

Inhibition of DNA Synthesis by Somatostatin in Rat Hepatocytes Stimulated by Hepatocyte Growth Factor or Epidermal Growth Factor

Norihiro Kokudo, MD, Piyush C. Kothary, MS, Frederic E. Eckhauser, MD, Ann Arbor, Michigan, Toshikazu Nakamura, PhD, Fukuoka, Japan, Steven E. Raper, MD, Ann Arbor, Michigan

The antiproliferative effects of somatostatin on hepatocytes stimulated by hepatocyte growth factor (HGF) or epidermal growth factor (EGF) were investigated using primary cultures of adult rat hepatocytes. Somatostatin inhibits HGF-induced (at a dose of 10 ng/mL) or EGF-induced (at a dose of 100 ng/mL) ^3H -thymidine incorporation into hepatocytes in a dose-dependent manner (10^{-10} to 10^{-8} M). This inhibition was confirmed by autoradiography. The effect of somatostatin was nontoxic as judged by preserved albumin synthesis, a marker for differentiated hepatocyte function. In the presence or absence of somatostatin, neither HGF nor EGF significantly altered intracellular cyclic adenosine monophosphate (cAMP). We conclude that somatostatin is a potent inhibitor of HGF- or EGF-induced deoxyribonucleic acid synthesis in adult rat hepatocytes. The mechanism of this inhibition appears to be independent of cAMP. The significance of somatostatin in liver regeneration has yet to be assessed.

The liver has a remarkable capacity to regenerate after hepatectomy or injury. In normal rats, resected liver grows rapidly, doubles in size within 48 hours, and resumes its original volume within 7 to 10 days [1]. There have been a variety of investigations on humoral factors that may trigger or promote liver growth. Insulin, glucagon, epidermal growth factor (EGF), norepinephrine, and vasopressin are all known hepatotrophic factors [2]. Recently, hepatocyte growth factor (HGF) has been discovered as a potent liver mitogen and has been postulated to play a major role in liver growth [3,4].

In contrast to uncontrolled or autonomous tumor growth, cellular proliferation associated with liver regeneration is strictly controlled and ceases once the original liver mass is restored. A variety of tissue specific substances that inhibit deoxyribonucleic acid (DNA) synthesis of the liver *in vitro* [5] or *in vivo* [6] have been suggested to explain this strict regulation. However, only transforming growth factor β [7,8] and heparin-binding growth factor type 1 [9] have been well characterized. Our knowledge of the regulating process of this phenomenon is still incomplete.

Somatostatin is known to inhibit the growth of a number of cell types such as gastrointestinal mucosa [10], exocrine pancreas [11], lymphocytes [12], and some tumors [13]. We have recently shown that somatostatin inhibits DNA synthesis in rat hepatocytes stimulated by insulin or cAMP analogue [14,15] and that the liver takes up intraportally injected somatostatin-14 in an efficient and saturable manner [16]. These findings, taken together with the fact that immunoreactive somatostatin levels measured in the portal vein are 10 times higher than in systemic circulation [17], have led us to hypothesize that somatostatin may play an important role in regulating liver regeneration. In this study, we used primary cultures of adult rat hepatocytes to investigate the effects of somatostatin on DNA synthesis stimulated by HGF or EGF.

MATERIAL AND METHODS

Materials: Synthetic somatostatin-14 (SS-14), human insulin, and epidermal growth factor fragment [Cys-(Acm^{20,31})]-EGF20-31 (EGF) [18] were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Human recombinant HGF (HGF) [4] was kindly supplied by Dr. Toshikazu Nakamura (Fukuoka, Japan). Collagenase (type I) was obtained from Sigma Chemical Co. (St. Louis, MO). Matrigel and Dispase were obtained from Collaborative Research Inc. (Bedford, MA). All chemicals used for sodium dodecyl polyacrylamide gel electro-

From the Department of Surgery (NK, PCK, FEE, SER), University of Michigan Medical Center, Ann Arbor, Michigan, and the Department of Biology (TN), Kyushu University, Fukuoka, Japan. This work was supported by National Institutes of Health grant 1 R29 DK42485-01 and a Career Development Award from the Society for Surgery of the Alimentary Tract.

