論文の内容の要旨

論文題目　STIMULATION OF PUTRESCINE PRODUCTION BY EPIDERMAL GROWTH FACTOR IN RAT LIVER AFTER PARTIAL HEPATECTOMY

上皮成長因子（ＥＧＦ）による
ラット部分切除肝の putrescine 産生促進

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緒言

Epidermal growth factor(EGF)は初代培養肝細胞の DNA合成を促進することが知られているが、in vivoでの肝再生に対する作用はいまだ不明である。細胞増殖には polyamineが必要とされるが、ラットの部分切除肝の再生では putrescineが必須 polyamineであることが明らかにされている。Putrescineは通常 ornithine decarboxylase(ODC)の作用により ornithineから合成され、さらに spermidineに変換されるが、肝部分切除(肝切)後早期にODC活性化が抑制されると、 spermidine N[1]-acetyltransferase(SAT)および polyamine oxidaseにより spermidineから逆に変換される代償的合成経路により供給される。肝ODC活性は肝切後14時間目までに2峰性に上昇するが、ピーク前に putrescineを投与

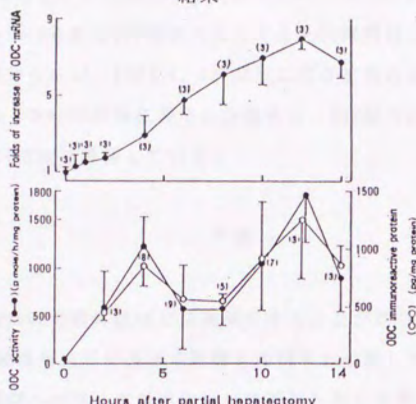
するといずれの場合にもDNA合成を促進することから、putrescine は肝再生の必須因子であるばかりでなく、再生をより促進する因子にもなると考えられている。一方、EGFは初代培養肝細胞のODC活性を上昇させることが知られている。そこで、EGFが肝切後の putrescineの産生を促進するか否か、およびその作用点を明らかにする目的で以下の実験を行った。

研究方法

実験I：ラットに肝切あるいは偽手術を施行後、4または8時間目に^{125}I-EGFを門脈内投与し、その1時間後に、glutaraldehyde溶液で灌流固定した肝右側葉で放射活性の測定および光顕 autoradiographyを行なった。

実験II：肝切ラットに次の様な処置をし、屠殺後摘出した肝で各種測定をした。1) 無処置の上、術直後から14時間目まで経時的に屠殺した。2) EGF(12.5μg/kg)を術直後から2時間毎に4または8時間目まで腹腔内投与し、6または10時間目に屠殺した。3) 同量のEGFを術直後に投与し、4時間後に屠殺した。4) actinomycin D(1mg/kg)を術後0または6時間目に、EGFまたは生食を0、2または6、8時間目に腹腔内投与し、4または10時間目に屠殺した。摘出肝のODCおよびSAT活性は各々OkaとPerryの方法および Libbyの方法、polyamine量は Oshimaの方法に準じて測定した。ODCmRNAは、guanidinium thiocyanate-hot phenol法にて全RNAを抽出し、マウス腎ODCcDNAを用いて Northern blot法により定量した。ODC蛋白量は polyclonal IgG抗マウス腎ODC抗体を用いたサンドイッチ法 radioimmunoassay(RIA)で測定した。

結果



Changes of hepatic ornithine decarboxylase (ODC) mRNA, immunoreactive protein and activity after partial hepatectomy in rats

実験Ｉ：^{125}I-EGFの取込みは肝切群では偽手術群より高かったが、肝切5と9時間目とでは差がなかった。Autoradiographic grainsは両群とも肝細胞内に存在し、そのうち核内に存在する grainsの比率は肝切群で有意に高かったが、肝切後の時間による差はなかった。

実験II：肝切後、ODCmRNA量は12時間目まで徐々に増加した。ODC活性と蛋白量は4、12時間目でいずれも２つのピークを形成し(図)、かつ両者間には正の相関関係が認められた($r=0.91, p<0.001$)。EGFを術直後に投与すると putrescine量、ODC活性は2時間目では対照に比べ差がなかったが、4時間目では有意に上昇した。EGFを術直後より2時間毎に投与すると、putrescine量、ODC活性は6、10時間目で、SAT活性は6時間目で有意に上昇した。EGF投与群で術後2時間目のODCmRNAは対照群の2.2倍であったが10時間目では差がなかった。ODC活性と蛋白量との間にはEGF投

与の如何に拘らず正の相関関係が認められた($r=0.81, p<0.001$)。

Actinomycin Dを肝切6時間目に投与すると10時間目のODC活性は対照の約50%に減少したが、EGFを6、8時間目に投与することにより回復した。Actinomycin Dを肝切直後に投与した場合は、EGF投与の有無によらず、4時間目のODC活性は著減していた。

考察

肝細胞膜のEGF受容体数は肝切後減少することが知られているので、EGFの部分切除後再生肝に及ぼす影響を検討するに際して、まず投与されたEGFの肝細胞への取込まれ方について検討した。予備実験で、^{125}I-EGFの取込は肝葉内の部位により差が認められたが、同一肝葉全体ではラット間で一定していた。そこで本実験では右側葉全体の放射活性で比較したところ、EGFは肝切後の時間によらず同程度に取込まれており、しかも autoradiographyより肝細胞内に存在し、核内へも同等に移行することも明らかとなった。

ラット肝のODCmRNA量と蛋白量の測定にはラット肝ODCcDNAと90%以上の相同性を有するマウス腎ODCcDNAおよびラット肝ODCと交差反応するとされる抗マウス腎ODC抗体を用いた。予備実験の結果、Northern blottingでODCmRNAに相当する2本のバンドが認められ、また、RIAの標準曲線も testosteroneにより誘導された高いODC活性を有するマウス腎のものと全く一致したことから、両測定は妥当なものと考えられた。

肝切ラットにEGFを投与するとODC活性の上昇に伴って putrescineが増量した。また、putrescine増量をもたらす肝切6時間目のSAT活性も上昇したことから、EGFは部分切除肝では ODCとSATの活性上昇を介して

putrescineを増量すると推定された。

肝切後のODCmRNA量は術後12時間まで漸増するのに対してODC活性と蛋白量は2峰性に上昇し、actinomycin Dによる活性の抑制効果も投与時期により差があったことから、肝切後のODC活性誘導の調節は時期により異なることが判明した。また、活性と蛋白量とには高い正の相関関係が認められたことから活性はほぼ蛋白量を反映しODC-antizyme complexなどの不活性型ODCの関与は少ないど思われた。EGFを投与すると、ODCmRNAは肝切２時間目で対照に比べ増量しており、４時間目の活性上昇に関与していると考えられた。10時間目ではODCmRNAに差を認めなかったが、これは対照群のmRNA量が既に最大値に達していたか、あるいは増加しているmRNA量に比べEGFによる増量が検出できるほどのものではなかった可能性がある。しかし10時間目の活性はEGFにより有意に上昇しており、また、actinomycin DによりODCmRNAの転写を抑えると半減する活性が、EGF投与で回復することから、EGFは転写後にも作用すると推定された。さらに活性と蛋白量の関係からEGFによる活性上昇には不活性型ODCの関与は少ないと思われた。

