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TRANSGENIC OVEREXPRESSION OF INSULIN RECEPTOR SUBSTRATE 1 IN HEPATOCYTES ENHANCES HEPATOCELLULAR PROLIFERATION IN YOUNG MICE ONLY

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Abstract

The insulin receptor substrate-1 (IRS-1) is a multisite docking protein which plays a central part in the signal transduction of growth factors such as insulin and insulin-like growth factors (IGF-1 and IGF-2). It is found to be frequently overexpressed in human hepatocellular carcinoma (HCC). To study IRS-1 over-expression in hepatocytes *in vivo*, transgenic mice overexpressing IRS-1 exclusively in hepatocytes were created which showed enhanced hepatocyte proliferation in young animals. In the present study, the phenotype of IRS-1 transgenic animals was characterized over a period of two years. The livers of transgenic and control mice were analyzed for IRS-1 expression and phosphorylation, activation of the downstream mitogen activated protein kinase (MAPK) cascade and phosphatidylinositol 3' kinase (PI3'K) and macroscopical and histological abnormalities. Enhanced hepatocyte proliferation observed in young IRS-1 transgenic animals was no longer detectable in adult mice. Despite constitutive overexpression and phosphorylation of IRS-1, MAPK and IRS-1 associated PI3'K activity were significantly reduced in older transgenic mice. Furthermore, no premalignant lesions or HCC were detected in IRS-1 transgenic animals up to the age of 24 months. Therefore, additional mechanisms such as enhanced growth factor expression **or impaired negative feedback control mechanisms** may augment IRS-1 overexpression in human hepatocarcinogenesis.

Keywords

hepatocellular carcinoma; growth factors; signal transduction; MAPK; PI3'K; oncogenesis

INTRODUCTION

Growth factor mediated signal transduction is of central importance to hepatocyte proliferation in the context of liver regeneration as well as for hepatocarcinogenesis (1-3). To analyze the effects of the overexpression of specific genes involved in growth factor mediated signaling, *in vivo* transgenic mouse models have been shown to be powerful tools. The expression of transgenes such as TGF α , TGF β , c-myc, HGF/c-met or viral proteins in

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the livers of transgenic mice have demonstrated their potential importance in liver regeneration or in the development of human hepatocellular carcinoma (HCC) (4).

We have shown previously that insulin receptor substrate-1 (IRS-1) becomes highly phosphorylated during liver regeneration and is frequently overexpressed in human HCC (5,6). IRS-1 is a multisite docking protein which belongs to the structurally related IRS family (IRS-1 to IRS-6). IRS-1 and IRS-2 show similar molecular structures and a broad tissue distribution. They are involved in the signal transduction of insulin, insulin like growth factors (IGF-1 and IGF-2), growth hormone, tumor necrosis factor α (TNF α) as well as several interleukines and interferons. Compared to the dominant function of IRS-1 and IRS-2, the role of the remaining IRS molecules (IRS-3 to IRS-6) for insulin and IGF mediated signal transduction is of minor relevance.

Following tyrosyl phosphorylation by activated receptor tyrosine kinases (**mainly insulin- and IGF-1 receptor**), IRS-1 can interact with the SH-2 domains of several signaling molecules such as Grb2, SH-PTP2 (Syp) and the p85 subunit of phosphatidylinositol 3' kinase (PI3'K) thereby transmitting and diversifying growth factor mediated signals (7). IRS-1 mediated signal transduction results in activation of the *Ras*-signaling pathway with subsequent activation of mitogen-activated protein kinases (MAPK), the PI3'K pathway and is required for the insulin mediated mitogenic signal transduction (8).

Overexpression of IRS-1 in NIH 3T3 cells resulted in malignant transformation, which required interaction both with Grb2 and SH-PTP2 (Syp) (9,10). Human breast carcinoma cells lost estrogen requirement for cell growth by overexpression of IRS-1 (11). HCC cells overexpressing IRS-1 were protected against transforming growth factor β (TGF β) induced apoptosis (12) and overexpression of a dominant negative mutated form of IRS-1 resulted in the reversal of the malignant phenotype in human HCC cells (13).