Requests for reprints should be addressed to Steven E. Raper, MD, Department of Surgery, 2922H Taubman Health Care Center, 1500 East Medical Center Drive, Ann Arbor, Michigan 48109-0331.

Presented at the 32nd Annual Meeting of the Society for Surgery of the Alimentary Tract, New Orleans, Louisiana, May 20-22, 1991.

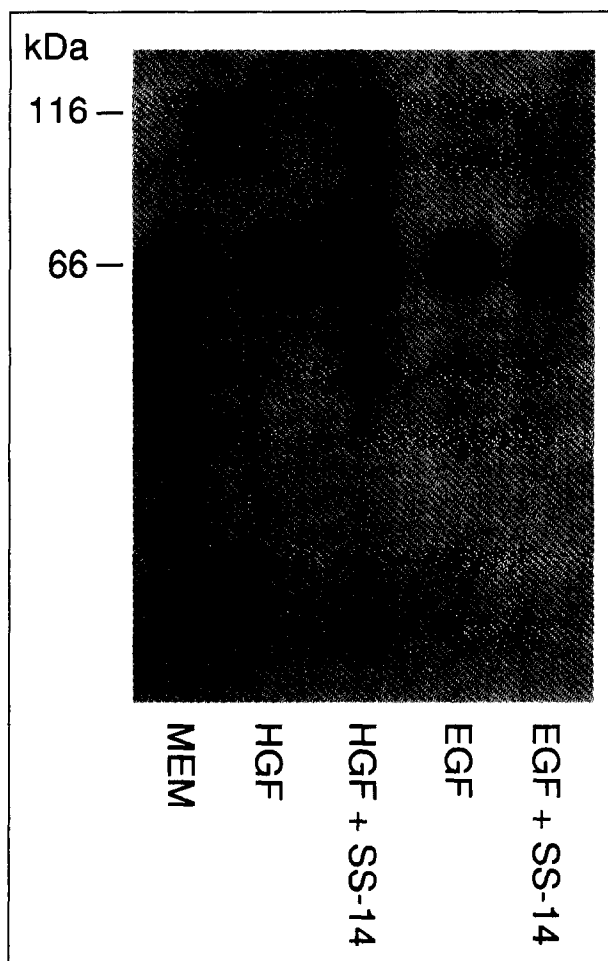


Figure 1. Immunoblot analysis of albumin secreted into the media by rat hepatocytes. The media (25 μ L) from hepatocyte culture dishes treated with appropriate reagents for 24 hours were subjected to electrophoresis (SDS-PAGE) and immunoblot analysis using antirat albumin antibodies (Organon Teknica Corp., Westchester, PA) [14,21]. MEM: media only, HGF: 10 ng/mL, EGF: 100 ng/mL, SS-14: 10^{-8} M. The figure is representative of three independent experiments. EGF = epidermal growth factor; HGF = hepatocyte growth factor; SS-14 = synthetic somatostatin-14; MEM = minimum essential medium; kDa = kilodaltons.

phoresis and immunoblot analysis were obtained from BioRad (Richmond, CA).

Hepatocyte isolation: The hepatocytes of Sprague-Dawley male rat (250 to 275 g) were isolated using a two-step collagenase perfusion technique described previously [14,15,19]. Briefly, rat liver was portally perfused for 10 minutes with Mg^{2+}/Ca^{2+} free Hank's buffer at 37°C followed by 8 to 10 minutes with Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY) containing 0.05% collagenase (type I). The liver was excised and placed in a Petri dish containing fresh MEM with collagenase. The liver capsule was stripped, and cells were released by gentle manipulation. The resulting crude preparation was filtered through 250- μ m nylon mesh, and the collagenase was inactivated with 0.5 mL of fetal calf serum. Centrifugal separation was employed to minimize contamination by nonparenchymal cells. Cell viability was tested by trypan blue exclusion (greater than