結論

部分切除肝におけるODC誘導の調節機構は肝切後の時期により異なっている。これに対して、EGFはODCmRNAの転写と転写後調節機構の両者に作用してODC蛋白の増量からその活性を上昇させ、一方、SAT活性も上昇させることにより putrescine量の増加をきたすと推定された。

Stimulation of Putrescine Production by Epidermal Growth Factor in Rat Liver after Partial Hepatectomy

上皮成長因子(EGF)によるラット部分切除肝のputrescine産生促進

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Stimulation of Putrescine Production by Epidermal Growth Factor

Introduction

Epidermal growth factor (EGF) is a single-chain polypeptide which has been shown to have a growth-promoting capacity in a variety of in vivo and in vitro systems (1-3). In the liver, although EGF can stimulate DNA synthesis by adult rat hepatocytes in primary culture (4,5), its continuous intraperitoneal infusion produces only a perceptible increase of DNA synthesis in normal rats (6).

Earp et al. reported that EGF receptors in hepatocyte plasma membrane are decreased in number, starting within 8 h and reaching the minimum around 36 h after two-thirds resection of the liver (Hx) (7). This may lead to the notion that EGF receptors are lost by internalization into hepatocytes as EGF-receptor complexes. However, no significant changes in plasma EGF levels during liver regeneration have been reported. Recent reports that the receptor of transforming growth factor α (TGF-α) is identical to EGF receptor (8) must also be taken into consideration, because TGF-α stimulates hepatocyte proliferation in vitro and its mRNA levels are increased in regenerating liver (9). Besides, exogenous EGF is shown to be translocated more abundantly to hepatocyte nuclei in rats after Hx relative to controls (10). Under these circumstances, it is a matter of interest to determine whether EGF promotes liver regeneration after Hx.

Polyamines have been suggested to play an important role in liver regeneration (11,12). The first step in polyamine biosyn-

thesis is conversion of ornithine to putrescine catalysed by ornithine decarboxylase (ODC), and putrescine is then converted to spermidine and spermine (12). The compensatory pathway of putrescine production via spermidine acetylation and oxidation by spermidine N^1-acetyltransferase (SAT) and polyamine oxidase is also shown to operate in the rat liver after Hx and injury (13, 14). Sato and Fujiwara showed that putrescine is an essential polyamine for liver regeneration after Hx in rats, and can be supplied sufficiently for DNA synthesis by the commpensatory pathway when ODC activity is inhibited at an early stage after Hx (15). They also showed that the increment of hepatic putrescine content occurs early after Hx in rats by administration of insulin and glucagon (16) which stimulate DNA synthesis in partially hepatectomized liver of rats with intact secretion of endogenous hormones (17), and hepatic DNA synthesis is promoted by exogenous putrescine (16).

EGF has been reported to increase ODC activity in experiments using hepatoma cells and primary cultured rat hepatocytes (18,19). In partially hepatectomized rat liver, hepatic ODC activity increases and shows two peaks of maximal activities (15). According to Sato et al. (16), glucagon enhances the first peak and insulin the second peak of ODC activity after Hx. This result may suggest that hepatic ODC induction during the course of regeneration after Hx is regulated by more than one mechanism.

The aim of the present study is to clarify the possibility that exogenous EGF stimulates hepatic putrescine production after

Hx and the sites of EGF action in liver regeneration.

Materials and Methods

Experiments

Seven-week-old male Sprague-Dawley rats (Charles River Japan, Atsugi, Japan) were housed in an air-conditioned and light-controled room, and fed a commercial pelleted diet and water *ad libitum*. Between 9:00 and 11:00 a.m., rats underwent Hx by the method of Higgins and Anderson (20), or sham operation under light ether anesthesia. They were kept fasting throughout the experiments, the designs of which are shown in Figure 1.

Experiment I: The partially hepatectomized and sham operated rats received an intraportal injection 4 or 8 h after operation of 111 ng (8.9×10^7 cpm) of ^{125}I-EGF. The radiolabeled EGF was produced by iodination of mouse EGF (Toyobo Co. Ltd., Osaka, Japan) with ^{125}I (New England Nuclear Co., Boston, U.S.A.) according to the method of Hunter and Greenwood (21). A specific activity of 5 MBq/μg, as a solution in 0.2 M phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA) was obtained. One hour following injection of ^{125}I-EGF, the liver was perfused via the portal vein for 2 min with about 20 ml of 0.1 M phosphate buffer, pH 7.4, followed by 10 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer at a flow rate of 5 ml/min under ether anesthesia. The liver was then excised for measurement of ^{125}I-EGF incorporation into the liver and autoradiographic examination.

Experiment II: The partially hepatectomized rats were divided into 4 groups. IIa: The first group received no treatment, and was sacrificed under ether anesthesia at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 14 h after operation. IIb: The second group received an intraperitoneal injection of 12.5 μg/kg body weight of mouse EGF, as a solution of 12.5 μg/ml in saline, every 2 h, starting at the time of the operation up to 2 h before sacrifice. IIc: The third group received the same amount of EGF only at the time of operation, and was sacrificed 4 h later. IId: The fourth group received an intraperitoneal injection of actinomycin D (Sigma Chemical Co., St. Louis, U.S.A.) at 0 or 6 h after operation as a solution in 10% ethanol in saline, at a dose of 1 mg/kg body weight, and sacrificed 4 h later. EGF was similarly injected twice at the time of actinomycin D administration and 2 h later. The partially hepatectomized rats given single or multiple intraperitoneal injections of the same volume of saline and/or 10% ethanol in saline instead of EGF and/or actinomycin D were used as control rats of the groups IIb, IIc and IId. In all rats, the liver was perfused with 20 ml of ice-cold saline at the time of sacrifice. The right lateral lobe of the excised liver was quickly frozen in liquid nitrogen and stored at -80°C until used for determinations of ODC and SAT activities and contents of polyamines, immunoreactive ODC protein and ODC mRNA.

To induce kidney ODC, 25 mg testosterone enanthate (Teikoku-zouki Co., Tokyo, Japan) was subcutaneously injected to 2-month-old male ICR mice fed a commercial pelleted diet and water _ad_

libitum 10 days before sacrifice as reported (22).

Measurement of ^{125}I-EGF incorporation into the liver

The entire right lateral lobe of the excised liver was put into vials, and its radioactivity was measured in a gamma-counter.

