Experimental Cell Research 341 (2016) 218–224

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Therapeutic approach to target mesothelioma cancer cells using the Wnt antagonist, secreted frizzled-related protein 4: Metabolic state of cancer cells

Vanathi Perumal [a,1], Sebastian Pohl [b,1], Kevin N. Keane [c,1], Frank Arfuso [b], Philip Newsholme [c], Simon Fox [a], Arun Dharmarajan [b,*]

[a] Molecular Pharmacology Laboratory, School of Pharmacy, Curtin Health Innovation Research Institute, Curtin University, Bentley, WA, Australia
[b] Stem Cell and Cancer Biology Laboratory, School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley, WA, Australia
[c] School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley, WA, Australia

ARTICLE INFO

Article history:
Received 18 December 2015
Received in revised form
5 February 2016
Accepted 7 February 2016
Available online 8 February 2016

Keywords:
Mesothelioma
Wnt
Secreted frizzled-related protein 4
Metabolism

ABSTRACT

Malignant mesothelioma (MM) is an aggressive cancer, characterized by rapid progression, along with late metastasis and poor patient prognosis. It is resistant to many forms of standard anti-cancer treatment. In this study, we determined the effect of secreted frizzled-related protein 4 (sFRP4), a Wnt pathway inhibitor, on cancer cell proliferation and metabolism using the JU77 mesothelioma cell line.

Treatment with sFRP4 (250 pg/ml) resulted in a significant reduction of cell proliferation. The addition of the Wnt activator Wnt3a (250 pg/ml) or sFRP4 had no significant effect on ATP production and glucose utilisation in JU77 cells at both the 24 and 48 h time points examined. We also examined their effect on Akt and Glycogen synthase kinase-3 beta (GSK3β) phosphorylation, which are both important components of Wnt signalling and glucose metabolism. We found that protein phosphorylation of Akt and GSK3β varied over the 24 h and 48 h time points, with constitutive phosphorylation of Akt at serine 473 (pAkt) decreasing to its most significant level when treated with Wnt3a+sFRP4 at the 24 h time point. A significant reduction in the level of Cytochrome c oxidase was observed at the 48 h time point, when sFRP4 and Wnt3a were added in combination.

We conclude that sFRP4 may function, in part, to reduce/alter cancer cell metabolism, which may lead to sensitisation of cancer cells to chemotherapeutics, or even cell death.

1. Introduction

Malignant mesothelioma (MM) is a particularly aggressive cancer that is characterised by rapid progression, late metastases, and poor prognosis [1]. Although this tumour is relatively uncommon, the incidence continues to rise in Australia as a consequence of high past asbestos use and the long latency period between exposure to asbestos and subsequent tumour development. MM is highly resistant to conventional forms of anti-cancer therapy, and both radiotherapy and chemotherapy have a limited effect [1]. There is an urgent need for more effective therapies for this cancer based upon identification of molecular targets through improved biological understanding of the disease. The Wnt signalling pathways play a key role in development and homoeostasis. There is convincing evidence in a number of different cancers that chronic activation of Wnt signalling is important in tumorigenesis, and this has fuelled interest in targeting this pathway [2–5]. Aberrations of Wnt signalling have been described in mesothelioma [6], and there is a need to identify the extent to which this pathway drives cell growth and survival in MM. The investigation of biological and immunological therapies for MM in preclinical models has been a major interest. In human and mouse MM cells, we have investigated regulators of the Wnt signalling pathway [7]. In particular, we have shown differential expression of secreted frizzled-related protein 4 (sFRP4) [6], which is a Wnt antagonist, and of longstanding interest to our group for its role in apoptotic signalling in morphogenesis, developmental biology, and neoplasia [8–11].

Cancer cells "rewire" metabolism to maintain continued proliferation. Changes in cellular metabolism occur down-stream of oncogene/tumour suppressors [12]. It has been demonstrated that tumour cells consume large amounts of glucose to yield ATP [13].

* Corresponding author.
E-mail address: a.dharmarajan@curtin.edu.au (A. Dharmarajan).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.yexcr.2016.02.008
0014-4827/

It is well established that deregulated pathways in cancer also impact on metabolism. The difference in metabolic properties of cancer cells and normal cells suggests that targeting metabolic dependence could be a selective approach to treat cancer patients.

In 1956, Warburg observed that the rate of glycolysis was abnormally high in cancer cells. This 'Warburg effect' indicates that cancer cells prefer glycolytic breakdown of glucose for energy, rather than mitochondrial oxidative phosphorylation [14]. Although the molecular mechanisms that define the Warburg effect are not yet fully understood, the increased glycolysis observed in cancer cells is well accepted to be important for the support of malignant phenotypes [15,16].

In this study, we are the first to report the effects of the Wnt-pathway inhibitor, sFRP4, on mesothelioma cancer cell metabolism via assessment of ATP production, glucose utilisation, and bioenergetics, together with downstream signalling molecules such as total and phosphorylated glycogen synthase kinase-3 beta (GSK3β), Protein kinase B (also known as Akt), and cytochrome C oxidase (COX IV).