To analyze the effects of IRS-1 overexpression in mouse hepatocytes, transgenic mice were constructed, which express the human IRS-1 under the transcriptional control of the human albumin promoter (6). IRS-1 was found to be highly overexpressed exclusively in the livers of the transgenic mice. The overexpressed IRS-1 became tyrosyl phosphorylated resulting in activation of both MAPK and PI3'K. Furthermore, in 3-month old transgenic animals we observed enhanced hepatocyte proliferation in comparison to nontransgenic littermates. In the present study we followed these mice for a period up to 24 months to characterize the long-term effect of IRS-1 overexpression in hepatocytes *in vivo*.

MATERIALS AND METHODS

IRS-1 transgenic mice

The construction of transgenic mice overexpressing the human IRS-1 gene exclusively in the liver under the control of the albumin enhancer/promoter element and determination of the genotype by PCR has been previously described (6). For analysis of long-term phenotypic changes, animals were observed for up to two years. At the age of 6 weeks, 3, 6, 9, 12 and 24 months, 5 transgenic mice and 5 control littermates were sacrificed. Mouse livers were placed on ice, examined for liver weight and macropathological abnormalities and immediately cut into several pieces. Liver pieces were either snap frozen in liquid nitrogen, lysed in ice cold Triton-lysis buffer as described previously (6) or fixed in Histochoice (Amresco, Solon, OH). Fixed tissue samples were embedded in paraffin. These protocols were approved by the subcommittee on Animal Studies at the Massachusetts General Hospital and conformed to guidelines established by the National Institutes of Health.

Immunoprecipitation and Immunoblot Analysis

Liver tissue lysates were centrifuged (10,000 g for 15 min at 4°C) and the protein concentration was determined (Protein assay, Bio Rad, Hercules, CA). Western blot analysis and immunoprecipitation experiments were performed as described previously (6). The following antisera and purified antibodies were used for immunoprecipitation and immunoblot analysis: Rabbit polyclonal anti-IRS-1 and anti-p85 PI3K antibodies (Upstate Biotechnology Inc., Lake Placid, NY), anti-phosphotyrosine mouse monoclonal antibody PY-20 (Signal Transduction, Lexington, KY), anti-Erk1 and anti-Erk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-active MAPK directed against phosphorylated MAPKs (Promega, Madison, WI).

Hepatocyte proliferation and liver histology

To measure DNA synthesis in transgenic and control mouse liver, 100 mg/kg of 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) were administered i.p.. In addition, mice were injected via the tail vein with 10 µCi of ³[H]-thymidine (DuPont, Boston, MA) and sacrificed 4h later. Genomic DNA was extracted from liver tissue as described. For measurement of ³[H]-thymidine incorporation, genomic DNA was added to scintillation fluid (Cytoscint, Fisher Scientific, Fair Lawn, NJ) and the amount of incorporated radioactivity was determined. Immunostaining for BrdU incorporation was performed on sections of paraffin embedded liver tissue using a specific anti-BrdU monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) and the Vectastain Kit (Vector laboratories, Burlingame, CA) as described before (6). For histological analysis, sections of liver tissue were stained with hematoxylin and eosin (H&E) followed by microscopic examination. Hepatocyte size was determined in microscopic high power fields analyzing only hepatocytes positive for nuclear staining using a morphometric software (analySIS, Softimaging Software GmbH, Germany).

IRS-1 associated PI3' Kinase activity

PI3'K activity was determined in immunoprecipitates generated with polyclonal antibodies to IRS-1 from 500 µg of liver lysate as described previously (14). In brief, the immunoprecipitates were incubated with 10 µg of phosphatidylinositol (Avanti, Alabaster, AL) in HEPES buffer (200 mM HEPES, 4 mM EGTA, and 4 mM sodium phosphate; pH 7.0) for 5 min at room temperature, followed by 10 min at 30°C upon addition of reaction buffer containing 5 µCi ³²ATP, 15 mM MgCl₂, 150 µM ATP, 1.5 mM Tris (pH 7.4) and 15 mM NaCl. The reactions were stopped by the addition of 1,2 M HCl (final concentration) followed by chloroform/methanol extraction. The phosphorylated lipids were analyzed by silica thin layer chromatography using gel plates precoated with 1% potassium oxalate (Merck, White House Station, NJ). The levels of PI3'K activity were determined using a phosphorimager (Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical analysis was performed using the Student T-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Phenotype of IRS-1 transgenic mice and hepatocyte proliferation

To analyze phenotypic abnormalities, groups of 5 IRS-1 transgenic animals and 5 nontransgenic littermates were sacrificed at 6 weeks, 3, 6 12, and 24 months of age. No differences were noticed with respect to body weight, animal survival and to the formation of extrahepatic malignancies (data not shown). As described before, overexpression of