Autoradiographic examination

The right lateral lobe of the excised liver was cut into slices about 1 mm thick, fixed in neutral buffered 7% formaldehyde solution for 2 h, rinsed overnight in 0.1 M phosphate buffer, pH 7.4, and embedded in paraffin. Thin sections were obtained and mounted on glass slides. After being dewaxed, they were kept at room temperature for 2 days. They were then dipped into 0.1% collodion solution in isoamyl acetate and coated with Kodak NTB-3 nuclear emulsion (Eastman Kodak Co., New York, U.S.A.). After exposure in the dark for 7 days, the slides were developed in Kodak D-19 developer (Eastman Kodak), kept overnight at 100° C, and stained with hematoxylin-eosin.

Grain quantitation of light autoradiography was performed at 1,000 x magnification. One section was obtained from each rat. Two of the fields containing 1050-1100 grains within hepatocytes were randomly selected for each section, and the number of the grains in hepatocyte nuclei were counted.

Assays for hepatic activities of ODC and SAT

Hepatic ODC activity was determined according to the method of

Oka and Perry (23) with some modifications. The excised liver was homogenized with 9 volumes of a buffer solution consisting of 50 mM Tris-HCl, pH 7.5, containing 5 mM dithiothreitol (Wako Pure Chemical Industries, Osaka, Japan), 40 mM pyridoxal phosphate, and 4 mM EDTA in an all-glass Tenbrock tissue grinder (Wheaton Scientific, Millville, N.J.). After centrifugation at 100,000 x g for 60 min at 4°C, the supernatant was collected. To 0.2 ml of the supernatant, 1 ml of the buffer solution without EDTA containing 18.5 kBq of DL-[1-^{14}C] ornithine hydrochloride (2.26 TBq/mol; Amersham International, London, U.K.) and 0.2 mM L-ornithine hydrochloride (Sigma Chemical) was added in a glass tube tightly capped with rubber, and the solution was incubated at 37°C for 60 min while being shaken continuously. After adding 0.5 ml of 50% trichloroacetic acid solution, the tube was shaken similarly for 60 min. Then $^{14}CO_2$ liberated from the reaction mixture was trapped with 0.5 ml of Protosol (New England Nuclear) in the center well. To the Protosol, 4 ml of Atomlight (New England Nuclear) was added, and the radioactivity was counted in a liquid scintillation counter. ODC activity in the mouse kidney was measured with the supernatant prepared from kidney homogenate with 9 volumes of the same buffer. Protein content of the supernatant was determined according to the method of Bradford (24).

Hepatic SAT activity was determined by the method of Libby (25) with some modifications. The excised liver was homogenized with 4 volumes of ice-cold 0.25 M sucrose containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$. The homogenate was cen-

trifuged at 100,000 x g for 60 min, and the supernatant was collected. To an aliquot of the supernatant at 100 μg of protein, 10 μmol of Tris-HCl, pH 7.8, 300 nmol of spermidine trihydrochloride (Sigma Chemical), and 1.48 kBq of [1-^{14}C] acetyl-CoA (1.78 TBq/mol; New England Nuclear) were added, and the final volume of the mixture was adjusted to 0.1 ml. After incubating the mixture at 30° C for 10 min, 20 μl of 1 M NH_2OH-HCl was added at 2° C, and then the mixture was kept at 100° C for 3 min. After centrifugation at 3,000 x g for 5 min, 0.05 ml of the supernatant was applied to cellulose phosphate paper disks. The disks were washed five times with distilled water and three times with ethanol. After drying in air, the disks were placed in vials containing 5 ml Atomlight. The radioactivity was counted in a liquid scintillation counter.

Assay for hepatic polyamine content

Hepatic polyamine content was determined by the method of Oshima (26) with some modifications. The excised liver was homogenized with 4 volumes of 0.5 N perchloric acid with a Polytron. After centrifugation at 10,000 x g for 20 min, the supernatant was collected and added to 4 volumes of 0.5% sulfosalicylic acid. After centrifugation at 3,000 x g for 15 min, the supernatant was applied to a cation exchange resin CK-10U (Mitsubishi Chemical Co., Tokyo, Japan) column, which was eluted with a linear gradient from 0.9 to 2.5 M NaCl in 0.33 M acetic acid buffer, pH 4.9, containing acetonitrile and NaOH at 0.7 ml/min,

55°C. The polyamines were quantitated by means of the O-phthalaldehyde reaction.

Assay for immunoreactive ODC protein

An aliquot of the solution of polyclonal IgG anti-mouse kidney ODC antibody raised in a rabbit (22), a gift of Dr. Shin-ichi Hayashi (Jikei University School of Medicine, Department of Nutrition, Japan), was diluted to 10 μg/ml in 0.1 M phosphate buffered saline (PBS), pH 7.0. Then, 100 μl of this solution was distributed into each well of a 96 well P.E.T.G. Assay Plate (Costar Europe Ltd., Badhoevedorp, the Netherlands), and kept at 4°C for 16 h. The solution was removed by pipetting, and each well was washed 4 times with 300 μl of 10 mM PBS, pH 7.0. Each well was, then, filled with 0.1 M PBS containing 25% Block Ace (Yukijirushi Nyugyou Ltd., Sapporo, Japan) and 0.1% sodium azide, and the plate was stored at 4°C for 16 h or more until use. Plates were washed 3 times with 300μl of 50 mM PBS, pH 7.0, containing 0.5% BSA prior to use. The assay samples used for ODC activity were diluted to 1:2 with 0.1 M PBS, containing 1 % BSA and 10 mM EDTA. 100 μl of these diluted samples was put into each well. After incubation at 4°C for 16 h, these samples were discarded, and each well was washed twice with 300 μl of 50 mM PBS, pH 7.0, containing 0.5% BSA and 4 times with 300 μl of 50 mM PBS. Then, 100 μl of 1 μg/ml iodinated polyclonal anti-ODC antibody solution in 50 mM PBS, containing 0.5 % BSA was put into each well; iodination was performed by the method of Hunter and

Greenwood (21). After incubation at 4°C for 16 h, the iodinated antibody solution was removed by suction, and the wells were washed 3 times with 300 μl of 50 mM PBS, containing 0.5% BSA, and 5 times with the same volume of 50 mM PBS. Each well was separated from the plate with a scalpel, and its radioactivity was counted in a gamma-counter.

When the serial dilution of supernatants from kidney homogenates of testosterone treated mice and those of rat liver homogenates prepared 4 h after Hx were assayed, there was an identical linear association of the radioactivity with ODC activity in a logistic scale, as shown in Figure 2. The immunoreactive protein concentration was calculated as described by Nishiyama *et al*. (22). 830 pg of rat liver ODC protein corresponded to ODC activity that will release 1 nmol of CO_2 from ornithine per hour. The lower limit of sensitivity of the method was 20 pg.

