

Abstract

Introduction/Background: The hepatocyte low-density lipoprotein receptor-related protein (LRP1) efficiently clears a wide variety of proteins from blood to liver. LRP1 is overexpressed on some hepatocellular carcinoma (HCC) cell lines and underexpressed on cirrhotic tissue. An LRP1 ligand, the receptor-associated protein (RAP), normally found within the cell, is endocytosed rapidly by LRP1 following intravenous injection, with primary accumulation in hepatocytes. A peptide version of human RAP has been developed (mRAP) that is easily manufactured, conveniently conjugated to other agents and retains high-affinity for LRP1.

Aims: First, we sought to test whether conjugation of mRAP to a cytotoxic agent enhanced the toxicity of the agent through a process requiring endocytosis by LRP1. Second, we tested whether conjugation of mRAP to a labeled protein increased accumulation of the labeled protein in the liver following intravenous injection. Finally, we determined LRP1 expression levels in a variety of liver samples.

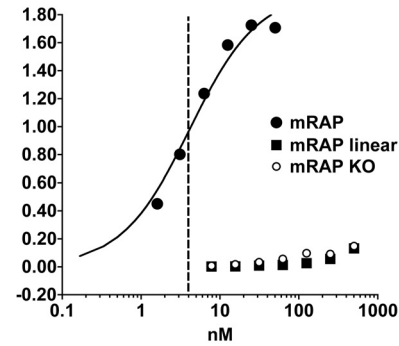
Methods: Cultured cell lines with differing levels of LRP1 expression were used to test the cytotoxicity of mRAP conjugates *in vitro*. Intravenous injection and whole-body autoradiography were used to test clearance of mRAP conjugates into the liver in rats and woodchucks. QPCR was used to test LRP1 mRNA expression levels in a variety of human tissue samples.

Results: Cytotoxic mRAP conjugates enhanced cell death by 4-fold over the unconjugated cytotoxic agent alone. This enhancement required LRP1. Labeled mRAP conjugates accumulated in the liver at levels between 4.5 (WHV-infected woodchuck) and 7 (normal rat) fold greater than unconjugated control. Among a variety of tissues, LRP1 expression levels were highest in liver. Among a variety of liver samples, expression levels were similar in normal, stage II HCC and diseased, but tumor-free, samples. LRP1 was strongly underexpressed in early-stage HCC and slightly underexpressed in late-stage HCC.

Conclusion: mRAP may provide a means of improving the efficacy of cytostatic and antiviral agents by increasing their level of accumulation in hepatocytes.

Receptor binding (Cont'd)

High affinity binding of a minimized receptor-associated protein (mRAP) peptide to LRP1 *in vitro*



Cell uptake

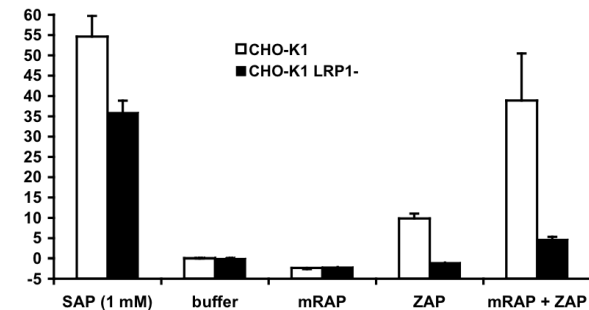
Binding of mRAP peptide to LRP1 on cells in culture

Wild-type and LRP1-deficient CHO-K1 were grown in BioWhittaker UltraCHO medium supplemented with 5% fetal bovine serum. Cells were seeded in 12-well tissue culture plates 48 hours prior to the experiment. The biotinylated mRAP peptide was combined with equimolar amounts of a conjugate between streptavidin and the bacterial toxin saporin ("ZAP", Advanced Targeting Systems, San Diego). The mixture was diluted into growth media to 100 nM and added to wells in duplicate. As controls, duplicate wells were incubated with mRAP peptide alone, the streptavidin-saporin conjugate alone, both at 100 nM concentrations, or with saporin alone at 1 μM (SAP). Cells were incubated for 48 hours at 37°C, 5% CO₂ in a humidified tissue culture chamber. Cell viability after this interval was determined with an MTT assay (Invitrogen, San Diego).