IRS-1 resulted in significantly enhanced relative liver weights also in 6 and 12-month-old animals (6,15). Histological examination did not demonstrate differences of liver architecture between IRS-1 transgenic animals and their control littermates. Hepatocytes of both genotypes showed no difference in cell size. Furthermore, no premalignant lesions or HCC were noticed even in mice 24 months of age (data not shown). To examine hepatocyte proliferation, genomic DNA was extracted from liver tissue and analyzed for ^3H -thymidine incorporation. At 3 months of age, we observed a significant increase of ^3H -thymidine incorporation in the liver DNA of IRS-1 transgenic animals in comparison to control littermates (1289 ± 370 versus 604 ± 89 counts / mg DNA, respectively; $p = 0.005$), whereas no difference could be observed between transgenic mice and control animals at 12 months of age (648 ± 284 versus 709 ± 279 counts / mg DNA) (Fig. 1 A). To be able to exclude proliferating cells in the liver other than hepatocytes (e.g. leukocytes) immunohistochemical analysis for nuclear BrdU incorporation was performed on liver sections after mice had received an intraperitoneal administration of BrdU to determine the number of S-phase hepatocytes. In 20 visual fields (representing 6000 hepatocyte nuclei) stained hepatocyte nuclei were counted. BrdU incorporation was significantly enhanced in hepatocytes of IRS-1 transgenic mice as compared to nontransgenic littermates at 6 weeks (34 ± 10 versus 20 ± 2) and 3 months of age (10 ± 4 versus 2 ± 1 , $p < 0.01$), whereas in older animals no difference between transgenic and control animals could be observed (Fig. 1 B).

IRS-1 mediated signal transduction in murine livers

The overexpression and phosphorylation of the human IRS-1 transgene driven by the human albumin promoter in liver tissue could be demonstrated in transgenic mice at all ages as compared to controls (Fig 2 A). Analysis of MAPK activation was performed by detection of MAPK phosphorylation in liver lysates using antibodies specific for phosphorylated MAPK. In liver lysates obtained from 6 weeks old IRS-1 transgenic animals enhanced phosphorylation of the MAPK ERK-1 and ERK-2 could be detected in comparison to nontransgenic control littermates (Fig. 2 B), but the extent of MAPK phosphorylation was found to be highly variable between single transgenic and nontransgenic mice. In adult mice (Fig. 2 B, right panel) no significant difference of MAPK phosphorylation could be detected. Expression levels of PI3'K in liver tissue showed no differences between transgenic and control mice and between 6 week old animals and 9-month old animals as determined by Western blot using antibodies specific against the p85 subunit of PI3'K (Fig. 2 C, upper left panel). Determination of p85 subunit of PI3'K associated with IRS-1 by Western blot following immunoprecipitation of IRS-1 showed enhanced binding of p85 to IRS-1 in liver lysates of transgenic animals as compared to control littermates (Fig. 2 C, upper right panel). The amount of PI3'K activity associated with IRS-1 was determined by an enzymatic assay. In 6-week old animals IRS-1 associated PI3'K activity was significantly enhanced in comparison to control littermates ($p = 0.004$), whereas in 9-month old animals the difference between IRS-1 transgenic mice and control littermates was found to be reduced and did not reach statistical significance (Fig. 2 C, lower panel).

DISCUSSION

IRS-1 is a multisite docking protein involved in several signal transduction pathways, which are associated with cell growth and cell proliferation and which are known to induce malignant transformation. IRS-1 has been found to be frequently overexpressed in human HCC (6,16) and IRS-1 overexpression resulted in malignant transformation of NIH 3T3 cells *in vitro* (9). There is also evidence that IRS-1 may be involved in tumor cell motility, adhesion and invasion (17,18). Therefore, we have evaluated the effect of IRS-1 overexpression for the development of HCC *in vivo* in a transgenic mouse model. In this

model IRS-1 is highly overexpressed exclusively in hepatocytes, which resulted in enhanced downstream signal transduction, increased relative liver size and enhanced hepatocyte proliferation (6). In the present study we performed a long-term observation of these animals to investigate whether IRS-1 overexpression in hepatocytes results in constitutively activated downstream signal transduction, continuous hepatocyte proliferation and the occurrence of premalignant lesions or of HCC in the livers of transgenic mice. IRS-1 was overexpressed and tyrosyl phosphorylated during the whole observation period. However, we observed a normalization of the rate of hepatocyte proliferation in transgenic mice above 3 months of age in comparison to wildtype control littermates. Furthermore, we observed no occurrence of premalignant lesions such as hyperplastic foci or the development of HCC in IRS-1 transgenic animals. To be able to explain the observed reduction of IRS-1 induced hepatocyte proliferation in older transgenic mice, we analyzed the activation of the downstream signaling pathways of IRS-1 in transgenic and wildtype control mice at different ages.