Assay for ODC mRNA

RNA was isolated by the guanidinium thiocyanate-hot phenol method (27) with some modifications. The liver was homogenized using a Polytron in 5 volumes of 4 M guanidine thiocyanate (Fluka Chemie AG, Buch, Switzerland) solution containing 0.1 M sodium acetate and 5 mM EDTA. After adding an equal volume of phenol-chloroform (1:1), the homogenate was vigorously shaken at 60°C for 15 min, centrifuged at 800 x g for 10 min at 4°C and the phenol-chloroform layer was discarded. This procedure was repeated twice, followed by extraction with chloroform alone twice.

After adding 2 volumes of ethanol, the remaining layer was kept at -20°C for 2 h, and its pellet was obtained by centrifugation at 4800 x g for 15 min at 4°C. The pellet was dissolved in 0.1 M Tris buffer, pH 7.4, containing 50 mM sodium chloride, 10 mM EDTA, 0.2% SDS and 200 μg/ml of proteinase K (Merck, Darmtadt, West Germany), and incubated at 37°C for 2 h. Then the extraction with phenol-chloroform and chloroform alone and the precipitation with ethanol were similarly carried out. The resultant pellet was dissolved in sterile distilled water and added with one-fourth volume of 10 M lithium chloride solution. After incubation at 4°C overnight, RNA was recovered by centrifugation at 18,000 x g for 20 min at 4°C. Isolated RNA was dissolved in sterile distilled water, and its concentration and purity were checked with a spectrophotometer. 10 μg of total RNA was denatured in 1X Goldburg buffer { 1X Goldburg buffer = 0.4 M sodium 3-[N-Morpholino] propanesulfonate (Wako Pure Chemical Industries), 50 mM sodium citrate, 5 mM EDTA, pH 7.2 } containing 6% formaldehyde and 50% deionized formamide (Merck), and applied to a 1% HGT agarose (FMC BioProducts, Rockland, U.S.A) gel formed in 1X Goldburg buffer with 6% formaldehyde. Size fractionation by electrophoresis was carried out in the same buffer. RNA was then transferred to a nylon filter (Amersham) by overnight blotting with 20X concentrated SSC buffer (1X SSC buffer = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) (28). The filter was baked at 80°C for 2 h, preannealed in prehybridization buffer {50% deionized formamide, 6X concentrated SSC buffer, 5X concentrated Denhardt's solution [

1X Denhardt's solution = 0.02% Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.02% polyvinylpyrrolidone, 0.02% BSA], 0.5% SDS, 100 μg/ml of denatured salmon sperm DNA} overnight at 42°C, and then annealed for 2 days at 42°C with 5 x 10^7 dpm of ^{32}P-plasmid (specific activity 2 x 10^9 dpm/μg of DNA) in hybridization buffer [prehybridization buffer containing 5% dextran sulfate (Pharmacia)]. The plasmid pODC934 was provided by Dr. Kiyoshi Nose, University of Tokyo, Institute of Medical Science, Japan); it contains, cloned in pBR322, a cDNA insert corresponding to mouse kidney ODC mRNA (29). Blots were washed once with 2X concentrated SSC buffer and 3 times with 0.2X concentrated SSC buffer at 50°C for 30 min. The hybridized RNA on the filter was then visualized by exposure to Kodak XAR-5 film (Eastman Kodak) at -80°C for 2 days using intensifying screens. The intensity of bands on the film was determined by scanning with a densitometer. Consistency of the amount of RNA applied to each lane was checked by determining the intensity of 28S rRNA visualized on the membrane by toluidine blue staining (30).

Results

^{125}I-EGF incorporation into the liver

Table 1 shows ^{125}I-EGF incorporation into the liver in partially hepatectomized and sham operated rats. The incorporation was about 2.4-fold higher in rats 5 h after Hx than in sham-operated rats. The incorporation 9 h after Hx was similar to that

at 5 h.

There were numerous autoradiographic grains throughout hepatocytes in both partially hepatectomized and sham operated rats (Figure 3). The ratio of the grains in hepatocyte nuclei to those within hepatocytes was significantly higher in partially hepatectomized rats than in sham operated rats. The ratio after Hx was similar at 5 and 9 h, as shown in Table 2.

Hepatic ODC activity and immunoreactive ODC protein and ODC mRNA contents after Hx in rats

Hepatic ODC activity increased progressively after Hx and reached an initial peak by 4 h. Activity decreased to 50% of its peak value at 6 and 8 h and then rose progressively to reach its maximal activity by 12 h (Figure 4). Immunoreactive ODC protein at 0 h was below the lower limit of the assay. Immunoreactive ODC protein correlated with enzymatic activity 2 to 14 h after Hx ($r=0.91$, $p<0.001$; Pearson's correlation coefficient), as shown in Figure 5.

There were two mRNA bands corresponding to 2.2 and 2.6 kb respectively. The smaller size mRNA was more prominent than the 2.6 kb mRNA. The ratio was approximately 2.4 to 1 (Figure 6). The intensity of each of the bands increased gradually from 1 h after Hx. By 4 h mRNA levels were 2.9-fold those at time zero and by 12 h 8.1-fold (Figure 4).

Effect of EGF on polyamine content and ODC and SAT activities in

partially hepatectomized liver

As shown in Table 3, when EGF was given only at the time of Hx, hepatic putrescine content and ODC activity were unchanged at 2 h, but significantly increased at 4 h compared to the control rats. When EGF was given every 2 h starting at the time of Hx, they were also significantly increased at 6 and 10 h. Hepatic SAT activity at 6 h was significantly increased compared to the control rats.

When actinomycin D was administered 6 h after Hx, hepatic ODC activity was reduced at 10 h compared to the control rats, but this reduction was significantly attenuated by giving EGF at 6 and 8 h. A similar attenuation was not seen by giving EGF at 0 and 2 h following actinomycin D administration at 0 h, as shown in Table 4.

Effect of EGF on ODC mRNA and immunoreactive ODC protein contents in partially hepatectomized liver

As shown in Table 5 and Figure 7, hepatic ODC mRNA content was significantly higher in EGF-treated rats relative to control rats 2 h after Hx, but not at 10 h, when the amount of ODC mRNA in control rats was 4-fold that at 2 h.

Hepatic immunoreactive ODC protein content changed correspondingly with ODC activity at 4, 6 and 10 h. There was a significant correlation between the content and activity ($r=0.87$, $p<0.001$; Pearson's correlation coefficient), as shown in Figure 8.