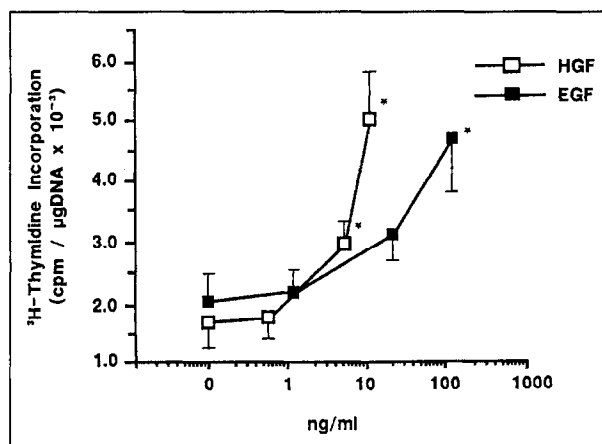


Figure 2. Dose-dependent stimulation of 3H -thymidine incorporation by HGF and EGF. Freshly isolated hepatocytes were plated as described in text. After a 24-hour attachment period, media was changed, and hepatocyte growth factor (HGF) (0.5 to 10 ng/mL) or epidermal growth factor (EGF) (1 to 100 ng/mL) was added. 3H -thymidine incorporation into deoxyribonucleic acid (DNA) was measured after 18-hour exposure to 3H -thymidine (2 μ Ci/dish). Points indicate the means of 6 to 10 dishes from three different experiments; bars, SEM. * $p < 0.05$ versus control (0 ng/mL of HGF or EGF).

90%). The hepatocytes were plated at a cell density of 10^5 per 16-mm tissue culture dish coated with 250 μ L (1:3 volume for volume) of Matrigel (laminin, heparan sulfate, type IV collagen) [20]. The medium was supplemented with insulin (100 ng/mL). To check the purity of the isolated hepatocytes and to exclude the possibility of toxicity by SS-14, we carried out an immunoblot analysis [21] of the media from hepatocyte culture dishes treated with HGF or EGF in the presence or absence of SS-14. The immunoblot analysis of the media using antirat albumin antibodies (Organon Teknica Corp., Westchester, PA) showed a single band of albumin, the molecular weight of which corresponded to chromatographically purified rat albumin (Organon Teknica Corp.) (Figure 1). These findings confirm that cultured hepatocytes in the present study were pure and functional.

Estimation of DNA synthesis: DNA synthesis was estimated by the rate of 3H -thymidine incorporation into DNA [22] and by autoradiographic measurement of the number of labeled nuclei.

After a 24-hour attachment period, media was changed and the appropriate reagents were added. The cell culture was maintained up to 3 days, and the media were changed daily. 3H -thymidine (2 μ Ci/dish) was added 18 hours before harvest, which was performed using Dispase (a neutral protease produced by *Bacillus polymyxa*) [23] for 2 hours at 37°C. Half of the harvested cells were processed for counting, and the other half were used to determine DNA content. The former half was filtered onto GF/C Whatman filters and rinsed with 1 mL of ice-cold 15% trichloroacetic acid (TCA) twice and with 70% ethanol once. Filters were dried and placed in 10 mL of scintillation cocktail (EcoLite, ICN Biochemicals, Inc., Irvine, CA) and were counted in a scintillation

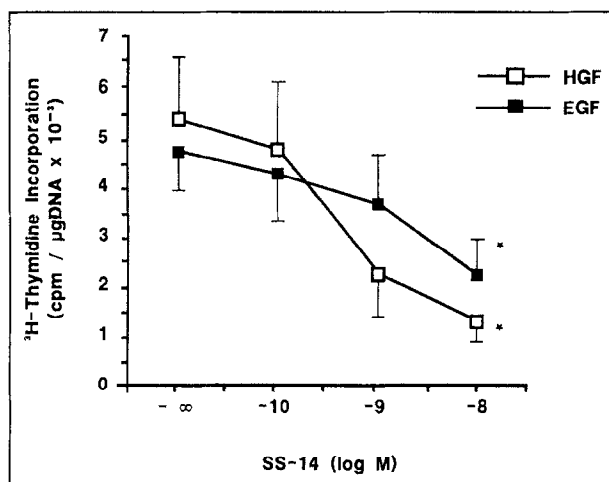


Figure 3. Effect of synthetic somatostatin-14 (SS-14) on deoxyribonucleic acid (DNA) synthesis stimulated by hepatocyte growth factor (HGF) or epidermal growth factor (EGF). Hepatocytes were treated with HGF (10 ng/mL) or with EGF (100 ng/mL) in the presence or absence of SS-14 (10^{-10} to 10^{-8} M). Methods for determining ^3H -thymidine incorporation were as for Figure 2. Points indicate the means from 6 to 8 dishes from two different experiments; bars, SEM. * $p < 0.05$ versus HGF- or EGF-stimulated ^3H -thymidine incorporation in absence of SS-14.