IRS-1 is linked to the *Ras* signaling pathway via its interactions with Grb2 and SH-PTP2 (Syp) which results in activation of the MAPK signaling cascade. Constitutive activation of the MAPK signaling cascade is sufficient to induce transformation of mammalian cells (19,20). *Ras* mutations frequently occur in mouse models of HCC but are rarely detected in human HCC (21). However, the downstream effectors of *Ras* such as the MAPK signaling cascade have been found to be similarly activated in mouse liver tumors with wildtype *Ras* and tumors harboring *Ras* mutations (22), in experimental rat hepatomas lacking *Ras* mutations (23) and in human HCC (24). These findings indicate the importance of enhanced activation of the MAPK signaling cascade for the malignant transformation of hepatocytes. In human HCC, as *Ras* mutations are rare, activation of the MAPK signaling cascade may therefore be predominantly induced by enhanced growth factor mediated signals. Constitutively enhanced growth factor signaling through pathways which involve tyrosyl phosphorylation of IRS-1 may therefore substitute for *Ras* mutations in HCC. A second major signal transduction pathway mediated by IRS-1 is the activation of PI3'K. The active catalytic subunit and also a gene derived from the regulatory subunit of PI3'K have been able to induce cellular transformation (25) and PI3'K has been identified as an oncogene in human cancers (26). Furthermore, PI3'K mediates tumor cell motility and invasion and downstream activation of AKT by PI3'K blocks apoptosis (27,28). Therefore, activation of PI3'K may add to the proliferative response following overexpression of IRS-1 and may also promote tumor development by the blockage of apoptosis.

In our transgenic mouse model, enhanced expression of IRS-1 resulted in activation of the MAPK and of the PI3'K cascades and in enhanced hepatocyte proliferation in young animals. However, in animals above 3 months of age no significant differences in MAPK phosphorylation could be observed between IRS-1 transgenic mice and wildtype control littermates, despite persistent IRS-1 overexpression and phosphorylation. Similarly we observed enhanced binding of the p85 subunit of PI3'K to IRS-1 and significantly increased IRS-1 associated PI3'K activity only in young IRS-1 transgenic mice. In older IRS-1 transgenic mice we observed reduced binding of p85 to overexpressed IRS-1 and also no significant differences of IRS-1 associated PI3'K activity between transgenic and control animals. Therefore, activation of the MAPK and PI3'K signaling cascade by overexpressed IRS-1 in hepatocytes *in vivo* seems to be restricted to young IRS-1 transgenic animals resulting in only a transient enhancement of hepatocyte proliferation.

A potential explanation for this phenomenon could be a reduction of available growth factors such as IGF-1, IGF-2 or growth hormone in adult mice as compared to young animals, although tyrosyl phosphorylation of the overexpressed IRS-1 could be detected also in adult mice. **However**, the presence of elevated levels of growth factors might induce

additional proliferative signals which may not involve IRS-1 and which might complement signaling pathways mediated by IRS-1. One example would be the direct phosphorylation of Shc by the IGF-1 receptor tyrosine kinase and to a lesser extent by the insulin receptor tyrosine kinase (29), which results in activation of the Ras/MAPK pathway independent of IRS-1.

Whether the combination of increased IRS-1 levels with enhanced IGF-2 expression and/or IGF-1 receptor upregulation is important for the malignant transformation of hepatocytes *in vivo* needs to be examined in the future. IGF-2 overexpression has been observed in human HCC and in human hepatoma cell lines. IGF-2 results in IRS-1 phosphorylation via its binding to the IGF-1 receptor promoting proliferative and antiapoptotic stimuli (30,31). To answer these questions using transgenic mouse models, IRS-1/IGF-2 transgenic mice could be created by crossbreeding our IRS-1 transgenic mice with mice overexpressing IGF-2 in the liver (32). Furthermore, the combination of enhanced IRS-1 levels with the overexpression of oncogenes such as v-Ha-Ras or SV-40 T antigen results in cooperative cellular transformation in cell culture systems (33,34) and therefore overexpression of IRS-1 in hepatocytes *in vivo* may need additional steps of transformation to result in a malignant phenotype.