Discussion

Because EGF receptors change in number during liver regeneration (7), it is of interest to determine whether EGF is taken up by hepatocytes after Hx. A recent report demonstrated that either intraportal EGF injection alone or Hx produced a similar 2-fold increase in retained internalized ^{125}I-EGF at 3 h post injection or Hx compared to 8 h, indicating a slower degradation rate at 3 h (31). Raper _et al_. showed that when ^{125}I-EGF was infused into either sham operated or hepatectomized rats, 60 to 70 % of the radioactivity isolated from liver nuclei represented immunoprecipitable EGF (10). The distribution of autoradiographic grains in hepatocytes after ^{125}I-EGF infusion has been reported to differ depending on lobular zones (10,32). In our preliminary experiments ^{125}I- EGF incorporation varried in different areas of the same liver lobe, but the total lobular uptake was reproducible between rats. Thus, we chose to determine the total radioactivity in one lobe to estimate the uptake of ^{125}I-EGF by hepatocytes, and used autoradiography only for confirmation of ^{125}I-EGF presence in hepatocytes and calculation of the uptake by the nuclei. Our results show that the total radioactivity in the right lateral lobe was altered to the same extent 5 and 9 h after Hx, and autoradiographic grains were present in hepatocytes of partially hepatectomized as well as sham operated rats. Although the ratio of EGF grains in hepatocyte nuclei to the total number of grains in hepatocytes was higher in partially hepatectomized

rats than in sham operated rats, the ratio was similar at 5 and 9 h after Hx. These results may indicate that the same amounts of EGF were present in hepatocytes and hepatocyte nuclei from 5 to 9 h after Hx, although the contributions of EGF degradation and receptor recycling to the dynamics of this process are unclear. It is unknown whether EGF exerts its action on hepatocytes via plasma membrane receptors and/or directly in hepatocyte nuclei. In any case, the present results indicate that exogenous EGF may continue to act on hepatocytes for at least 9 h following Hx.

In the measurement of hepatic ODC mRNA in rats, we used cDNA pODC934, a mouse probe for ODC. This probe can be assumed to measure rat ODC mRNA because mouse-rat cDNA homology is known to be over 90% in the coding region (33). Our results also showed two mRNA bands of 2.6 and 2.2 kb corresponding in size to ODC mRNAs (33). As an antibody for radioimmunoassay to determine rat liver ODC immunoreactive protein, we employed a polyclonal antibody to mouse kidney ODC. It seemed no problem to apply this antibody to measurement of rat liver ODC immunoreactive protein from the fact that the standard curve of radioimmunoassay using this antibody obtained with rat liver and mouse kidney was identical, as shown in Figure 2. This antibody was reported to disclose a single fused precipitin line against adjacently placed ODC from rat liver as well as mouse kidney upon Oudin double immunodiffusion analysis (22), and other investigators have also reported that ODCs from mouse kidney and rat liver exhibit a very similar immunological cross-reactivity to rabbit antibody raised

against the mouse enzyme (34,35).

Hepatic ODC activity changed in a highly close association with ODC immunoreactive protein. This may imply that ODC activity reflects to ODC protein production in partially hepatectomized rat liver. ODC immunoreactive protein increased rapidly up to 4 h, and fluctuated thereafter despite a gradual increase of ODC mRNA by 12 h after partial hepatectomy. ODC mRNA increased by 4 h to 2.9-fold compared to that at time zero, whereas the increase in ODC immunoreactive protein was more than 20-fold. The increase in ODC immunoreactive protein would appear to have come as a result of alternating of translational or post-translational regulation in addition to increased ODC mRNA. On the contrary, hepatic ODC mRNA increased by 12 h to 2.8-fold compared to that at 4 h, whereas ODC immunoreactive protein showed no significant change. Furthermore, the suppression of hepatic ODC activity 4 h after actinomycin D administration differed depending on the time after Hx. A similar suppression by actinomycin D in partially hepatectomized rat liver has been observed by other investigators (36,37). Thus, it is evident that the regulation in ODC induction varied in the process of liver regeneration after Hx. Similar inconsistency in ODC activity, protein and mRNA has been reported in other systems (38), although, on searching the literature, Hirvonen exceptionally reported that hepatic ODC mRNA changed showing two peaks correspondingly with ODC protein rather than with its activity after Hx in rats (39).

Exogenous EGF significantly increased hepatic putrescine con-

tent 4, 6 and 10 h after Hx, with concomitant elevations of hepatic ODC activity. It also increased hepatic SAT activity at 6 h. The enhancement of SAT activity at this time after Hx correlates with a significant increase in hepatic putrescine content (15). Thus, the observed increment in hepatic putrescine content would appear to have occured as a result of the elevated ODC and SAT activities.

Hepatic ODC mRNA content was significantly increased at 2 h after EGF treatment compared to the control levels. This increment of ODC mRNA content could have contributed to the increased ODC activity. A similar observation was reported in *in vitro* experiments using cell lines other than hepatocytes (40,41). Hepatic ODC activity, however, was not increased at 2 h. A significant increase was found at 4 h, when EGF was similarly administered. Although the reason for this is not clear from the present experiment, it would be explicable on the basis of a hypothesis that EGF also stimulates ODC protein degradation. ODC protein degradation might be too large to cause a detectable increase in ODC activity at 2 h. At 10 h, a significant increase in ODC mRNA content compared to controls was not seen. At this time, ODC mRNA content in controls was about 4-fold that seen at 2 h. It is possible that Hx had already increased the ODC mRNA to its maximum at 10 h or that the increase in ODC mRNA induced by EGF, if any, was too small to make a significant difference from the already elevated control levels. Nevertheless, the ODC activity was significantly increased albeit also to a lesser relative

degree than at 4 h and 6 h (Table 3). EGF dosing at 6 and 8 h also prevented the reduction in ODC activity at 10 h produced by inhibition of mRNA transcription with actinomycin D, which had been given at 6 h. The dosage of actinomycin D employed was thought to be almost enough to inhibit mRNA transcription, because ODC activity at 4 h was depressed to about one-twelfth control levels when dosed at 0 h, and EGF dosing at 0 and 2 h did not attenuate this reduction (Table 4). These results suggested posttranscriptional effects of EGF. On the other hand, there was a good correlation between the ODC activity and immunoreactive ODC protein content at 4, 6 and 10 h, irrespective of EGF treatment, suggesting that the contribution of an inactive ODC protein such as an ODC-antizyme complex (42,43) was unlikely. Thus, it is reasonable to assume that posttranscriptional alteration of ODC synthesis, via changes in stabilization of mRNA, mRNA translation, or both, also contributed to the increased ODC activity after EGF treatment.

In conclusion, hepatic ODC induction during the course of regeneration after Hx in rats may be regulated by different mechanisms. Exogenous EGF may stimulate hepatic putrescine production after Hx in rats by increasing ODC mRNA content and alterating posttranscriptional regulation of ODC synthesis as well as enhancing SAT activity.

The mechanisms which produced these alterations in ODC synthesis and SAT activation by EGF are unclear from the present study, and will require additional investigation.

