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World J Gastroenterol 2003;9(2):271-275
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

Study on the mechanism of epidermal growth factor-induced proliferation of hepatoma cells

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Received: 2001-04-11 **Accepted:** 2002-03-18

Abstract

AIM: Many growth factors, such as epidermal growth factor (EGF), are associated with the carcinogenesis. EGF plays its role in the proliferation of hepatoma cells through binding with EGF receptor (EGFR) and a series of signal transduction. But the postreceptor pathway is still not clear. In the present experiment, we studied the effect of tyrosine kinase, protein kinase C, Na^+/H^+ exchange, calmodulin and voltage-dependent Ca^{2+} channel on EGF-induced hepatoma cell proliferation.

METHODS: Hepatoma cell line SMMC7721 was cultured in RPMI1640 serum-free medium. In order to study the effect of thyrosine kinase, protein kinase C, Na^+/H^+ exchange, calmodulin and voltage-dependent Ca^{2+} channel on human heptoma cell proliferation induced by epidermal growth factor (EGF), DNA synthesis rate of hepatoma cells was measured by the method of ^{3}H-TdR incorporation.

RESULTS: EGF (10^{-9} M) stimulated the proliferation of heptoma cells significantly (^{3}H-TdR incorporation was 1 880±281 cpm/well, $P<0.05$), and this effect was significantly inhibited by tyrosine kinase inhibitor genistein (^{3}H-TdR incorporation was 808±209 cpm/well, $P<0.001$). Calmodulin inhibitor W-7, protein kinase C inhibitor H-7 and Na^+/H^+ exchange inhibitor amiloride individually had significant inhibiting effect on EGF-induced proliferation of hepatoma cells (^{3}H-TdR incorporation was 978±87.3 cpm/well, 1 241±147 cpm/well, 1 380±189 cpm/well, respectivly, $P<0.001$, $P<0.01$, $P<0.05$), but they all had no effect on the basal level proliferation of cultured hepatoma cells (^{3}H-TdR incorporation was 1 284±260 cpm/well, 1 179±150 cpm/well, 1 392±152 cpm/well, respectivly, ^{3}H-TdR incorporation of the control was 1 353±175 cpm/well, $P>0.05$). Voltage-dependent Ca^{2+} channel inhibitor verapamil had no inhibition on EGF-induced proliferation of hepatoma cells (^{3}H-TdR incorporation was 1 637±133 cpm/well, $P>0.05$), it also had no effect on the basal level proliferation of cultured hepatoma cells (^{3}H-TdR incorporation was 1 196±112 cpm/well, $P>0.05$).

CONCLUSION: Our data suggest that tyrosine kinase, Ca^{2+}-calmodulin-dependent pathway, protein kinase C and Na^+/H^+ exchange play a critical role in EGF-induced proliferation of hepatoma cells and that the effect of EGF is independent of voltage-dependent Ca^{2+} channel.

Wu WB, Wu Y, Wang JL, Lin JS, Yuan SY, Li A, Cui WR.Study on the mechanism of epidermal growth factor-induced proliferation of hepatoma cells. *World J Gastroenterol* 2003; 9(2): 271-275
http://www.wjgnet.com/1007-9327/9/271.htm

INTRODUCTION

Most hepatocellular carcinoma (HCC) is associated with cirrhosis, a condition in which liver cell necrosis coexists with inflammation and hepatocellular regeneration. HCC arising in cirrhotic livers is exposed to a number of growth factors mediating hepatocellular regeneration. Epidermal growth factor (EGF) is one of them.

EGF is a single strand polypeptide composed of 53 amino acid and has implicated in the regulation of a wide variety of physiological and pathological processes including embryo genesis, growth, tissue repair, regeneration and neoplasia. EGF inhibits gastric acid secretion and has a cytoprotective effect on stomach tissue, EGF has been of clinical interest for the treatment of acid hypersecretion and the healing of ulcers. EGF has been implicated as a hepatotrophic factor during liver regeneration, has protective effect during hepatic damage through inducing the DNA synthesis of hepatic cells[1-4]. EGF also induces DNA synthesis of hepatoma cells and promotes the occurrence and expansion of hepatoma[5,6].

