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Research Article

Insulin-like growth factor-I stimulates H_4II rat hepatoma cell proliferation: Dominant role of PI-3′K/Akt signaling

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ARTICLE INFORMATION

Article Chronology:
Received 17 June 2004
Revised version received
23 November 2005
Accepted 10 January 2006
Available online 17 February 2006

Keywords:
H4II cells
Rat hepatoma
Proliferation
DNA replication
Insulin-like growth factor-I/-II
Erk-1/Erk-2
PI-3′K/Akt signaling
mTOR
Signaling cross-talk

ABSTRACT

Although hepatocytes are the primary source of endocrine IGF-I and -II in mammals, their autocrine/paracrine role in the dysregulation of proliferation and apoptosis during hepatocarcinogenesis and in hepatocarcinomas (HCC) remains to be elucidated. Indeed, IGF-II and type-I IGF receptors are overexpressed in HCC cells, and IGF-I is synthesized in adjacent non-tumoral liver tissue. In the present study, we have investigated the effects of type-I IGF receptor signaling on H_4II rat hepatoma cell proliferation, as estimated by ^3H-thymidine incorporation into DNA. IGF-I stimulated the rate of DNA synthesis of serum-deprived H_4II cells, stimulation being maximal 3 h after the onset of IGF-I treatment and remaining elevated until at least 6 h. The IGF-I-induced increase in DNA replication was abolished by LY294002 and only partially inhibited by PD98059, suggesting that phosphoinositol-3′ kinase (PI-3′K) and to a lesser extent MEK/Erk signaling were involved. Furthermore, the 3- to 19-fold activation of the Erks in the presence of LY294002 suggested a down-regulation of the MEK/Erk cascade by PI-3′K signaling. Finally, the effect of IGF-I on DNA replication was almost completely abolished in clones of H_4II cells expressing a dominant-negative form of Akt but was unaltered by rapamycin treatment of wild-type H_4II cells. Altogether, these data support the notion that the stimulation of H_4II rat hepatoma cell proliferation by IGF-I is especially dependent on Akt activation but independent on the Akt/mTOR signaling.

Introduction

Hepatocellular carcinoma (HCC) accounts for >80% of liver cancers worldwide, is one of the most fatal cancers, and arises from multiple risk factors [1]. In this connection, understanding the molecular mechanisms that underlie imbalanced proliferation/apoptosis processes in the course of hepatocarcinogenesis is needed [2,3]. Such an imbalance may result from the loss of coordinated response to growth factors and cytokines [4,5] among which the insulin-like growth factors (IGF-I and -II) stand as suitable candidates.

The IGFs are synthesized and secreted in extracellular fluids by fetal as well as adult hepatocytes [6,7], and their interaction with type-I IGF receptor (IGF-IR) plays a pivotal role in the proliferation of a variety of cell types [8–10], in the control of cell cycle progression in G_1 [11], in the regulation of the early phases of tumorigenicity [12,13], in the maintenance of the tumorigenic phenotype [14–17], and in the prevention of apoptosis [18–21].

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doi:10.1016/j.yexcr.2006.01.007

Although the low abundance of IGF-IR in normal hepatocytes [22] suggests only weak IGF effects in adult liver, several evidence support the putative contribution of the IGFs and of IGF-IR to dysregulated hepatocyte proliferation in vivo.

First, significant increases in IGF-II and in IGF-IR genes expression were observed in human cirrhotic liver and in primary liver cancers vs. normal adult liver [22–26], and hepatic IGF-II gene expression was reactivated during hepatocarcinogenesis in transgenic mice [27]. In both species, overexpression of IGF-II in HCC was concomitant with the reactivation of a fetal pattern of gene expression [23–25] and was correlated with increased rates of cell mitotic activity, but not with changes in apoptosis [25,27,28]. Second, in hepatitis-B-infected liver at various stages of the disease and in hepatoma cells, expression of HBx was often associated with that of IGF-II and IGF-IR [29–31]. Moreover, the higher level of IGF-IR in HBx-expressing hepatoma cells enhances the mitogenic effect of the IGFs [30,31]. These observations suggest that IGF-II gene overexpression and correlated peptide secretion may play a role (i) in the premalignant proliferation observed in hepatitis B and C virus-related chronic liver diseases and (ii) in tumor cell proliferation in HCC.

