Abstract

AIM: To explore the possibility that liver regeneration could trigger PDX-1 expression, and hence insulin production. Twenty-four hours after surgical liver removal, regeneration was active as demonstrated by the increased proliferating cell nuclear antigen; however, all the other checked genes (involved in insulin gene expression): PC-1, Ngn3, NeuroD1, Btc, PDX-1 and Ins-1, were not related to the molecular events caused by this process. The only marker detected in regenerating liver was E47: a transcription factor of the basic helix-loop-helix family known to be expressed ubiquitously in mammalian cells. In the rat pancreas, almost all of the tested genes were expressed as shown by RT-PCR, except for Ngn3, which was silenced 2 d after birth. Therefore, the molecular events in liver regeneration are not sufficient to promote PDX-1 expression. DNA methylation is a known mechanism to achieve stable repression of gene expression in mammals: Hxk 2 gene is silenced through this mechanism in normal hepatocytes. The administration of 5-aza-dC to cultured cells is in fact able to upregulate Hxk 2 mRNA. We investigated whether PDX-1 silencing in liver cells could be exerted through methylation of Cpg islands in both the promoter and the gene coding regions. The results show that the drug increased the expression level of the Hxk 2 control gene but failed to rescue the expression of PDX-1, thus DNA demethylation is not sufficient to override repression of the PDX-1 gene.

CONCLUSION: During liver regeneration, PDX-1 gene is not reactivated. Demethylation does not de-repress PDX-1 gene expression. Therefore gene silencing is not achieved through this epigenetic mechanism.

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Key words: Transcription factor PDX-1; Hepatectomy; Liver regeneration; Quantitative real time polymerase chain reaction; DNA methylation

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Pillich RT, Scarsella G, Risuleo G. Regeneration and DNA demethylation do not trigger PDX-1 expression in rat hepatocytes. World J Biol Chem 2010; 10(9): 281-285 Available from: URL: http://www.wjgnet.com/1949-8454/full/v1/9/281.htm DOI: http://dx.doi.org/10.4331/wjbc.v1.i9.281

INTRODUCTION

Diabetes mellitus is a metabolic disease that is characterized by persistent hyperglycemia resulting from defects in insulin secretion and/or action[1,2]. When the amount of glucose in the blood increases, the release of insulin from the pancreas is triggered. This hormone removes glucose from the blood and stimulates the liver to metabolize glucose, thus controlling the level of sugar in the organism. In diabetic patients, the blood sugar levels remain high. This might derive from lack of insulin production, from insufficient levels of the hormone or from its diminished effectiveness. This peptide hormone is primarily involved in the glucose metabolism, and is produced by the pancreatic β cells that, also due to autoimmune responses, are destroyed in type 1 diabetes[3]. Insulin is a very potent regulator because it can exert its specific action at a blood concentration as low as 10⁻⁸ mol/L[4]

A number of different transcription factors are involved in the control of insulin expression and/or regulation[5]. The better studied is possibly PDX-1[6], which acts synergistically with E47, a member of the basic helix-loop-helix (bHLH) family of transcription factors: synergism requires DNA binding and activation domains of both PDX-1 and bHLH proteins. It has also been shown that synergistic transactivation results from the co-expression of E47, PDX-1 and NeuroD1[7]. The PDX-1 protein contains 283 amino acids with a predicted molecular weight of 31 kDa. Like most other transcription factors, PDX-1 is characterized by a modular architecture with separate functional domains. However, in spite of the vast body of literature on the function of PDX-1, its actual role in the activation/regulation of the insulin gene is controversial. In any case, it is clear that post-translational modification in fact shifts PDX-1 activation/regulation of the insulin gene expression. The SUMO modification also affects negatively the metabolic function also in animal models. The liver is considered a good candidate for the expression of insulin in patients with type 1 diabetes. However, this transcription factor is not expressed in the liver, and therefore, transformation of hepatocytes with exogenous PDX-1 gene copies is necessary. The rationale of this work was to assess whether endogenous PDX-1 expression could be spontaneously reactivated during liver regeneration in partially hepatectomized rats, and to verify whether PDX-1 silencing in normal hepatocytes might be achieved through DNA methylation.

MATERIALS AND METHODS

Clone-9 rat hepatocytes (American Type Culture Collection, ATCC No. CRL-1439) were maintained in DMEM-F12 (Gibco) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and gentamicin (100 μg/mL) when applicable. Cells were split every 3-4 d at a 1:10 ratio for no more than 10-15 times. Rat insulinoma RIN 1046-38 cells were maintained in M-199 supplemented with 10% FBS, penicillin and streptomycin. Glucose was added to a final concentration of 11.1 mmol/L. Cells were split every 4-5 d at a ratio of 1:5. In regeneration studies, the lateral and medial liver lobes of adult male Wistar rats (200-250 g) were surgically removed, which caused a 70% loss of liver mass[8]. In methylation experiments, 5-aza-deoxycytidine (5-aza-dC) was used at a final concentration of 5 μmol/L and treatment was performed as described by Goel and collaborators[9]. Total RNA purification was achieved using the Nucleospin RNA extraction kit (Macherey-Nagel). cDNA synthesis was performed using random primers, 1 μg total RNA as template and 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using a Geneamp 2400 (Applied Biosystems) and ExTaq DNA polymerase (Takara). PCR primers were designed using the Primer3 online software. Quantitative real time PCR (qRT-PCR) was performed using a Biorad iCycler and the SYBR Green technology; primers for qRT-PCR were designed using the Beacon Designer 6 software. All PCR primers have already been validated and used in a previous study[10]. SDS-PAGE and western blotting were performed according to standard procedures. Antibodies were purchased from Sigma (β-actin, cat. No. A-4700) and Santa Cruz Biotechnology (proliferating cell nuclear antigen; PCNA, cat No. SC-25280).

