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Knockdown of LRP/LR induces apoptosis in pancreatic cancer and neuroblastoma cells through activation of caspases

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ABSTRACT

The 37 kDa/67 kDa laminin receptor (LRP/LR) serves various physiological and pathological roles such as enhancing tumour-related processes including metastasis, angiogenesis, cellular viability and telomerase activation in cancerous cell lines. The present study investigates the effect of siRNA mediated downregulation of LRP/LR on pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells. MTT and BrdU assays revealed that siRNA mediated downregulation of LRP resulted in a significant reduction in cell viability and cell proliferation. In addition, knock-down of LRP resulted in phosphatidylserine externalization, diminished nuclear integrity and significantly enhanced caspase-3 activity, which is indicative of apoptosis. LRP downregulation resulted in a significant increase in caspase-8 activity in IMR-32 cells and enhanced caspase-8 and 9 activity in AsPC-1 cells. These data recommend siRNA mediated knock-down of LRP as a potential therapeutic avenue for the treatment of pancreatic cancer and neuroblastoma.

1. Introduction

Laminins are glycoproteins that play a major role in the formation of the extracellular matrix [1]. Both integrin and non-integrin laminin receptors have been identified as mediators used by laminins to carry out their functions, which include cell migration [2], adhesion [3], growth [4], cell differentiation, proliferation [5] and cell signaling [6]. One major receptor and binding partner for laminin is the 37 kDa/67 kDa laminin receptor precursor/high-affinity laminin receptor (LRP/LR)[7].

LRP/LR is a major extracellular matrix/non-integrin laminin receptor with high specificity and affinity for laminin-1 [8]. LRP/LR is predominantly found as a transmembrane receptor but also localizes in the cytosol [9] and the nucleus [10] – facilitating translational processes and the maintenance of nuclear structures, respectively. It plays a role in physiological processes such as cellular adhesion, migration, proliferation, maintenance of cellular viability [11], cell cycle [12], protein synthesis [12] and processing of ribosomal RNA [13].

Numerous studies have implicated LRP/LR as a key contributor to the pathogenesis of certain viral and bacterial infections [14,15], prion-protein related diseases such as Transmissible Spongiform Encephalopathies [16], neurodegenerative diseases such as Alzheimer's disease [17,18], and several cancer types [19–22]. Blockage of the interaction between Laminin-1 and LRP/LR by use of the anti-LRP/LR specific antibody IgG1-iS18 has been shown to diminish levels of adhesion and invasion of various cell lines in vitro [19–25]. Therefore, it is evident that LRP/LR is crucial for metastatic processes and is a promising target for metastatic cancer therapeutics. In addition to metastasis, LRP/LR is also suggested to enhance tumour angiogenesis [26] and recently Naidoo et al. [27] and Otgaar et al. [28]elucidated a novel role for LRP/LR in mediating telomerase activity through the enhancement of hTERT activity. In particular, knock-down of LRP/LR resulted in impediment of telomerase activity in metastatic breast cancer cells, combating the pro-tumourigenic activities of LRP/LR [27]. Very recently, we showed that LRP::FLAG enhances telomerase activity concomitant with reduced senescence markers, indicating that LRP plays a profound role in the ageing process [28]. Due to the involvement of LRP/LR in these aforementioned numerous tumourigenic processes, the role of this receptor in cell survival and viability has become an attractive topic of research [29]. Recent studies showed reductions

Abbreviations: AsPC-1, Pancreatic cancer cells; BCA, bicinchoninic acid; BrdU, Bromodeoxyuridine; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked Immunosorbent Assay; EMEM, Eagle Minimum Essential Medium; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; HRP, Horse radish peroxidase; hTERT, Human telomerase reverse transcriptase; IgG, Immunoglobulin class G; IMR-32, neuroblastoma cells; IgG1-iS18, Full Length Immunoglobulin Antibody; LR, Laminin Receptor; LRP, Laminin Receptor Precursor; PAGE, Polyacrylamide gel electrophoresis; PCA, Protocatechuic acid; siRNA, small interfering RNA; SDS, Sodium Dodecyl Sulphate

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in the viability of liver (Hep3b) [30], lung (A549) [31], cervical (HeLa) [31], breast (MCF-7 and MDA-MB231) [32] and oesophageal cancer (WHCO1) [32] cells after siRNA-mediated downregulation of LRP/LR – thus suggesting that LRP/LR plays a pivotal role in the maintenance of tumour cell viability. Furthermore it has been shown that LRP/LR plays an important role in apoptosis [30–32].

Apoptosis is a process of programmed cell death necessary for the maintenance of tissue homeostasis and embryonic development [33]. Three main types of biochemical changes have been observed in apoptosis, namely caspase activation, DNA/protein breakdown, and membrane changes that result in phagocytosis [34]. There are two main pathways by which caspases can be activated and thereby induce apoptosis, namely the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [34]. Recent studies made use of siRNA technology to downregulate LRP/LR and assess the effects of this down-regulation on apoptosis [31,32]. siRNA-mediated LRP/LR down-regulation was shown to significantly reduce cellular viability of lung (A549) and cervical cancer (HeLa) cells and increase the levels of caspase-3 activity in both cell lines, thus suggesting that LRP/LR is pivotal in the maintenance of cellular viability as well as the evasion of apoptosis in tumour cells.