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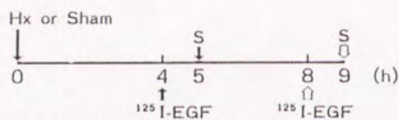
Experiment I



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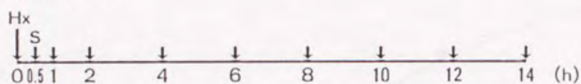

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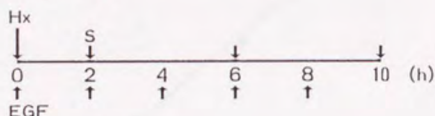

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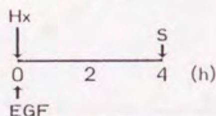

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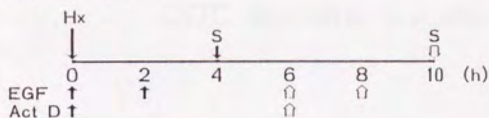


Figure 1. Experimental designs. Hx = partial hepatectomy, Sham = sham operation, S = sacrifice, EGF = administration of epidermal growth factor or saline, ^{125}I-EGF = administration of ^{125}I-labeled epidermal growth factor, Act D = administration of actinomycin D or 10% ethanol in saline.

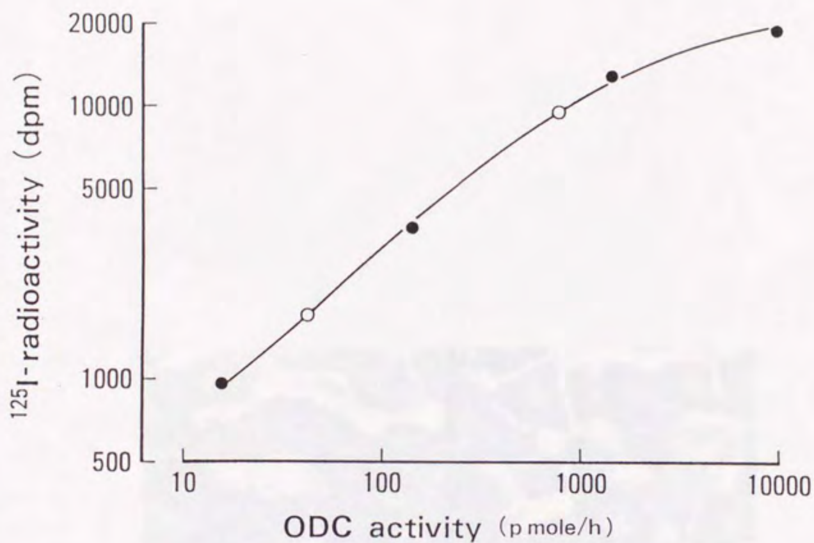


Figure 2. Standard curve for mouse kidney and rat liver ornithine decarboxylase (ODC) protein by solid phase radioimmunoassay. Closed circles indicate kidney extract from mouse dosed with testosterone. Open circles indicate liver extract from a rat 4 h after partial hepatectomy.

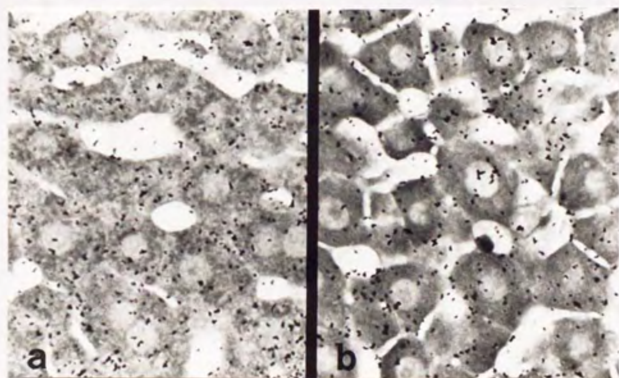


Figure 3. Autoradiographs of rat hepatocytes 1 h after injection of ^{125}I-EGF. x400. Numerous grains are seen throughout hepatocytes including the nuclei. (a) Sham operated rats and (b) rats 9 h after partial hepatectomy.

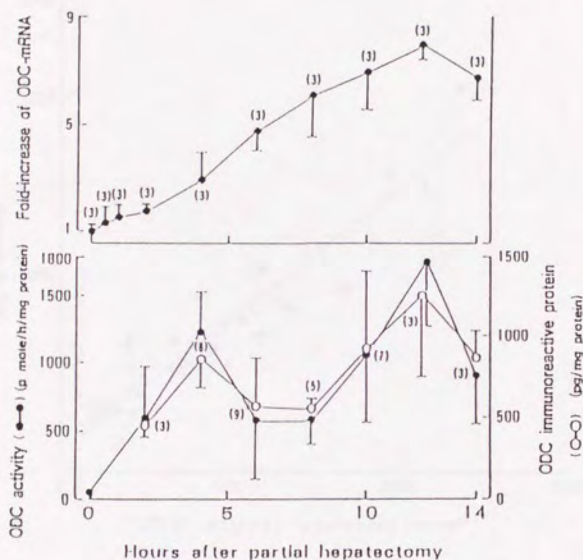


Figure 4. Changes of hepatic ornithine decarboxylase (ODC) mRNA and immunoreactive protein contents and ODC activity after partial hepatectomy in rats. Figures in parentheses indicate numbers of rats. Error bars correspond to ±SD.

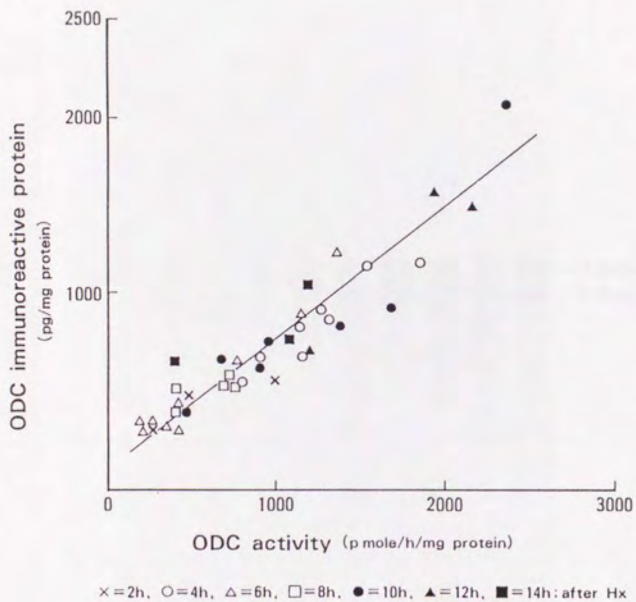


Figure 5. Correlation between hepatic ornithine decarboxylase (ODC) activity and immunoreactive protein content after partial hepatectomy (Hx) in rats.