EGF exerts its biologic effects by binding with the EGF receptor (EGFR), a type of transmembrane glucoprotein with a molecule of 170kD. The primary structure of its intramembranous part is associated with the expression of oncogene erb-B. The intracellular part of EGFR has tyrosine kinase activity. EGFR is overexpressed in various carcinoma, such as hepatoma[7-9], gastric carcinoma[10-14], colon carcinoma[15-20], lung carcinoma[21-23] and prostatic carcinoma[24-28], and also associated with the histologic types and invasiveness of the tumor[29-32]. Many growth factors have the effect of promoting cells proliferation and malignant transformation by binding with EGFR such as EGF, heparin binding EGF (HB-EGF)[33,34], transforming growth factor β (TGF-β)[35,36] and hepatopoientin[37,38]. After the integration of HBV-X gene into hepatic cells, EGFR was activated and overexpressed, which induced liver carcinogenesis[39].

In general, EGF induces tumor cell division and proliferation through a series of signal transduction by binding with EGF receptor (EGFR) and activating its thyrosine protein kinase and phosphorating itself and its protein substrate[40]. This postreceptor singnal transduction is associated with phosphatidylinositol pathway[41]. But the exact mechanism is still not clear.

In this report, we have characterized the anti-tumor activity of tyrosine kinase, calmodulin, protein kinase C, Na^+/H^+ exchange and voltage-dependent Ca^{2+} channel inhibitors. The SMMC7721 cell line cultured in serum-free medium was used in this study.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from sigma chemical CO. (St. Louis. Mo. USA) unless otherwise stated. ^{3}H-TdR was purchased from the Institute of Nuclear Power of China. Verapamil, streptomycin, penicillin and 0.5 % hydrocortisone were clinical medicines.

Cell culture

SMMC7721 cells were stored in our laboratory and cultured in RPMI1640 serum-free medium. Briefly, SMMC7721 cells were grown in RPMI1640 supplemented with 5 μg/ml transferrin, 20 μU/ml insulin, 0.4 μg/ml hydrocortisone and 100 μg/ml streptomycin and penicillin.

Experiment program

Eleven groups were separated in the experiment: (1) control, (2) EGF (10^{-9}M), (3) W-7, (4) verapamil, (5) W-7+EGF, (6) verapamil+EGF, (7) genistein+EGF, (8) H-7, (9) H-7+EGF, (10) amiloride, (11) amiloride+EGF.

DNA synthesis rate of hepatoma cells

The DNA synthesis rates were measured by the method of ^{3}H-TdR incorporation. The hepatoma cells were seeded into 96 well plates at a density of 1×10^4/well and incubated with serum-free RPMI1640. After 24 hours, fresh medium was changed, and reagents added. After incubation for 18 hours the medium was then replaced with fresh medium containing ^{3}H-TdR 0.5 μci/ml for another 6 hours. The cells were finally lysed with 0.33 mol/L HCl, ^{3}H-TdR incorporation was determined with a β-counter.

Statistical analysis

Two-side *t* test was used to examine the significant difference between groups.

RESULTS

Effect of genistein on EGF-induced growth of hepatoma cells

Genistein is a tyrosine kinase inhibitor. EGF (10^{-9}M) stimulated the growth of hepatoma cells significantly compared with the control ($P<0.05$). EGF-induced ^{3}H-TdR incorporation was reduced from 1 880±281 cpm/well to 808±209 cpm/well by genistein ($P<0.001$, Figure 1).