Third, IGF-I gene expression is enhanced in non-burdened liver lobes of rats that had developed HCCs in one lobe after inoculation of H_4II rat hepatoma cells [32]. In these animals, the serum level of IGF-I remained unchanged [32]. Similarly, the level of IGF-I mRNA is lower in human HCC than in adjacent, non-tumoral liver tissue [33], and accordingly, serum IGF-I levels are reduced in virus-negative and virus-positive HCC patients [34,35]. These observations suggest that IGF-I may play a paracrine role in tumoral liver tissue and synergize with IGF-II in hepatocarcinogenesis or in HCCs cell proliferation. The putative role of IGF-I in HCC was further supported by a case report [36]: HCC was diagnosed in a patient with partially treated acromegaly and high serum levels of IGF-I.

Fourth, that paracrine IGF-I and overexpressed IGF-II may be involved in hepatocarcinogenesis, or in HCC cell proliferation was also suggested by the following observations. Insulin receptor substrate-1 (IRS-1) an adapter molecule in IGF-IR-mediated signaling is overexpressed in human HCCs and expressed at lower level in adjacent, non-tumoral liver [37,38]. Accordingly, targeting IRS-1 overexpression in the liver of transgenic mice yields constitutive activation of mitogen activated protein kinases (MAPK) and of phosphatidylinositol-3′ kinases (PI-3′K) signaling cascades and leads to increased DNA synthesis [38]. Finally, antisense oligonucleotides anti-IGF-II and anti-IGF-I selectively inhibit the growth of human hepatoma cell lines overexpressing IGF-II [39] and decreased the growth rate of rat HCC in vivo [40,41], respectively.

In the present study, we have investigated the effect of IGF-IR signaling on hepatoma cells proliferation, using H_4II rat hepatoma cells as a model system. H_4II cells were treated with recombinant human IGF-I rather than with IGF-II since, contrarily to IGF-II, IGF-I does not bind to type A insulin receptor (IR-A) and thus allows to study the effects mediated by IGF-IR per se. In addition, quiescent and growing H_4II cells express IGF-IR [42] and exhibit distinct responses to insulin and IGF-I [43]. We report that treatment of H_4II cells with physiological concentrations of IGF-I triggers DNA replication, and that this effect occurs within a few hours after the onset of IGF-I treatment. Such enhancement of DNA replication was found to be mediated by both the MAPK and PI-3′K signaling cascades, the effect of the latter being dominant over that of the former. Moreover, our data showed that IGF-I-enhanced cell proliferation was dependent on Akt activation but independent on mTOR signaling.

Materials and methods

Reagents

Rabbit polyclonal anti-Erk-1/Erk-2, pErk-1/pErk-2 (Thr^{202}/Tyr^{204}), Akt and pAkt (Ser^{473}) antibodies were obtained from cell signaling. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Immunotech. Recombinant human IGF-I (rhIGF-I) was from R&D Systems. LY294002, PD98059, and rapamycin were obtained from Calbiochem.

Plasmids

$pCEP_4$ and pcDNA3 expression vectors were obtained from Invitrogen and R&D, respectively. pCMV6-DN-Akt which encodes for HA-tagged, catalytically inactive Akt (K79M) was provided by Dr. D. Tsichlis.

Oligonucleotides

The oligonucleotides primers used for PCR were designed within the pCMV6 vector (5′-GATCTGGTACCACGCGTATC-3′) and in Akt cDNA sequences (5′-TGCAGCCAACCCTCCTTCAC-3′).

Cell culture

H_4II cells, a well-differentiated line of rat hepatoma cells, were cultured in HAM-F12:DMEM (1:1) supplemented with 5% fetal calf serum, 100 UI × ml^{-1} penicillin and 100 μg × ml^{-1} streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell transfection

Exponentially growing H_4II cells were collected, resuspended in 1× PBS, 10 mM HEPES, pH 7.4. 25 × 10^6 cells were cotransfected by electroporation (230 V, 960 μF; Biorad Gene Pulser) with 10 μg of $pCEP_4$ and with 40 μg either of pCMV6-DN-Akt or of empty vector, then grown in culture medium supplemented with 400 μg × ml^{-1} hygromycin. After 3 weeks, hygromycin-resistant colonies were isolated and checked for DN-Akt transgene integration.

PCR

Genomic DNA was prepared from transfected H_4II cells [44], and 1 μg samples were submitted to PCR amplification in a reaction mixture (50 μl) containing 10 mM Tris–HCl, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, 2.5 U Platinum Taq DNA polymerase. Amplification was carried out as follows: 94°C, 1 min; 60°C, 1 min; 72°C, 1 min (30 cycles), and amplicons were analyzed by 2% agarose gel electrophoresis.