2. Materials and methods

2.1. Cell culture and treatment

The malignant mesothelioma cell line JU77, originally derived from pleural effusions of different patients presenting with malignant pleural mesothelioma, was used in this study [17]. The cells were cultured in RPMI – 1640 (Hyclone) with 10% FBS (Bovogen), penicillin (100IU/ml), and streptomycin (100 μg/ml) (Life Technologies). JU77 cells were seeded in 6-well plates (Corning) at a density of 30,000/cm^2, and treated with recombinant Wnt3a (250 pg/ml) and sFRP4 (250 pg/ml) (R&D Systems) for 24 and 48 h.

2.2. Morphology

Phase contrast images were obtained following the different treatments using an Olympus bright field microscope.

2.3. MTT assay

Cell proliferation in response to various treatments was quantitated using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay. Cells were seeded into 96-well plates at a density of 10,000–20,000 cells/well, depending on the experiment. Following 24 h incubation, treatments were added and cells incubated for a further 24–48 h (depending on experiments). The MTT assay was performed as previously described [18], and absorbance was read at 595 nm with a microplate reader (Enspire, Perkin Elmer). For each treatment, the mean absorbance for the assay replicates (3 wells) was calculated. The data are expressed as mean ± standard deviation for at least 3 independent experiments, and plotted using Prism for Windows v 6.07 (Graphpad Software, CA, USA).

2.4. Viability assay

Trypan blue is a stain used to selectively stain dead cells blue. To minimise operator bias, trypsinised cells were stained with trypan blue, and live and dead cells enumerated using Invitrogen Countess™ Automated Cell Counter chambers [19].

2.5. Measurement of cellular ATP and cellular glycolytic activity

Relative cellular ATP content was measured by the luciferase-based Cell Titer-Glo Luminescent Cell Viability kit (Promega), with modifications from the manufacturer's protocol. Briefly, cells were plated in 96-well plates at 5000 cells per well to allow for attachment overnight. At the desired harvest time, an equal volume of the single-one-step reagent provided by the kit was added to each well and rocked for 15 min at room temperature. Cellular ATP content was measured and quantified using an ATP standard curve and a luminescent plate reader (Enspire, Perkin Elmer).

Glycolytic activity was determined by measuring glucose consumption. Cells were seeded in 96-well cell culture microplates at 5000 cells per well per 100 μl according to their growth rate, and allowed to attach overnight. Fresh medium was replaced the next morning along with treatment conditions and incubated for 24 or 48 h prior to the assay. Cell culture medium with no cells present was used to calculate the initial level of glucose in the medium (11.1 mM). The glucose consumed from the cell culture medium was measured using the Amplex Red Glucose kit (Invitrogen, Molecular Probes). The samples were diluted 150 fold and the assay was performed according to the manufacturer's instructions. The fluorescence emission was measured at 590 nm following excitation at 550 nm using a Perkin Elmer microplate reader. The values were calculated according to the standard curve, and then subtracted from the base line to obtain values of glucose consumption from the growth culture medium by the cells [20]. Data were then presented as a percentage of the initial glucose concentration.

2.6. Seahorse XF^{e}96 measurements and analysis

The Seahorse Bioscience XF^{e}96 Flux analyser and the Mito Stress Test kit (Seahorse Biosciences) were used according to manufacturer's instructions. In brief, cells were seeded into 96 well plates at a density of 5,000 cells/well and allowed to adhere overnight (cell density was previously optimised for oxygen consumption rates (OCR) and proton production rates (PPR)). Prior to Seahorse analysis, the culture medium was changed to serum-free DMEM (pH 7.4) containing 1 mM sodium pyruvate, 2.5 mM Glucose, and without sodium bicarbonate, and plates were then incubated for 60 min at 37 °C in a non-CO_2 incubator. Following instrument calibration, the Mito Stress Test was performed using the following injection strategy: DMEM (2.5 mM glucose) with or without Wnt3a, sFRP4, or Wnt3a and sFRP4 in combination, oligomycin (2 μM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, 0.4 μM), and finally, rotenone/antimycin A in combination (1 μM). OCR and PPR were determined using three 3.5 min assay cycles of mix, and measurement undertaken following each injection. Normalisation of data was performed by determining the cell density using the neutral red uptake assay after the stress test, and measurements were compared to ensure there were no significant changes in relative cell numbers among treatment groups. The concentrations of all inhibitors used were optimised to ensure the lowest concentration was used to produce the maximum effect.

Basal respiration was calculated by subtracting the minimum OCR following addition of rotenone/antimycin A (non-mitochondrial respiration) from the last OCR measurement recorded prior to the addition of oligomycin. Proton leak was calculated by subtracting the minimum OCR following the addition of rotenone/antimycin A (non-mitochondrial respiration) from the minimum OCR measurement recorded after addition of oligomycin. OCR related to ATP production (turnover) was calculated by subtracting the proton leak from the basal respiration. Coupling efficiency percentage was calculated by dividing the ATP turnover-dependent OCR by the basal respiration and multiplying by 100. Max respiration was determined by subtracting the non-mitochondrial respiration from the maximum OCR following the addition of FCCP. The spare respiratory capacity was calculated by subtracting the basal OCR from the maximum OCR after FCCP addition. The