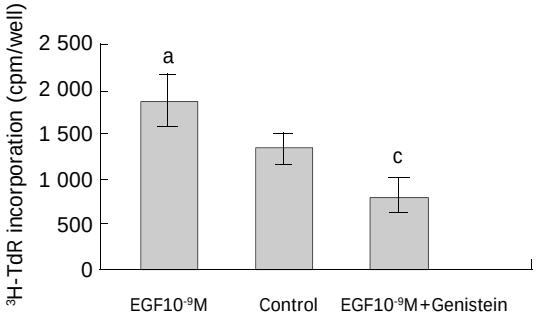

Figure 1 Effect of genistein on EGF-induced ^{3}H-TdR incorporation into hepatoma cells. $n=6$, $\bar{x}\pm s$, $^aP<0.05$, *vs* control; $^cP<0.001$, *vs* EGF 10^{-9}M.

Effect of W-7 on EGF-induced growth of hepatoma cells

W-7 is a calmodulin inhibitor. W-7 (25 μmol/L) alone had no effect on ^{3}H-TdR incorporation, the incorporation of W-7 group and control were 1 284±260 cpm/well and 1 353±175 cpm/well, respectively ($P>0.05$). When W-7 was added with EGF (10^{-9}M), the effect of EGF on the growth of hepatoma cells was inhibited significantly, the incorporation (978±87.3 cpm/well) was significantly lower than that of EGF (10^{-9}M) group (1 880±281 cpm/well) ($P<0.001$, Figure2).

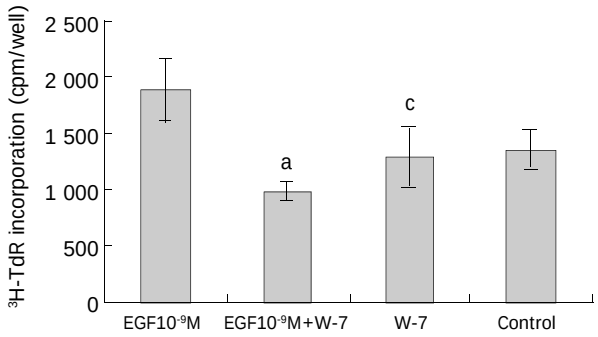

Figure 2 Effect of W-7 on EGF-induced ^{3}H-TdR incorporation into hepatoma cells. $n=6$, $\bar{x}\pm s$ $^aP<0.001$ *vs* W-7 group; $^cP>0.05$ *vs* control.

Effect of verapamil on EGF-induced growth of hepatoma cells

Verapamil is voltage-dependent Ca^{2+} channel inhibitor. As shown in Figure3, verapamil (100 μmol/L) alone had no effect on ^{3}H-TdR incorporation, the incorporation of verapamil group and control were 1 196±112 cpm/well and 1 353±175 cpm/well, respectively ($P>0.05$). When verapamil (100 μmol/L) was used with EGF (10^{-9}M), the incorporation was 1637±133 cpm/well, which was not significantly different from the incorporation with EGF (10^{-9}M) alone (1 880±281 cpm/well, $P>0.05$).

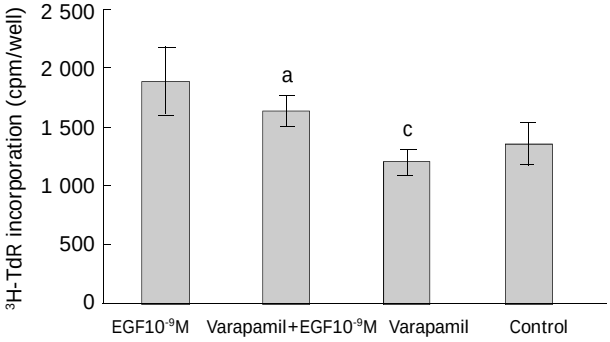

Figure 3 Effect of verapamil on EGF-induced ^{3}H-TdR incorporation into hepatoma cells. $n=6$, $\bar{x}\pm s$ $^aP>0.05$ *vs* EGF10^{-9}M group; $^cP>0.05$ *vs* control.