Finally, the effects of regulatory mechanisms need further investigation, which may be able to reduce the signaling function of IRS-1 in the sense of a negative feedback control mechanism (35). It could be shown that TNF- α decreases the tyrosine kinase activity of the insulin receptor (36). Furthermore, enhanced serine phosphorylation of IRS-1 leads to impairment in the ability of IRS-1 to activate downstream signaling pathways (37) and c-Jun amino-terminal kinases (JNK) interfere with insulin mediated signal transduction (38). Furthermore it could be recently demonstrated that IRS-1 translocates to the nucleus in various cell lines. Nuclear IRS-1 influences cell cycle genes and interacts with other pathways such as the estrogen receptor (39,40), which may lead to further modification of the effects of IRS-1 overexpression in hepatocytes.

In addition, liver growth may be controlled via enhanced apoptosis. In this regard, significant upregulation of Fas expression was observed in the livers of IRS-1 transgenic mice which resulted in enhanced sensitivity of hepatocytes to intravenously injected activating anti-Fas (Jo-2) (15). Therefore, enhanced apoptosis via the Fas/FasL pathway might represent an important mechanism to control liver growth and malignant transformation in the hepatocytes of IRS-1 transgenic animals.

In conclusion, our findings demonstrate that the overexpression of IRS-1 in hepatocytes *in vivo* results in transiently enhanced hepatocyte proliferation in young mice, induction of HCC did not occur. Therefore further investigations are necessary to characterize additional factors acting synergistically with IRS-1 in the malignant transformation of hepatocytes *in vivo*.

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Abbreviations

IRS-1	insulin receptor substrate-1
IGF	insulin like growth factor

HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
MAPK	mitogen-activated protein kinase
PI3'K	phosphatidylinositol-3' kinase
PAGE	polyacrylamide gel electrophoresis
TGF	transforming growth factor
TNF	tumor necrosis factor

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Fig. 1. Hepatocyte proliferation

(A) Liver DNA synthesis was measured by the incorporation of ^3H -thymidine into total liver DNA following tail vein injection of $10\ \mu\text{Ci}$ ^3H -thymidine. At 3 months of age, tritium incorporation was found to be significantly ($p = 0.005$) enhanced in IRS-1 transgenic mice, whereas no difference was found at the age of 12 months. Data are expressed as mean $\pm$ SD. (B) Immunohistochemical analysis of BrdU incorporation into hepatocyte nuclei showed a significantly higher number of S-phase hepatocyte nuclei in IRS-1 transgenic mice at the age of 6 weeks and 3 months ($p < 0.01$), whereas no difference was found in older mice.

Fig. 2. A: IRS-1 expression and tyrosyl phosphorylation in transgenic mice and control littermates

(A) Marked overexpression of the tyrosyl phosphorylated transgene in the livers of IRS-1 transgenic mice could be demonstrated by Western blot in mice of all age groups analyzed as compared to the low levels of IRS-1 expression in control littermates. **B: MAPK activation.** Enhanced MAPK phosphorylation could be detected by Western blot using monoclonal antibodies specific for the phosphorylated MAP-kinases ERK1 and ERK2 in 6 weeks old animals (left panel), but significant difference in MAPK phosphorylation could be detected in adult animals at the age of 9 months (right panel). **C: IRS-1 associated PI3'K activity.** Equal expression levels of PI3'K in liver tissue in 6 weeks and 9-month old animals was demonstrated by Western blot using antibodies specific against the p85 subunit of PI3'K (upper left panel), but the amount of p85 subunit of PI3'K associated with IRS-1 as determined by Western blot following immunoprecipitation of IRS-1 was enhanced in transgenic mice, especially in 6 week old animals (upper right panel). Enzymatic PI3'K activity associated with IRS-1 as measured by an enzymatic assay in liver lysates of 6 weeks old animals and 9-month old animals. The IRS-1 associated PI3'K activity was significantly enhanced in comparison to control littermates in 6 weeks old mice ($p = 0.004$), whereas in 9 months old animals the difference was reduced and did not reach statistical significance (lower panel).