The mRAP peptide alone had no significant effect on cell survival, regardless of the cell-type used. The streptavidin-saporin conjugate alone reduced viable cell number by approximately 10% for wild-type CHO-K1, with no effect on LRP-deficient CHO-K1 cells. The combination of mRAP and the cytotoxic conjugate reduced viable cell number by nearly 40% for wild-type CHO-K1, with only a 5% loss for the LRP-deficient CHO-K1. Saporin alone (1 μM) resulted in losses of about 55% (wild-type) and 35% (LRP-deficient).

Conclusion: The mRAP peptide can significantly enhance the effect of a bioactive agent in an LRP1-dependent manner.

Enhancement of efficacy through LRP1-dependent endocytosis of a RAP peptide conjugate



Biodistribution

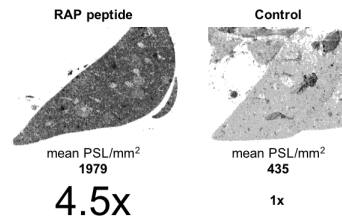
Accumulation of mRAP peptide in liver

Rat studies were performed at Charles River Laboratories in Montreal, Canada. Woodchuck studies were performed at the NRC Institute of Biodiagnostics in Winnipeg, Canada. Biotinylated mRAP peptide or buffer were combined with ³⁵S-SLR-streptavidin (0.7 mCi/mL, 300 Ci/mmol, GE Healthcare) and dialyzed against phosphate-buffered saline (PBS) with D-tube™ dialysis cassettes (14 kD MWCO, EMD Biosciences). Chronically WHV-infected Eastern woodchucks or male Sprague-Dawley rats (6-8 weeks) were injected with test materials through peripheral veins. Animals were sacrificed thirty minutes post-injection with pentobarbital (200 mg/kg). All subjects were treated in accordance with the guidelines set by the Canadian Council on Animal Care for the humane treatment of laboratory animals. Carcasses were frozen, embedded in carboxymethylcellulose and sectioned for analysis by semi-quantitative whole-body autoradioluminography (QWBA) using a Fuji BAS-2500 phosphorimager. Clearly delineated areas within assayed organs for each animal were selected for luminescence analysis (Fuji Image Reader v1.1 and Fuji Image Gauge v3.12). Values are reports in units of photostimulated luminescence per unit area (PSL/mm²).

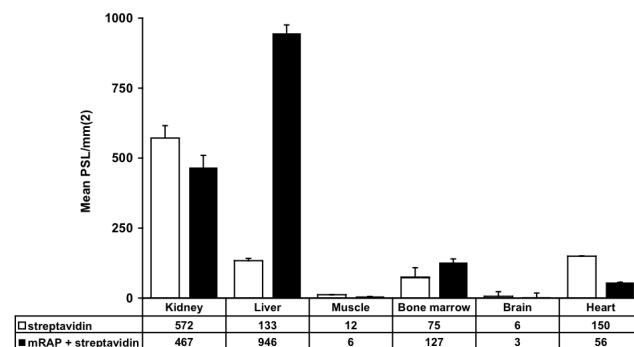
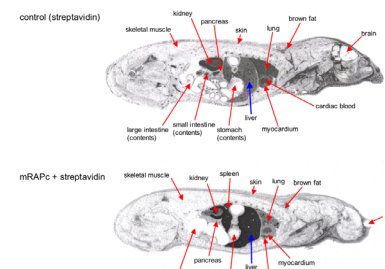
The mRAP peptide, conjugated to labeled streptavidin, distributed to the liver at levels over 4-fold greater than streptavidin alone and 7-fold greater than streptavidin alone, with similar or lower levels compared to control in all other tissues tested. It is notable that high levels of competing LRP1 ligands in the blood were apparently unable to block liver uptake of the peptide complex, an observation made previously, as well as here, for intravenously-administered full-length RAP (1).

Conclusion: The mRAP peptide is primarily cleared to the liver after intravenous injection.