counter (Beckman LS 6,000LL, Beckman Instruments, Inc., Fullerton, CA). A modified diphenylamine reaction for desoxypentose was performed to measure DNA content [24]. ^3H -thymidine incorporation was expressed as counts per minute (cpm)/ μg DNA.

The labeling index, i.e., the percentage of total nuclei labeled with ^3H -thymidine, was measured as follows. After 18-hour exposure to ^3H -thymidine (2 μCi /dish), hepatocytes were washed twice with cold phosphate-buffered saline and fixed in 4% paraformaldehyde for 10 minutes, coated with Kodak NTB-3 emulsion (Eastman Kodak, Rochester, NY) and exposed for 10 days before development in Kodak Dektol. The percentage of morphologically defined hepatocytes with labeled nuclei was determined by phase contrast microscopy.

Cyclic AMP assay: Freshly isolated hepatocytes ($10^5/\text{mL}$) were incubated in glass tubes with the appropriate reagents for 5 minutes. Following incubation, the test tubes were placed in an ice-water bath for 10 minutes, vortexed gently, and centrifuged at 1,800g for 10 minutes at 4°C. The pellet was deproteinized with 15% TCA, ether extracted, and lyophilized. Cyclic AMP levels were measured by a competitive protein binding assay using an Amersham kit (Amersham Life Science Products, Arlington Heights, IL).

Statistical analysis: All values shown represent the mean \pm standard error of the mean (SEM). Wilcoxon signed rank test was used for comparing group means. The level of significance was established at $p < 0.05$.

RESULTS

Effect of HGF and EGF on hepatocyte DNA synthesis: HGF and EGF stimulated ^3H -thymidine incorpo-

TABLE I
Effect of SS-14 on HGF- and EGF-Stimulated Labeling Index

Reagents	Labeling Index (%)*
Control	26.0 \pm 3.2
HGF (10 ng/mL)	50.5 \pm 5.1 [†]
+SS-14 (10 nM)	22.2 \pm 3.8 [‡]
EGF (100 ng/mL)	49.0 \pm 5.3 [†]
+SS-14	24.8 \pm 4.7 [‡]

HGF = hepatocyte growth factor; EGF = epidermal growth factor; SS-14 = synthetic somatostatin-14.

*The labeling index, i.e., the percentage of nuclei labeled with ^3H -thymidine, was measured as described in "Materials and Methods." Data are the mean \pm SEM from 9 to 12 determinations from two different experiments.

[†] $p < 0.05$ versus control.

[‡] $p < 0.05$ versus HGF- or EGF-stimulated labeling index.

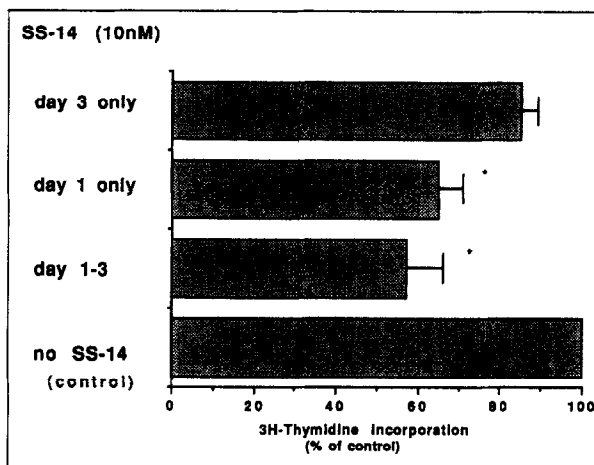


Figure 4. Effect of timing of synthetic somatostatin-14 (SS-14) exposure on deoxyribonucleic acid (DNA) synthesis. Hepatocyte cultures were maintained for 3 days in the presence of epidermal growth factor (EGF) (100 ng/mL), and ^3H -thymidine incorporation (cpm/ μg DNA) during the last 18 hours was measured. The cells were exposed to SS-14 (10^{-8} M) at various intervals as indicated on the ordinate. Values are expressed as % of control (no SS-14). Columns indicate the means from 8 dishes from two different experiments; bars, SEM. * $p < 0.05$ versus control.