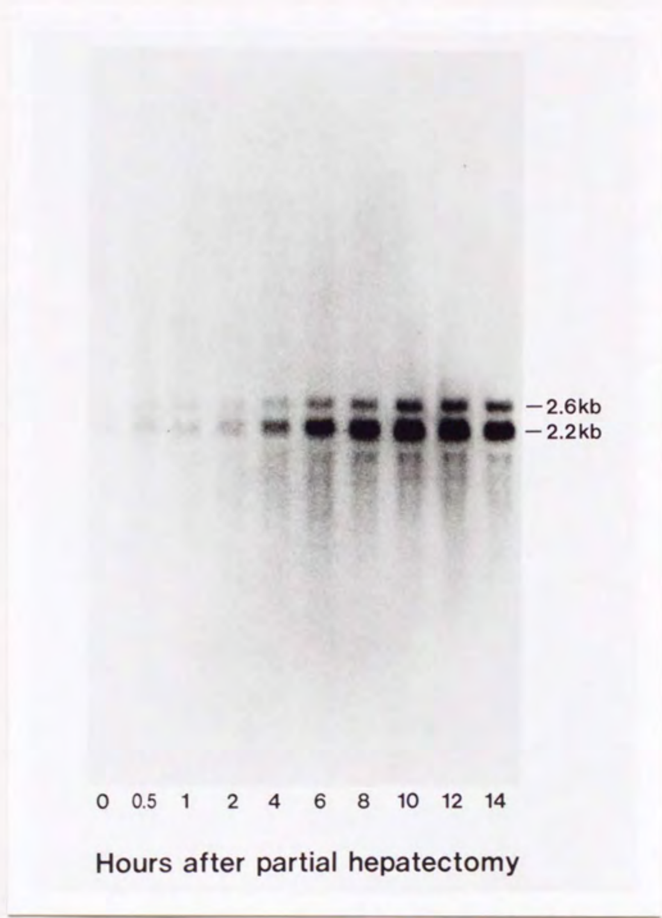


Figure 6. Northern blot analysis of hepatic ornithine decarboxylase (ODC) mRNA after partial hepatectomy in rats.

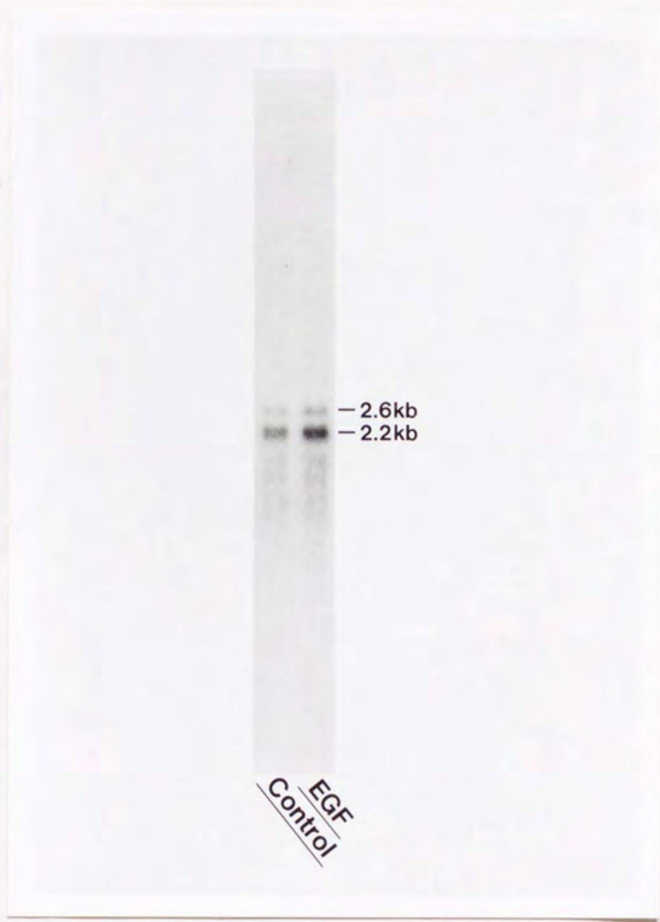


Figure 7. Northern blot analysis of hepatic ornithine decarboxylase (ODC) mRNA 2h after partial hepatectomy in control or EGF-treated rats.

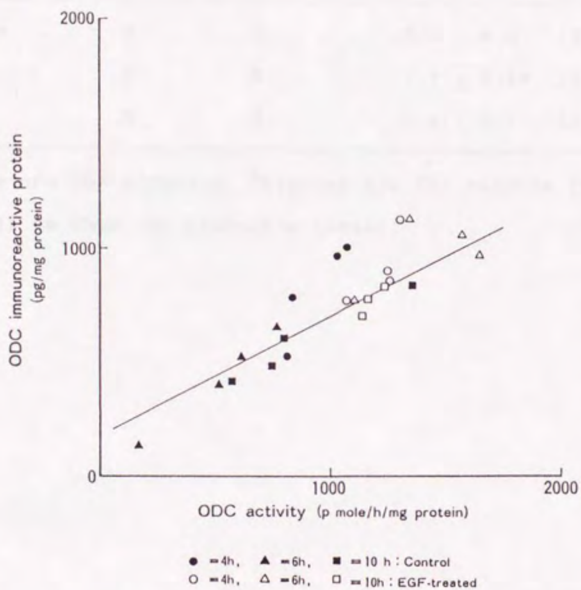


Figure 8. Correlation between hepatic ornithine decarboxylase (ODC) acitvity and immunoreactive protein content in rats treated with EGF and control rats after partial hepatectomy (Hx).

Table 1 Incorporation of ^{125}I-mouse EGF into rat liver after partial hepatectomy (Hx) or sham operation

Groups	Hours after operation	No. of rats	^{125}I-EGF incorporation (cpm/g wet wt liver x10^6)	(% of injected dose)
Sham	5	3	3.3 ± 0.6	5.4 ± 1.3[a]
Hx	5	3	7.7 ± 0.1*	13.2 ± 0.6*
Hx	9	2	6.4 , 6.7	12.1 , 12.2[b]

[a]Values are the means±SD. [b]Figures are the results from each rat.
*$p<0.001$ vs Sham, by Student's *t*-test.

Table 2 Quantitative autoradiography of ^{125}I-mouse EGF uptake by hepatocytes in rats after partial hepatectomy or sham operation

Groups	Hours after operation	^{125}I-EGF uptake nuclei/hepatocytes (%)
Sham	5	14 ± 3[a]
Hx	5	28 ± 1*
Hx	9	26 ± 3*

[a]Values are the means±SD of 4 fields in 2 rats. *$p<0.01$ vs Sham, by Student's *t*-test.

Table 3 Effect of mouse EGF on polyamine content and activities of ornithine decarboxylase (ODC) and spermidine N^1-acetyltransferase (SAT) in rat liver after partial hepatectomy (Hx)

Groups	Time[a] of EGF doses	Time[a] of determination	No. of rats	Polyamines[b] (nmole/g wet wt) Put	Spd	Spm	ODC (pmole/h/mg protein)	SAT
Control	0	2	10	32 ±7	1127 ±94	836 ±88	222[c] ±52	
EGF	0	2	10	38 ±12	1217 ±198	798 ±40	166 ±61	
Control	0	4	4	109 ±24	1308 ±85	840 ±37	942 ±137	
EGF	0	4	4	201* ±67	1160 ±25	770 ±57	1217* ±100	
Control	0-4	6	4	272 ±120	1279 ±101	828 ±98	532 ±291	768 ±174
EGF	0-4	6	4	446* ±69	1134 ±111	604 ±398	1422** ±464	1218* ±174
Control	0-8	10	9	223 ±45	1475 ±198	904 ±102	810 ±191	
EGF	0-8	10	9	359** ±75	1554 ±120	904 ±101	970* ±122	

[a]Hours after partial hepatectomy. [b]Put indicates putrescine, Spd spermidine, and Spm spermine. [c]Values are the means±SD. *$p<0.05$, **$p<0.01$ vs Control, by Student's *t*-test.

