Research Article

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M6P/IGF2R modulates the invasiveness of liver cells via its capacity to bind mannose 6-phosphate residues

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Background & Aims: The mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R), a multifunctional protein, plays a central role in intracellular targeting of lysosomal enzymes and control of insulin-like growth factor II (IGF-II) bioactivity. Importantly, the gene encoding this receptor is frequently inactivated in a wide range of malignant tumors including hepatocellular carcinomas. Thus, M6P/IGF2R is considered a putative liver tumor suppressor. The aim of this study was to establish the impact of the receptor on the invasive properties of liver cells.

Methods: Reconstitution experiments were performed by expression of wild type and mutant M6P/IGF2R in receptor-deficient FRL14 fetal rat liver cells. RNA interference was used to induce M6P/IGF2R downregulation in receptor-positive MIM-1–4 mouse hepatocytes.

Results: We show that the M6P/IGF2R status exerts a strong impact on the invasiveness of tumorigenic rodent liver cells. M6P/IGF2R-deficient fetal rat liver cells hypersecrete lysosomal cathepsins and penetrate extracellular matrix barriers in a cathepsin-dependent manner. Forced expression of M6P/IGF2R restores intracellular transport of cathepsins to lysosomes and concomitantly reduces the tumorigenicity and invasive potential of these cells. Conversely, M6P/IGF2R knock-down in receptor-positive mouse hepatocytes causes increased cathepsin secretion as well as enhanced cell motility and invasiveness. We also demonstrate that functional M6P-binding sites are important for the anti-invasive properties of M6P/IGF2R, whereas the capacity to bind IGF-II is dispensable for the anti-invasive activity of the receptor in liver cells.

Conclusions: M6P/IGF2R restricts liver cell invasion by preventing the pericellular action of M6P-modified proteins.

Keywords: Cathepsin; Hepatocellular carcinoma; Lysosome; Matrix degradation; Cell invasion.

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Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; IGF-II, insulin-like growth factor II; M6P, mannose 6-phosphate; M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor II receptor; MPR46, 46-kDa mannose 6-phosphate receptor; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, short interfering RNA.

Introduction

Liver cancer represents worldwide one of the most frequent malignancies [1]. Hepatocellular carcinomas (HCCs) are the most common primary liver tumors. Although many improvements have been made in terms of diagnosis and treatment, HCCs are associated with poor clinical prognosis [2]. Aggressive HCCs have the capacity to penetrate extracellular matrix (ECM) barriers and spread into the surrounding parenchyma, leading to intrahepatic metastasis and portal venous invasion [3].

Different proteinases are involved in the breakdown of ECM components during tumor invasion and metastasis, including plasminogen activators, matrix metalloproteinases, and cathepsins [4–6]. Hepatocytes are known to produce substantial amounts of the lysosomal proteinases cathepsin B, cathepsin D, and cathepsin L [7]. As typical for lysosomal enzymes, the N-glycan moieties of cathepsins are modified during their biosynthesis with mannose 6-phosphate (M6P) residues which permit interaction with the main lysosomal sorting receptors, the 300-kDa mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) and the 46-kDa mannose 6-phosphate receptor (MPR46) [8].

M6P/IGF2R is a multifunctional receptor involved in (a) transport of newly synthesized M6P-tagged lysosomal proteins from the Golgi network to lysosomal compartments, (b) endocytosis of extracellular M6P-tagged lysosomal enzymes, (c) proteolytic activation of transforming growth factor β, and (d) regulation of the bioavailability of IGF-II [8–10]. To perform all these important functions, the receptor depends on multiple ligand-binding sites.

For some M6P/IGF2R ligands, the receptor domains accommodating the respective binding sites have been identified. Furthermore, point mutations have been described which specifically interfere with the interaction of the receptor with IGF-II or M6P-tagged proteins [11–14].

Several lines of evidence support the hypothesis that loss of M6P/IGF2R function is associated with liver tumor progression. The gene encoding M6P/IGF2R has been shown to undergo frequent loss of heterozygosity in human HCCs and adenomas, with concomitant inactivating mutations in the remaining allele [15,16]. It has been demonstrated that the receptor gene is lost early in liver tumorigenesis, which suggests that loss of M6P/IGF2R may represent an initiation event [17]. Given this clinical significance, it comes as a surprise that the impact of the M6P/IGF2R status on the properties of hepatocytes and HCC cells has not yet been firmly established. However, studies in choriocarcinoma and breast cancer cells have demonstrated that a decrease in M6P/IGF2R expression enhances tumor cell growth [18,19], whereas overexpression of the receptor causes the opposite effect [20,21].