Therefore, the present study aimed to assess the role of LRP/LR on the viability and survival of highly aggressive pancreatic cancer (AsPC-1) and neuroblastoma (IMR-32) cells by using an siRNA approach. Additionally, both the intrinsic and extrinsic apoptotic pathways were evaluated following siRNA mediated downregulation of LRP/LR. This study showed that LRP/LR knockdown significantly reduced the viability of pancreatic cancer and neuroblastoma cells, and suggests that the observed reduction in cellular viability is due to apoptosis.

2. Materials and methods

2.1. Cell culture

AsPC-1 and IMR-32 cells were obtained from the Fox Chase Cancer Centre (USA). AsPC-1 cells were cultured in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES and sodium pyruvate. IMR-32 cells were cultured in Eagle Minimum Essential Medium (EMEM) supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine and 1% non-essential amino acids. Cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2 and were sub-cultured when 80% confluency was reached.

2.2. siRNA-mediated down-regulation of LRP/LR

Cells were seeded into 6 well plates and were transfected at approximately 50–70% confluency. Transfections were performed using siRNA-LAMR1 to downregulate LRP/LR and siRNA-scrambled was employed as a negative control (Dharmacon). The Dharmafect1 Transfection reagent was used according to the manufacturer's instructions. Additionally, AsPC-1 and IMR-32 cells were transfected with Lipofectamine 3000 reagent (Thermo Scientific) and either esiRNA-RPSA (targeted to LRP/LR) or esiRNA-RLUC (negative control) (Sigma-Aldrich), in order to confirm that downregulation was not due to off-target effects. Thereafter, the plates were incubated at 37 °C for a period of 72 h prior to downstream experiments being performed.

2.3. Western blotting

Endogenous LRP/LR levels as well as siRNA-treated LRP/LR levels were determined by employing Western blotting. In order to prepare cell lysates, cells were incubated in 500 µl of lysis buffer (10 mM Tris/HCl pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.05% (w/v) deoxycholic acid (DOC), 0.5% (v/v) Nonidet-P40) for 15 min at 4 °C. This was followed by centrifugation at 14,000 rpm for 2 min, after which the supernatant containing the extracted proteins was retained. Cell lysates were separated on 12% SDS-PAGE gels (Bio-Rad). Proteins were transferred to PVDF membranes for 45 min at 300 V and 350 A. Thereafter, the membranes were blocked for 1 h using blocking buffer (3% BSA in PBS-Tween) followed by an 1 h incubation of the membranes in the primary antibody (anti-LRP/LR specific antibody IgG1-iS18) diluted 1:5000 in blocking buffer. Three washes in washing buffer (10 min each) were performed before incubation of the membranes in secondary antibody (anti-human IgG-HRP conjugated antibody) diluted 1:10,000 in blocking buffer for 1 h. Three washes were again performed as previously mentioned before chemiluminescent substrate was added for detection of proteins. X-ray films were used to capture the chemiluminescent reaction. β-actin (mouse monoclonal anti-β-actin peroxidase; Sigma) served as a loading control. Densitometry was performed to quantify protein levels with ImageJ™ software.

2.4. MTT assay

Both AsPC-1 and IMR-32 cells were seeded at a density of 1×10^4 cells per well in a 24-well plate and transfected 24 h later. Seventy two hours post transfection, 100 µg of MTT was added to each well, and incubated for 2 h at 37 °C. Post incubation, the MTT-containing medium was discarded from each well and the remaining formazan crystals were dissolved in 500 µl of DMSO. The absorbance was measured at 570 nm using an ELISA plate reader. Controls included untreated cells, negative control siRNA treated cells, and positive control protocatechuic acid (PCA) treated cells.

2.5. BrdU assay

BrdU assays were performed using a BrdU Cell Proliferation Kit (Calbiochem) according to the manufacturer's instructions. In brief, cells were seeded at a density of 1×10^4 cells in a 24-well plate and transfected. Post transfection (72 h), 80 µl of a working stock of BrdU was added to each well followed by a 24 h incubation period at 37 °C. Following aspiration of contents, 800 µl of fixative solution was added to each well and incubated for 30 min at room temperature. After removal of the fixative, cells were subjected to an hour incubation in 100 µl of anti-BrdU antibody. Cells were washed thrice with wash buffer and incubated for 30 min, at room temperature, with 400 µl of the peroxidase anti-mouse IgG HRP-conjugated antibody. After cells were washed three times, 400 µl of substrate solution was added followed by a subsequent incubation period of 15 min. Absorbance was measured within 30 min of addition of stop solution (400 µl) at dual wavelengths of 450 and 540 nm. Untreated cells were used as a control and siRNA-scr and PCA were used as negative and positive controls, respectively.

2.6. Confocal microscopy

AsPC-1 and IMR-32 cells were seeded onto coverslips at a density of 1×10^4 cells prior to siRNA transfection. Thereafter, 72 h post transfection, cells were fixed in 4% paraformaldehyde for 15 min followed by three washes with PBS. Post washing, cells were incubated with Hoechst 33342 nuclear stain diluted in PBS (1:100) for 5–10 min in the dark. The coverslips were then washed 2–3 times in PBS and thereafter, coverslips were mounted (cell side down) onto a clean microscope slide using mounting fluid. After setting for 45 min in the dark, slides were stored at 4 °C until ready to view with a confocal microscope (Zeiss LSM 780). Controls included untreated cells, cells treated with siRNA-scr (negative control) and PCA (positive control).

2.7. Annexin V-FITC/7AAD assays

The Annexin V-FITC/7-AAD assay (Beckman Coulter) was performed according to the manufacturer's instructions. Briefly, post siRNA