Effect of H-7 on EGF-induced growth of hepatoma cells

H-7 is a specific inhibitor of protein kinase C. H-7 (50 μmol/L) alone had no effect on the basal level ^{3}H-TdR incorporation, the incorporation of H-7 group and control were 1 179±150 cpm/well and 1 353±175 cpm/well, respectively ($P>0.05$). When H-7 was used together with EGF (10^{-9}M), the effect of EGF on the proliferation of hepatoma cells was inhibited significantly, the incorporation (1 241±147 cpm/well) was significantly lower than that of EGF group (1 880±281 cpm/well, $P<0.01$) (Figure4).

Effect of Na^+/H^+ exchange on EGF-induced growth of hepatoma cells

Amiloride is an inhibitor of Na^+/H^+ exchange. As shown in Figure 5, amiloride (0.1 mmol/L) alone had no effect on ^{3}H-TdR incorporation, the incorporation of amiloride group and control were 1 392±152 cpm/well and 1 353±175 cpm/well, respectively ($P>0.05$). When amiloride (0.1 mmol/L) was used

with EGF (10^{-9}M), the effect of EGF on the proliferation of hepatoma cells was inhibited significantly, the incorporation (1 380±189 cpm/well) was significantly lower than that of EGF group (1 880±281 cpm/well, $P<0.05$) (Figure 5).

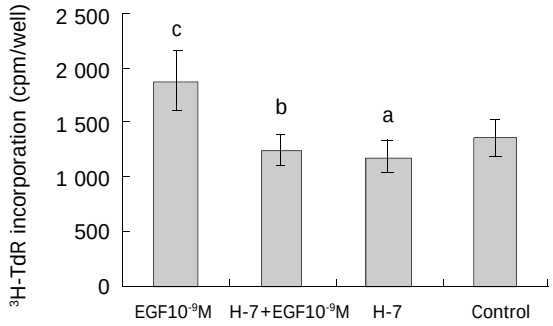

Figure 4 Effect of H-7 on EGF-induced ^{3}H-TdR incorporation into hepatoma cells. n=6, $\bar{x}\pm s$, $^aP>0.05$ compared with control; $^bP<0.01$ *vs* EGF 10^{-9}M group; $^cP<0.05$ *vs* control.

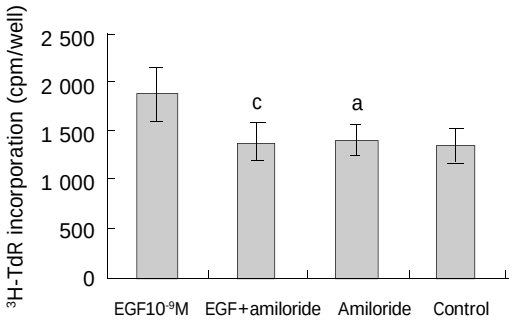

Figure 5 Effect of amiloride on EGF-induced ^{3}H-TdR incorporation into hepatoma cells. n=6, $\bar{x}\pm s$, $^aP>0.05$ *vs* control; $^cP<0.05$ *vs* EGF (10^{-9}M) group.

DISCUSSION

In this study, we demonstrated that EGF (10^{-9}M) had strong stimulation on the growth of hepatoma cells. It is well documented that EGF binds with the EGFR and activates the tyrosine kinase of the receptor. In this paper, the tyrosine kinase inhibitor genistein effectively blocked the growth of hepatoma cells induced by EGF.

Introcellular Ca^{2+} is one of critical factors maintaining the intracellular homeostasis. The concentration of Ca^{2+} in the cell plasma is about 10^{-7} mol/L, which is far lower than extracellular concentration of 10^{-3} mol/L. The enhancement of the intracellular Ca^{2+} concentration is achieved in two ways: one is the membrane depolarization, the other is the release from the intracellular stores intervened by phosphatidylinositol 1,4, 5-triphosphate (IP_3). When the concentration of the intracellular Ca^{2+} rises to 5 folds above normal, the Ca^{2+} pump will be activated and put the Ca^{2+} out of the cell. Intracellular Ca^{2+} is the cofactor of many critical enzymes and functional proteins, and is an important intracellular messenger.

