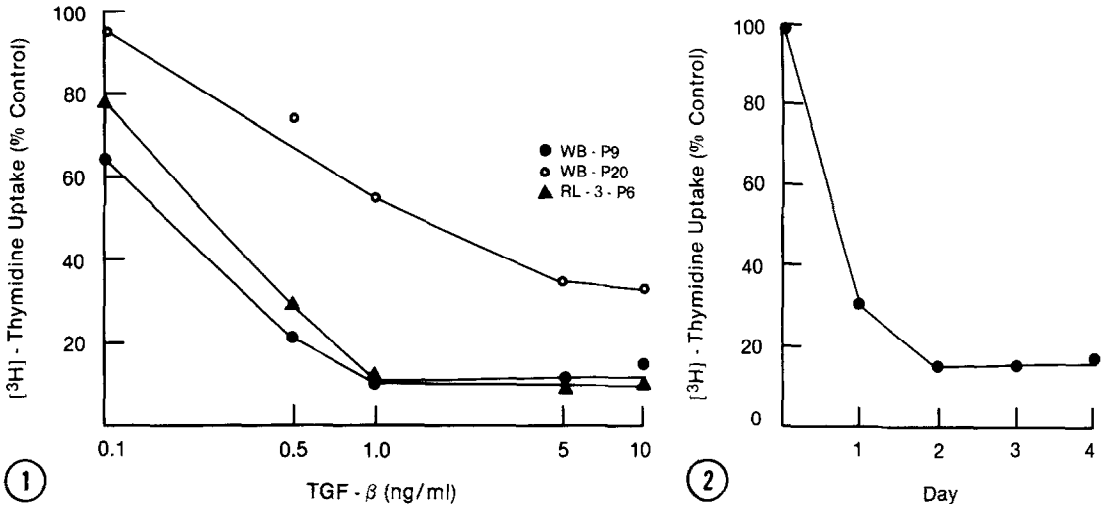


Figure 1. Inhibition of DNA synthesis in non-confluent and logarithmically growing cultures of rat liver epithelial cells which were exposed for 4 days to varying concentrations of TGF-β. $^{3}$H-Tdr uptake was for 1 hr; P indicates the passage number of the cell lines. 2 to $5x10^{4}$ cells were plated in each 60 mm tissue culture plate, and the medium was changed 2 days later to medium with or without (controls) TGF-β.

Figure 2. Inhibition of proliferation of WB-344 cells at passage 11 after exposure to 1 ng/ml of TGF-β for 4 days.

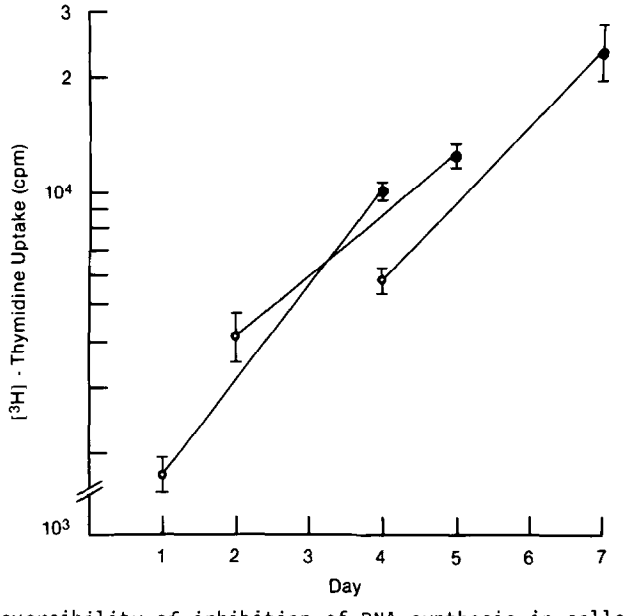


Figure 3. Reversibility of inhibition of DNA synthesis in cells which were suppressed by TGF-β for 1 to 4 days. $5x10^{4}$ cells were plated in 60 mm tissue culture dishes. Two days later, media in all the plates were changed to that containing 1 ng/ml TGF-β. (o) $^{3}$H-Tdr uptake was performed daily during the subsequent 4 days and concurrently the media of triplicate sets of plates were changed to media without TGF-β (o). Assays of $^{3}$H-Tdr uptake were again performed after the cells were allowed to recover from TGF-β effect for 3 days (●). Values represent the means and the standard deviations of triplicate studies.