Experiments in transgenic mice have indicated that the antitumorigenic properties of M6P/IGF2R could be linked to its capacity to downregulate the biological activities of IGF-II [22]. However, other studies indicate that failure to express M6P/IGF2R may result in an increase of the proteolytic load in the pericellular environment and thus enhance the invasive capacity of tumor cells [23–25]. Thus, it seems that multiple M6P/IGF2R ligands play a role in tumor formation and metastasis, possibly in a tissue-specific manner. In this study, we have now assessed the impact of M6P/IGF2R on the growth, motility and invasiveness of liver cells.

Materials and methods

A detailed account of the methodology used in this study can be found in the Supplementary Materials and methods.

Results

FRL14 cells display features typical for early-stage fetal hepatocytes

There is now compelling evidence to suggest that in some liver pathologies HCCs derive from liver stem/progenitor cells or immature hepatoblasts. Hence, HCC cells often more closely resemble fetal than adult hepatocytes in terms of their gene expression pattern [26]. To permit detailed studies on a cellular level, substantial efforts have been undertaken to establish transformed hepatocyte-like cell lines from fetal rat liver of different gestational stages. One such cell line, FRL19, has been previously derived from a prenatal rat liver at day 18.5 of gestation and shown to express several hepatocyte markers, including the late-onset enzymes tyrosine aminotransferase and alpha-glutathione S-transferase [27,28]. We have now generated a cell line derived from day 13.5 fetal rat hepatocytes. The morphology of these FRL14 cells reflects their origin from the hepatocyte lineage. Furthermore, FRL14 cells express early-stage hepatocyte markers such as transferrin, while late-onset genes are not expressed (Supplementary Fig. 1).

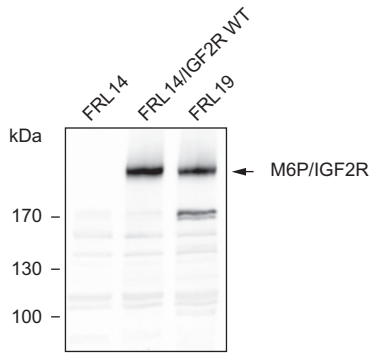

Fig. 1. M6P/IGF2R status of FRL14 cells. Membrane protein extracts (40 μg) of FRL14, FRL14/IGF2R wt, and FRL19 cells were subjected to SDS–PAGE and then immunoblotted with anti-M6P/IGF2R antibodies. The migration positions of selected molecular mass standards are indicated.

In vitro invasion of M6P/IGF2R-deficient FRL14 cells is strongly inhibited by cysteine cathepsin inhibitors

FRL14 cells are capable of migrating across ECM barriers as typical for malignant cancer cells. Since such invasive properties frequently depend on ECM proteolysis, the contribution of matrix-degrading proteinases to the invasive potential of FRL14 cells was assessed by invasion assays performed in the presence of different synthetic and endogenous proteinase inhibitors (Supplementary Fig. 2). The strongest effect was caused by the general lysosomal cysteine proteinase inhibitor E-64 (60% reduction). Inhibition of FRL14 invasion by the more selective E-64 derivative CA-074 (40%) and the matrix-metalloproteinase inhibitor GM6001 (46%) was less pronounced. When the efficacy of physiological proteinase inhibitors was tested, it was found that the potent serine proteinase inhibitor aprotinin had a much weaker effect on cell invasion (22% reduction) than the cysteine proteinase inhibitor cystatin C (49% reduction). We have earlier observed a similar proteinase inhibition profile for the invasive properties of M6P/IGF2R-deficient murine squamous cell carcinoma cells [25]. When FRL14 extracts were immunoblotted with antibodies recognizing rat M6P/IGF2R, it became evident that these cells lack expression of the receptor (Fig. 1).

Ectopic expression of M6P/IGF2R restores the intracellular retention of lysosomal enzymes and decreases the invasive potential of FRL14 cells in vitro

Receptor-deficient FRL14 cells were stably transfected with human wild type *M6P/IGF2R* cDNA to assess the impact of the M6P/IGF2R status on their cellular properties. Two clones were selected for further studies, FRL14/IGF2R wt-1 and FRL14/IGF2R wt-2. By comparison with receptor-positive HeLa human cervical carcinoma cells, the M6P/IGF2R content of FRL14/IGF2R wt-1 and FRL14/IGF2R wt-2 cells was estimated to be 2.5 and 2.1 pmol/mg total cell protein, respectively (Supplementary Table 1). Hence, the receptor level of the selected clones was within the physiological range [29]. The subcellular localization of M6P/IGF2R was assessed by immunofluorescence microscopy. As expected, the ectopically expressed receptor was found to reside in the Golgi apparatus (Supplementary Fig. 3).