Studies demonstrate that in hepatoma cells EGF and other Ca^{2+} mobilizing hormones stimulate the rapid formation of IP_3 and a concomitant rise in cytosolic Ca^{2+}, apparently mobilized from intracellular stores[42]. Ca^{2+}-binding protein regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis, and inhibits Ca^{2+}-activated DNA fragmentation. Regucalcin may play a physiological role in the control of over-prolifertive cells[43].

In this experiment, the calmodulin inhibitor W-7 was used with EGF, and it significantly inhibited the growth of hepatoma cells induced by EGF, but it showed no effect on the growth of hepatoma cell when used alone. On the contrary, voltage-dependent Ca^{2+} channel inhibitor verapamil had no effect on the growth of hepatoma cells induced by EGF. It is suggested that Ca^{2+}-calmodulin-dependent pathway plays a critical role on the proliferation of heptoma cells induced by EGF, and the effect of EGF is independent of voltage-dependent Ca^{2+} channel.

In the signal transduction pathway of phosphatidylinositol, activators bind with the cell surface receptor and activate phospholipase C (PLC)-β through G protein. PLC catalyzes dehydration of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diglyceride (DG). IP_3 promotes the release of Ca^{2+} from endoplasmic reticulum. With the participation of Ca^{2+}, DG activates PKC, and PKC urges the phosphoration of tyrosine which activates oncogene erb-B with productions of protein Erb-B2 and Erb-B3[44]. In this study, PKC inhibitor H-7 used alone had no significant effect compared with control, but when used with EGF, it significantly inhibited the mitogenic effect of EGF on hepatoma cells. The result suggests the activation of PKC is an important link in the effect of EGF, and also implies that the effect of EGF is associated with phosphatidylinositol signaling pathway.

Intracellular pH (pHi) is another critical factor maintaining the intracellular homeostasis. At present, many studies found that pHi is associated with the pathogenesis of many diseases and mitosis of many kind of cells. Na^+/H^+ exchange is an important mechanism of pHi modulation. Activation of Na^+/H^+ exchange is the trigger of cellular protein and DNA synthesis, and is of fundamental importance for tumor growth. An increased influx of Na^+ through the stimulation of Na^+/H^+ exchange plays a key role during early phases of the cell cycle. Na^+/H^+ exchange messenger RNA expression may reach 10 times higher in HepG2 than in normal hepatocytes. EGF can increase baseline pHi in a dose-dependent manner. This effect was completely inhibited by pretreatment with amiloride[45]. In this experiment, we found that Na^+/H^+ exchange inhibitor amiloride (0.1 mmol/L) alone showed no effect on the basal level ^{3}H-TdR incorporation of hepatoma cells. On the contrary, when it was used with EGF (10^{-9}M), the stimulatory effect of EGF was significantly inhibited. This suggests that the activation of Na^+/H^+ exchange may be one pathway of the effect of EGF.

Recent studies have shown that Ca^{2+}/calmodulin-dependent protein kinase IV was overexpressed in HCC with high activity and might be involved in the development of HCC[46]. The effect of EGF on the hepatoma cells was associated with protein kinase C and calmodulin. Protein kinase C can activate Na^+/H^+ exchange. After the phosphorylation of EGFR, the proliferation signal was sent into the nucleus through STAT3 (signal transducers and activators of transcription 3)[47]. The mechanism of this signal transduction is very complicated. It is connected with the activation of oncogene ras[48,49], erb-B[44], cyclinD1 and c-Myc[50,51]. The effect of cell proliferation induced by EGF is associated with the MAPK (mitogen-activated protein kinase) pathway. The interactions between them demand more intensive studies.

In conclusion, our data suggest that PKC, calmodulin and Na^+/H^+ exchange could play a critical role in the growth of some hepatic tumors and that the inhibition of PKC, calmodulin or Na^+/H^+ exchange activity through pharmacological or genetic interventions, could theoretically be a useful strategy to reduce the growth rate of hepatocellular carcinoma.

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Edited by Pang LH