ration into hepatocytes in a dose-dependent manner (0.5 to 10 ng/mL and 1 to 100 ng/mL, respectively, Figure 2). Of the tested doses, 10 ng/mL of HGF and 100 ng/mL of EGF were most effective, and these concentrations were used thereafter.

Effect of SS-14 on DNA synthesis stimulated by HGF and EGF: Increasing concentrations of SS-14 (10^{-10} M to 10^{-8} M) progressively inhibited hepatocyte DNA synthesis stimulated by HGF and EGF (Figure 3). Since the optimal effect of SS-14 was reached at 10^{-8} M, this concentration was used for all remaining experiments. No significant morphological changes in hepatocytes exposed to 10^{-8} M of SS-14 were observed. Further, SS-14 significantly decreased the labeling index of the hepatocytes stimulated by HGF or EGF (Table I).

This also confirms the inhibitory effect of SS-14 on DNA synthesis stimulated by HGF or by EGF.

Effect of timing of SS-14 exposure on DNA synthesis: To determine whether a critical period exists during which hepatocytes are sensitive to the antiproliferative effect of SS-14, hepatocytes stimulated with EGF were treated with SS-14 (10^{-8} M) at different intervals. In these studies (Figure 4), hepatocyte cultures were maintained for 3 days after attachment, and ^3H -thymidine incorporation during the last 18 hours was measured. The cells were exposed to SS-14 at various intervals as indicated on the ordinate. Exposure of cells to SS-14 between days 1 and 3 produced the highest reduction ($57\% \pm 9\%$ of control, $p < 0.05$) in DNA synthesis. Removal of SS-14 after day 1 also significantly inhibited DNA synthesis ($65\% \pm 6\%$, $p < 0.05$). Addition of SS-14 on day 3 did not significantly inhibit EGF-stimulated ^3H -thymidine incorporation.

Intracellular cAMP: The effects of HGF (10 ng/mL) and EGF (100 ng/mL) on intracellular cAMP levels in the presence or absence of SS-14 were determined in freshly isolated hepatocytes. Five minutes after the addition of HGF or EGF, there was no significant change in intracellular cAMP (2.04 ± 0.52 pmol/tube and 2.76 ± 0.48 pmol/tube, respectively; $n = 5$) compared with control levels (2.29 ± 0.49 pmol/tube, $n = 9$). In the presence of SS-14 (10^{-8} M), cAMP levels deviated slightly (HGF + SS-14, 2.10 ± 0.58 pmol/tube; EGF + SS-14, 2.35 ± 0.19 pmol/tube; $n = 5$), but these changes were not statistically significant.

COMMENTS

The present study shows that SS-14 inhibits HGF- or EGF-induced DNA synthesis in adult rat hepatocytes in a dose-dependent manner. This inhibition was apparently nontoxic as evidenced by the observation that albumin synthesis, a marker for differentiated hepatocyte function was maintained in cells treated with SS-14 (Figure 1) [14]. SS-14 exerted its inhibitory action when present even only on day 1 of a 3-day culture but had no significant effect when added on day 3 only. This suggests that somatostatin inhibits the early proliferative response of hepatocytes, and this is supported by the observation that somatostatin blocked EGF-induced centrosomal separation, a biological marker of early G1 phase in gerbil fibroma and HeLa cells [13].