Table 4 Effect of actinomycin D (Act D) and mouse EGF on hepatic ornithine decarboxylase (ODC) activity after partial hepatectomy in rats

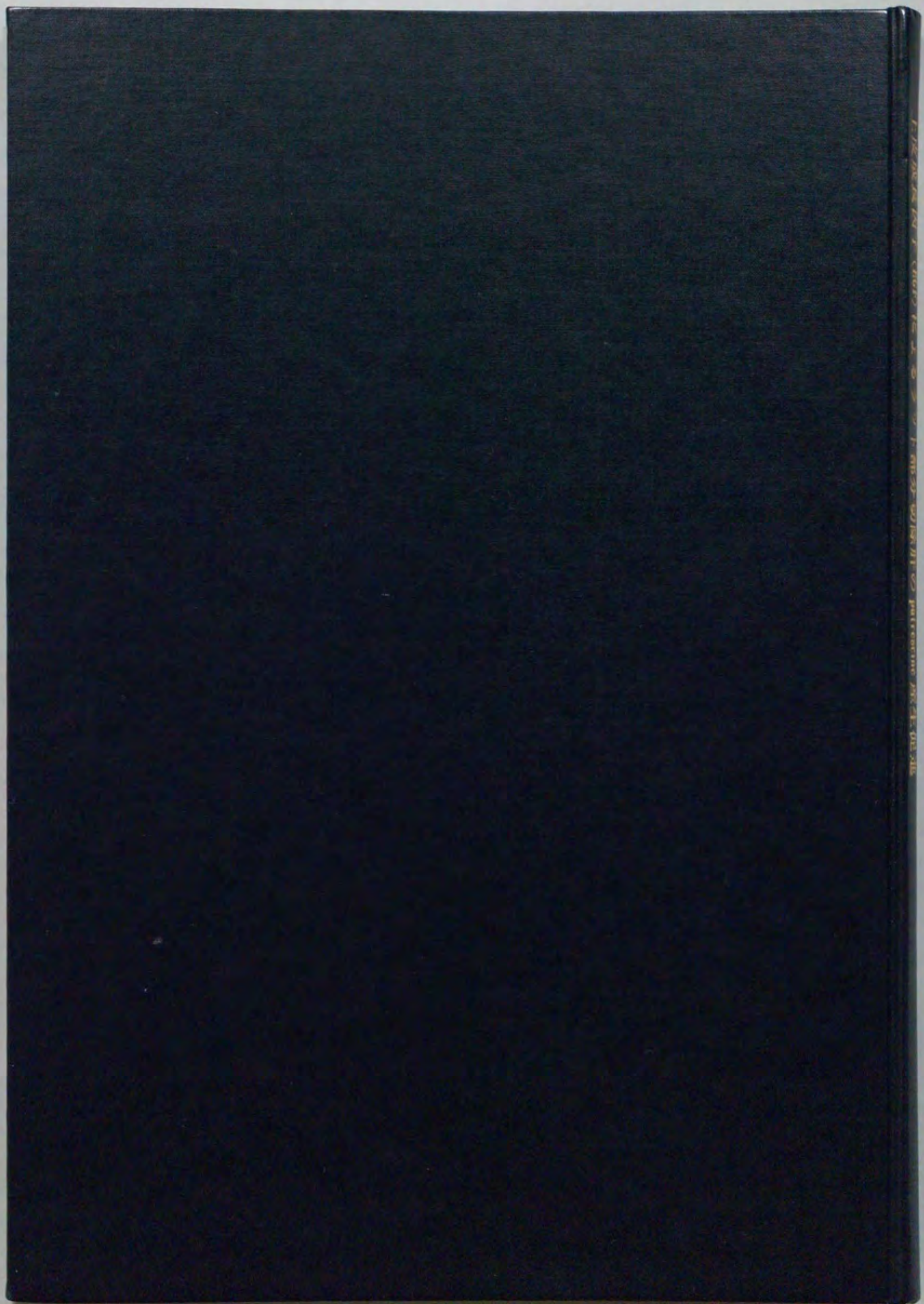
Groups	Time[a] of Act D dose	Time[a] of ODC determination	ODC (pmole/h/mg protein)
Control	0	4	1405 ± 619 (4)[b]
Act D	0	4	120 ± 21† (4)
Act D + EGF	0	4	156 ± 46† (4)
Control	6	10	1403 ± 235 (7)
Act D	6	10	705 ± 190† (7)
Act D + EGF	6	10	1216 ± 545* (7)

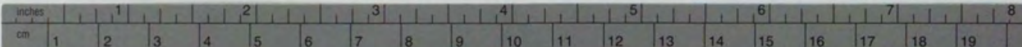
[a]Hours after partial hepatectomy. [b]Values are the means±SD, and figures in parentheses are the number of rats. †$p<0.01$ vs Control, *$p<0.05$ vs Act D, by Student's *t*-test.

Table 5 Effect of mouse EGF on ornithine decarboxylase (ODC) mRNA content in rat liver after partial hepatectomy (Hx)

Groups	Hours after Hx	ODC mRNA (fold induction[a])
Control	2	1.0 ± 0.1[b]
EGF	2	2.2 ± 0.6*
Control	10	4.0 ± 0.8
EGF	10	5.2 ± 1.2

[a]Fold induction of control groups 2 h after Hx. [b]Values are the means±SD of 7 rats. *$p<0.01$ vs Control, by Student's *t*-test.





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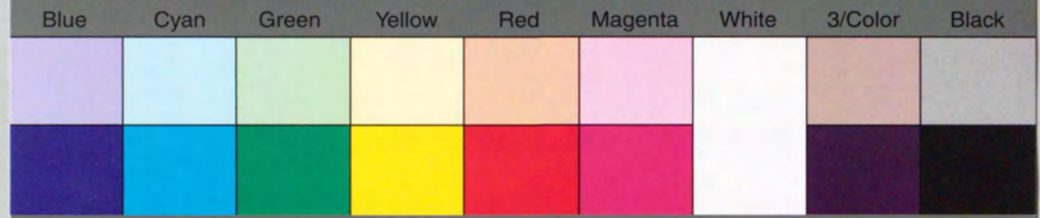

Kodak Gray Scale

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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19