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RESULTS

PDX-1 and the regenerating liver

Figure 1 panel A shows that 24 h after surgical resection of the liver, regeneration was fully operational. This was demonstrated by the increased levels of PCNA shown in panels B and C. All the other genes involved in insulin gene expression that were checked (PC-1, Ngn3, NeuroD1, Btc, PDX-1 and Ins-1) did not show any relationships with the molecular events caused by this process. In fact, the only marker that could be detected in the regenerating liver was E47; a transcription factor that belongs to the bHLH family that is known to be expressed ubiquitously in mammalian cells. As a control, in the pancreas, almost all of the tested genes were expressed and could be identified by a band after RT-PCR; the only exception was Ngn3 that was silenced 2 d after birth in rats.

DNA methylation

As reported by Goel and collaborators[11], DNA methylation is known to be a mechanism that is commonly used to achieve stable repression of gene expression in mammalian cells: the Hxk 2 gene is silenced through this mechanism in the normal hepatocyte cell line Clone-9 (K-9). The administration of 5 μmol/L 5-aza-dC to K-9 cells in culture is in fact able to upregulate Hxk 2 mRNA transcription by about threefold compared to untreated cells. This drug can be incorporated into nascent DNA but cannot be methylated by cellular methylases; therefore, the gene expression pattern is altered and the expression of silenced genes is reactivated. As a result, we decided to check whether PDX-1 silencing in liver cells could be exerted through methylation of CpG islands in both the promoter and the gene coding regions. We cultured wild-type K-9 cells in the presence or absence of 5 μmol/L 5-aza-dC for 4 d and prepared cDNA from total RNA to be used for QRT-PCR; the Hxk 2 gene was used as a positive control and GAPDH as a reference gene. Figure 2 shows that, as expected, the drug treatment increased the expression level of the Hxk 2 control gene, but failed to rescue the expression of PDX-1, which indicated that the DNA demethylation status induced by the drug was not sufficient to override repression of the PDX-1 gene.

DISCUSSION

Liver regeneration is a complex process that involves reactivation of cell proliferation and expression of fetal markers such as α-fetoproteins that are normally silent in adult tissue[12-15]. The protein PDX-1 is an important transcription factor that is expressed during gut endoderm differentiation and organ formation. This was the rationale to explore the possibility that liver regeneration could also trigger PDX-1 expression, and hopefully, insulin production.

Regeneration was fully operational after partial surgical
ablation of the liver; this was demonstrated by the increased levels of PCNA. Other genes involved in insulin expression did not show any correlation with the molecular events that derived from the enhancement of tissue proliferation. In fact, the only marker that could be detected in the regenerating liver was E47; a transcription factor that belongs to the bHLH family, which is known to be expressed ubiquitously in mammalian cells. In the pancreas of the same animals used as controls, on the contrary, almost all tested genes were expressed and could be identified by RT-PCR. The only gene that was not expressed is Ngn3 but it is known that this gene is silenced 2 d after birth in rats. Therefore, in the light of the results reported in this brief communication we can confidently state that the molecular events that occur during liver regeneration are not per se sufficient to promote PDX-1 expression; a key transcription factor in insulin regulation.

DNA methylation is a commonly accepted mechanism for the stable repression of gene expression in mammalian cells. The Hxk 2 gene is silenced through this mechanism in the normal hepatocytes. To explore the possibility that the expression of PDX-1 might also be blocked through DNA methylation, we grew K-9 cells in the presence of 5-aza-dC; a de-methylating agent that is incorporated into nascent DNA but cannot be methylated by cellular methylases. Treatment with this drug upregulates Hxk 2 mRNA transcription, thus, the gene is actually repressed by methylation, but its function can be rescued after exposure to 5-aza-dC. We checked whether PDX-1 silencing in liver cells could be exerted through methylation of CpG islands in both the promoter and the gene coding regions. We cultured wild-type K-9 cells in the presence or absence of 5 μmol/L 5-aza-dC; the Hxk 2 gene was used as positive control and GAPDH as a reference gene. Treatment with
5-asa-dC increased expression of the Hxk 2 control gene but failed to rescue the expression/function of PDX-1. The overall meaning of this result is that DNA demethylation induced by the drug is not sufficient to override repression of the PDX-1 gene.

The data presented in this short paper clearly indicate that the molecular events that occur during liver regeneration are not sufficient to reactivate spontaneously PDX-1 gene expression. In addition, the use of a demethylating agent in cultured rat hepatocytes is not able to de-repress PDX-1 gene expression, which indicates that silencing of the gene is unlikely to be achieved through this epigenetic mechanism. In conclusion, the results presented here clearly suggest that, because neither molecular mechanism is able to re-activate PDX-1 gene function, the repression of this gene must be ascribed to a different and possibly more complex phenomenon. The peptide hormone insulin is primarily involved in glucose metabolism, and is produced by pancreatic β cells that, also due to autoimmune responses, are destroyed in type 1 diabetes. However, the reactivation at liver level of the enzyme PDX-1 for therapeutic purposes does not seem to be an immediately attainable target.

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S- Editor Cheng JX  L- Editor Kerr C  E- Editor Zheng XM