The mechanism by which somatostatin inhibits cell proliferation is unknown. In previous studies using a number of cell types including hepatocytes, somatostatin has been shown to inhibit various cellular events by attenuating the production of cellular cAMP [25]. We have shown in isolated hepatocytes that somatostatin exerts at least part of its antiproliferative effects via the adenylate cyclase system [14,15]. Tsuzaki *et al* [26] reported that somatostatin inhibits DNA synthesis in FRTL5 line of rat thyroid follicular cells stimulated by thyroid-stimulating hormone (TSH), which is known to act via the adenylate cyclase system. EGF has not been shown to alter cellular cAMP in hepatocytes [27]. After binding to its cell surface receptor, EGF rapidly activates the receptor's

intrinsic tyrosine kinase. Although the kinase activity is assumed to be important in signal generation, the mechanism coupling this early response and delayed responses such as DNA synthesis is unknown [28]. Human lung fibroblast-derived mitogen (a variant of HGF) has recently shown to induce rapid tyrosine phosphorylation in human mammary epithelial cells [29]. However, little is known about signalling mechanisms for HGF action. Since there were no alterations in intracellular cAMP levels as shown in this study, it appears that the inhibitory action of somatostatin on DNA synthesis stimulated by HGF or EGF is mediated via cAMP independent mechanism. Further study of other signal transduction systems may yield more information about mechanisms of hepatocyte growth regulation.

The significance of somatostatin for growth control *in vivo* has yet to be assessed. Somatostatin is widely distributed in the gastrointestinal system and is produced in the pancreas as well as the gut [30]. Patel *et al* [17] have reported that immunoreactive somatostatin levels identified in the portal vein are more than 10 times higher than those identified in the systemic circulation. Although the predominant effect of somatostatin is presumed to be paracrine, this peptide may directly influence the liver by way of the portal circulation.

We conclude that somatostatin inhibits the early phase of hepatocyte proliferation stimulated by liver mitogens HGF and EGF. Somatostatin may be important in the modulation of normal liver growth and repair, perhaps to prevent uncontrolled hepatocyte proliferation.

REFERENCES

- Higgins GM, Anderson RM. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 1931; 12: 186-202.
- Fausto N. Hepatic regeneration. In: Zakim D, Boyer TD, editors. *Hepatology: a textbook of liver disease*, Vol 1. Philadelphia: WB Saunders, 1990: 49-65.
- Nakamura T, Teramoto H, Ichihara A. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc Natl Acad Sci USA* 1986; 83: 6489-93.
- Nakamura T, Nishizawa T, Hagiya M, *et al*. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989; 342: 440-3.
- Otsuka H, Terayama H. Inhibition of DNA synthesis in ascites hepatoma cells by normal liver extract. *Biochim Biophys Acta* 1966; 123: 274-85.
- Makowka L, Falk RE, Falk JA, *et al*. The effect of liver cytosol on hepatic regeneration and tumor growth. *Cancer* 1983; 51: 2181-90.
- Carr BI, Hayashi I, Braum EL, Moses HL. Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type β transforming growth factor. *Cancer Res* 1986; 46: 2330-4.
- Russell WE, Coffey RJ, Ouellette AJ, Moses HL. Type β transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat. *Proc Natl Acad Sci USA* 1988; 85: 5126-30.
- Kan M, Huang J, Mansson P-E, Yasumitsu H, Carr B, McKeehan WL. Heparin-binding growth factor type 1 (acidic fibroblast growth factor): a potential biphasic autocrine and paracrine regulator of hepatocyte regeneration. *Proc Natl Acad Sci USA* 1989; 86: 7432-6.