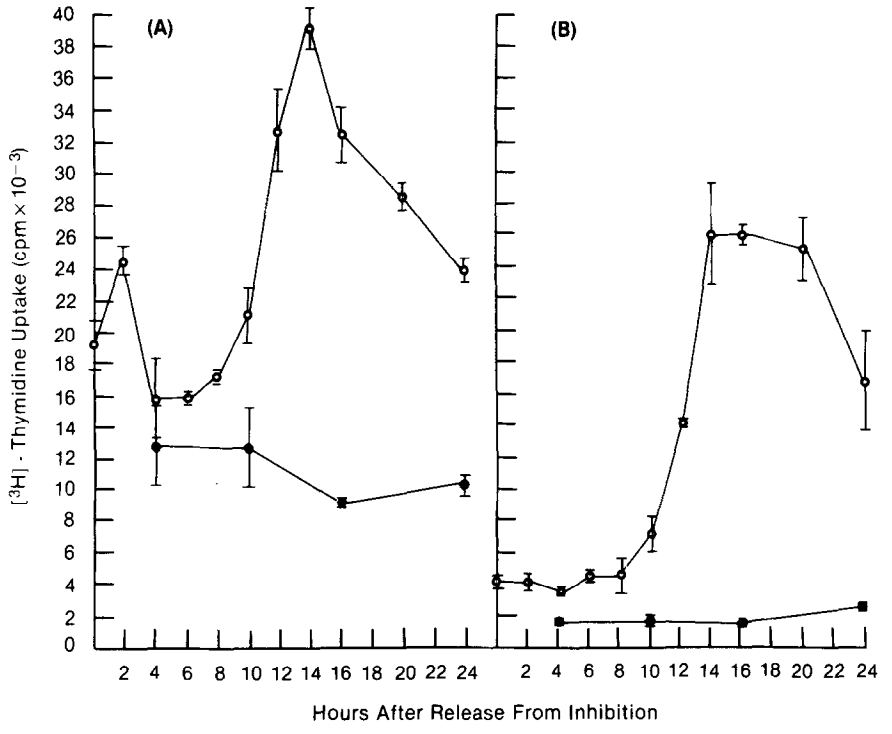


Figure 4. Recovery of DNA synthesis following the removal of TGF-β (A) and the addition of serum (B) to cells which were rendered quiescent in serum-free medium. Inhibition of proliferation by either TGF-β or serum-free medium was for 3 days. $^{3}$H-Tdr uptake was for 0.5 hr. (o) indicates cells which had been released from inhibition and (●) indicates cells which continued to be inhibited. Values represent the means and standard deviations of triplicate studies.

(Figure4A). A parallel experiment in which DNA synthesis was re-initiated in cells which had been made quiescent by serum-deprivation was also performed (Figure4B). These experiments indicated that serum-deprived, quiescent WB-344 cells re-inititated DNA synthesis approximately 10-12 hr after exposure to serum, whereas release from inhibitory effect of TGF-β resulted in an immediate, but transient, wave of DNA synthesis, which was followed by a second wave of DNA synthesis of larger magnitude 10-12 hr later.

## DISCUSSION

The data reported here confirm that TGF-β is a powerful inhibitor of cellular proliferation of cultured normal rat liver epithelial cells (7), and that this inhibitory effect is completely reversible when TGF-β is removed. This finding contradicts the observation of McMahon et al (7), who

reported that inhibition of colony forming ability of cultured hepatic epithelial cells by TGF-β exposure was irreversible. Our observations agree with the results obtained in human prokeratinocytes (2). The demonstration of 2 waves of DNA synthesis after release from inhibition suggests that hepatic epithelial cells were blocked at 2 cell cycle points: at the $G_1$/S border and in the $G_0$ or early $G_1$ phase. Hepatic epithelial cells became less sensitive to the inhibitory effect of TGF-β with increasing passage in vitro. The mechanism of this change is unclear, but may be related to a step or steps in transformation of these cells. With increasing passages cultured rat liver epithelial cells may transform spontaneously (8), and neoplastically transformed cells have been reported to resist the inhibitory effect of TGF-β on cellular proliferation (1,2,7).

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## REFERENCES

1. Sporn, M.B., Roberts, A.B., Wakefield, L.M., and Assoian, R.K. (1986) Science 233: 532-534.

2. Shipley, G.D., Pittelkow, M.R., Wille Jr., J.J., Scott, R.E., and Moses, H.L. (1986) Cancer Res.: 2068-2071.

3. Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K., Ischihara, A. (1985) Biochem. Biophys. Res. Commun. 133: 1042-1050.

4. Heimark, R.L., Twardzik, D.R., and Schwartz, S.M. (1986) Science 233: 1078-1080.

5. Williams, G.M., Weisburger, E.K., and Weisburger, J.H. (1971) Exp. Cell Res. 69: 106-112.

6. Tsao, M.-S., Smith, J.D., Nelson, K.G., and Grisham, J.W. (1984) Exp. Cell Res. 154: 38-52.

7. McMahon, J.B., Richards, W.L., del Campo, A.A., Song, M.-K.H., and Thorgiersson, S.S. (1986) Cancer Res. 46 4665-4671.

8. Schaeffer, W.I. (1980) Ann. N.Y. Acad. Sci. 349: 165-182.