Enhancement of delivery to liver through conjugation to a RAP peptide: Woodchuck



Enhancement of delivery to liver through conjugation to a RAP peptide: Rat



LRP1 expression

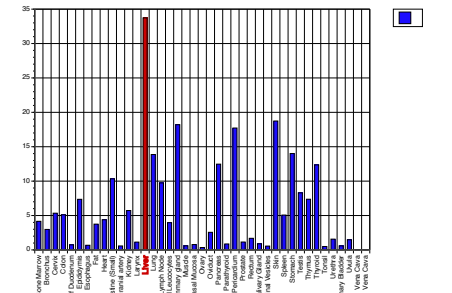
Expression of LRP1 mRNA in human tissues

An Origene™ liver cancer disease panel cDNA array was assayed with LRP1 primers by Taqman™ QPCR. Well-to-well variation in cDNA quantities were normalized to β-actin. Linearity and specificity of amplification was validated on bonafide sequence standards prior to assay.

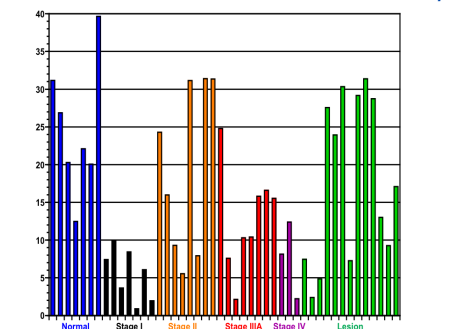
The highest LRP1 mRNA expression levels were measured in normal liver tissue. Similar levels were observed in stage II HCC and diseased, but tumor-free, liver tissue. LRP1 mRNA was underexpressed in stage I, IIIA and IV HCC.

Conclusion: LRP1 mRNA expression levels support targeting of this receptor for delivery of antiviral agents and some antitumor agents to liver, provided that tumor sensitivity to drug is sufficiently differentiable from that of normal tissue.

Expression of LRP1 mRNA in normal human tissues



Expression of LRP1 mRNA in normal and diseased liver samples



Introduction

An intracellular human chaperone protein, the receptor-associated protein (RAP), has multiple, high-affinity binding sites within LRP1 on hepatocytes. LRP1 directs endocytosis and transport of exogenous RAP to the lysosome, where the protein is rapidly degraded (1). Capture efficiency of RAP by the liver is enhanced by an initial, low-affinity binding step to abundant cell-surface heparin sulfate proteoglycan on hepatocytes (2,3). Nearly quantitative delivery of RAP-conjugated chemotherapeutics or antivirals to liver after intravenous administration would significantly reduce the systemic toxicities and increase efficacy, reducing risk to the patient.

In order to develop a hepatotropic vehicle to enhance drug delivery to liver, we have focused on a 67-amino acid peptide comprising the highest affinity receptor-binding region within RAP (4,5,6) This peptide, mRAP, is stabilized by a non-native internal disulfide bond linking the termini. We have sought to determine the suitability of this peptide as a liver targeting agent.

Receptor binding

Binding of mRAP peptide to LRP1 *in vitro*

Solid phase binding assays. Recombinant human LRP1 cluster II (R&D Systems, amino acids 786-1165, with C-terminal Fc tag, 1 μg) was used to coat Nunc Maxisorp™ 96-well plates in TBS pH 8 supplemented with 5 mM CaCl₂ (TBSC) overnight at 4°C. Peptides were incubated with the immobilized receptor at a range of concentrations for 2 hours in blocking buffer supplemented with 0.05% Tween-20 at room temperature. Wells were washed with TBSTC and bound peptides detected with streptavidin-HRP (Pierce). Color was developed using TMB reagents (BioRad, Hercules, CA). Absorption at 450 nm was measured with a microplate spectrophotometer (Molecular Devices, Palo Alto).

The cyclized mRAP peptide bound with high-affinity in a calcium-dependent fashion with a K_d of 4 nM. The mRAP linear peptide had no measurable affinity for LRP1 cluster II at the concentrations tested. As expected, the mRAP KO peptide, containing two mutations previously shown to block LRP1 binding, did not bind saturably to the receptor at the concentrations tested.

Conclusion: The 67 amino acid cyclic mRAP peptide binds tightly to a receptor found at high density on hepatocytes, LRP1.

References

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