10. Lehy T, Dubrasquet M, Bonfils S. Effect of somatostatin on normal and gastric-stimulated cell proliferation in the gastric and intestinal mucosae of the rat. *Digestion* 1979; 19: 99-109.
11. Morriset J. Somatostatin: a potential antigrowth factor for the exocrine pancreas. *Regul Pept* 1984; 10: 11-22.
12. Pawlikowski M, Stepień H, Kunert-Radek J, *et al.* Immunomodulatory action of somatostatin. *Ann NY Acad Sci* 1987; 496: 233-9.
13. Mascardo RN, Sherline P. Somatostatin inhibits rapid centrosomal separation and cell proliferation induced by epidermal growth factor. *Endocrinology* 1982; 111: 1394-6.
14. Raper SE, Kothary PC, Kokudo N. Somatostatin-14 blocks the hepatotrophic effects of insulin in the rat. *J Surg Res* 1991; 50: 386-90.
15. Kokudo N, Kothary PC, Eckhauser FE, Raper SE. Inhibitory effects of somatostatin on hepatocyte proliferation are mediated by cyclic AMP. *J Surg Res* 1991; 51: 113-8.
16. Raper SE, Kothary PC, Kokudo N, DelValle J. The liver plays an important role in the regulation of somatostatin-14 metabolism in the rat. *Am J Surg* 1991; 161: 184-9.
17. Patel YC, Wheatley T, Fitz-Patrick D, Brock G. A sensitive radioimmunoassay for immunoreactive somatostatin in extracted plasma: measurement and characterization of portal and peripheral plasma in the rat. *Endocrinology* 1980; 107: 306-13.
18. Komoriya A, Hortsch M, Meyers C, Smith M, Kanety H, Schlessinger J. Biologically active synthetic fragments of epidermal growth factor: localization of a major receptor-binding region. *Proc Natl Acad Sci USA* 1984; 81: 1351-5.
19. Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976; 13: 29-83.
20. Bissell DM, Arenson DM, Maher JJ, *et al.* Support of cultured hepatocytes by a laminin-rich gel. *J Clin Invest* 1987; 79: 801-12.
21. Renat J, Reiser J, Stark GR. Transfer of proteins from gels to diazobenzylmethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci USA* 1979; 76: 3116-20.
22. McGowan JA, Strain AJ, Bucher NL. DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: effect of epidermal growth factor, insulin, glucagon, and cyclic-AMP. *J Cell Physiol* 1981; 108: 353-63.
23. Matsumura T, Yamanaka T, Hashizume S, Irie Y, Nitta K. Tissue dispersion, cell harvest and fluid suspension culture by the use of bacterial neutral protease. *Jpn J Exp Med* 1975; 45: 377-82.
24. Volkin E, Cohn WE. Estimation of nucleic acids. *Methods Biochem Anal* 1956; 1: 287-305.
25. Koch BD, Dorflinger LJ, Schonbrunn A. Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin. *J Biol Chem* 1985; 260: 13138-45.
26. Tsuzaki S, Moses AC. Somatostatin inhibits deoxyribonucleic acid synthesis induced by both thyrotropin and insulin-like growth factor-I in FRTL5 cells. *Endocrinology* 1990; 126: 3131-8.
27. Choudhury MH, Agius L. Epidermal growth factor counteracts the glycogenic effect of insulin in parenchymal hepatocyte cultures. *Biochem J* 1987; 247: 307-14.
28. McCune BK, Earp HS. The epidermal growth factor receptor tyrosine kinase in liver epithelial cells. *J Biol Chem* 1989; 264: 15501-7.
29. Rubin JS, Chan AM-L, Bottalo DP, *et al.* A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc Natl Acad Sci USA* 1991; 88: 415-19.
30. Reichlin S. Somatostatin. *N Engl J Med* 1983; 309: 1495-501.

DISCUSSION

William C. Meyers (Durham, NC): We've just completed a similar experiment and found similar results. Sandostatin (Sandoz, Ltd., Basle, Switzerland) does inhibit regeneration in the 70% hepatectomy model of Michalopoulos (*Cancer Res* 1989; 49: 3314-20), and also BRDU (bromodeoxyuridine) incorporation. When the administration of Sandostatin is stopped, regeneration goes back to the previous curve. Have you had any experience with an *in vivo* model to confirm your results?

David Allison (Baltimore, MD): You've shown that thymidine incorporation has been altered. As you know, thymidine incorporation can be influenced by the pool levels of thymidine in a cell. Do you have any cytophotometric evidence to show that these differences in thymidine incorporation actually relate to differences in DNA synthesis?

Norihiko Kokudo (closing): We have not done any experiments using Sandostatin (octreotide) *in vivo*. Goldman (*Surg Forum* 1978; 29: 402-4) reported that intravenous infusion of somatostatin inhibited liver regeneration after partial hepatectomy in rats. Since somatostatin inhibits insulin and glucagon *in vivo*, which are known hepatotrophic factors, it is very difficult to prove whether there is a direct inhibitory effect of somatostatin or not.

Dr. Allison, the histologic evidence we have relates to the significant changes seen in the nuclear labeling index (Table I). The predominance of silver grains over the nuclei precludes the presence of intracellular cytoplasmic pools of tritiated thymidine as a source of error.