Blood Cells, Molecules, and Diseases 45 (2010) 201–209

Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

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

Notch protection against apoptosis in T-ALL cells mediated by GIMAP5 ☆

Nicholas Chadwick [a,*], Leo Zeef [b], Virginia Portillo [a], Joanna Boros [b], Sarah Hoyle [a], Jaap C.L. van Doesburg [a], Anne-Marie Buckle [a]

[a] Faculty of Life Sciences, Manchester Interdisciplinary Biocenter, University of Manchester, Manchester M1 7DN, UK
[b] Michael Smith Building, University of Manchester, Manchester M1 7DN, UK

ARTICLE INFO

Article history:
Submitted 13 April 2010
Revised 14 June 2010

(Communicated by A. Townsend, M.D., Ph.D., F.R.C.P., F.R.S., 5 July 2010)

Keywords:
Notch
T-ALL
Apoptosis
GIMAP5

ABSTRACT

Recent studies have highlighted the role of Notch signalling in the development of T cell acute lymphoblasic leukaemia (T-ALL). Over-expression of Notch3 and gain of function mutations in the *Notch1* gene have been reported. The aims of this study were to determine the effect of Notch signalling on apoptosis in human T-ALL cell lines and to identify targets of Notch signalling that may mediate this effect. Functional studies showed that inhibition of Notch signalling using gamma secretase inhibitors promoted glucocorticoid-induced apoptosis in cells carrying gain of function mutations in *Notch1*. Moreover, ectopic expression of constitutively activated Notch provided protection against glucocorticoid-induced apoptosis, indicating that signalling via Notch may also contribute to the development of T-ALL by conferring resistance to apoptosis. Microarray analysis revealed that GIMAP5, a gene coding for an anti-apoptotic intracellular protein, is upregulated by Notch in T-ALL cell lines. Knockdown of GIMAP5 expression using siRNA promoted glucocorticoid-induced apoptosis in T-ALL cells carrying gain of function mutations in *Notch1* and in T-ALL cells engineered to express ectopic constitutively activated Notch indicating that Notch signalling protects T-ALL cells from apoptosis by upregulating the expression of GIMAP5.

Introduction

Dysregulation of the Notch signalling pathway has been identified in patients with T cell acute lymphoblastic leukaemia (T-ALL). The human homologue of *Drosophila* Notch was first identified as a chromosomal translocation in a rare subset of patients with T-ALL [1]. Mice transgenic for the Notch1 intracellular domain (ICD) [2] develop aggressive T cell lymphomas, revealing the potential of aberrant Notch signalling to induce T cell neoplasia. More recently, a study by Bellavia *et al.* showed over-expression of Notch3 and the pre-T cell receptor alpha to be a characteristic of all T-ALL samples [3]. Weng *et al.* have identified mutations in the heterodimerisation domain of Notch1 which result in ligand-independent activation, and in the PEST domain, which result in prolonged activity of the cleaved form of Notch1 [4]. These gain-of-function mutations in Notch1 were found to be present in half of the T-ALL patients studied, and in many of the cell lines derived from T-ALL patients.

The functional consequences of aberrant Notch1 signalling in the context of T-ALL include the induction of proliferation [4,5] and the inhibition of apoptosis [6,7]. Notch has been shown to promote proliferation by the upregulation of c-myc [8,9] and cyclin D1 [10], while mechanisms of Notch-protection against apoptosis include downregulation of the glucocorticoid receptor [11], and activation of the PI-3 K/AKT pathway [6,7,12]. The latter mechanism has been shown to lead to mTOR activation and p53 inhibition in breast cancer cell lines, while inhibition of this pathway promoted apoptosis in human and mouse T-ALL cell lines [6].

To date the studies of Notch signalling have focussed on Notch1 and the comparative effects of Notch1 and Notch3 have not yet been studied. Both Notch1 and Notch3 are expressed during normal thymocyte development where Notch signalling is thought to act synergistically with signalling through the pre-TCR alpha chain to mediate positive selection [13]. Also, Deftos *et al.* have demonstrated Notch-associated resistance to glucocorticoid-mediated apoptosis in a mouse CD4/CD8 double-positive (DP) thymocyte cell line [14] and in primary DP thymocytes [14].

In this study, we investigated the role Notch in T-ALL cell apoptosis and found that inhibiting endogenous Notch signalling using gamma secretase inhibitors (GSIs) promoted apoptosis. We also found that ectopic expression of either Notch1 or Notch3 protected T-ALL cells from apoptosis. We showed that expression of GIMAP5 (a GTPase associated with intracellular membranes) is upregulated by Notch signalling and that knockdown of this gene using siRNA attenuates Notch-associated protection against apoptosis. These data provide evidence of a novel mechanism mediating Notch protection against apoptosis in T-ALL.

☆ Dedication: This manuscript is dedicated to the memory of Anne-Marie Buckle.

* Corresponding author. Manchester Interdisciplinary Biocenter, University of Manchester, Manchester M1 7DN, UK. Fax: +44 161 236 0409.
E-mail address: n.chadwick@manchester.ac.uk (N. Chadwick).

1079-9796/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcmd.2010.07.006

Materials and Methods

Plasmid constructs, cell lines and retroviral transductions

"ΔE" constructs of Notch were generated which lack extracellular ligand binding domains and are activated constitutively by gamma secretase at the plasma membrane. N1ΔE (base pairs 5143-7671) and N3ΔE (base pairs 4942-7045) cDNAs were cloned into the bicistronic retroviral vector, pMX-eGFP (a kind gift from T. Kitamura, Tokyo, Japan). Similarly, Notch1 ICD, which is constitutively active and gamma-secretase-independent, was cloned into pMX as described previously [15]. Dominant-negative Mastermind-Like-1 (DN-MAML1) cDNA, coding for aa13-74 in pMSCV was a kind gift from J.Aster, Harvard, USA). Wild-type GIMAP5 in pcDNA3 and retroviral pJIM-GIMAP5-GFP fusion constructs were kind gifts from S. Dorskeland, (Bergen, Norway). Retrovirus was produced using the Phoenix amphotropic packaging cell line. Empty pMX or pMSCV vector was used to make negative control virus. T-ALL cell lines used were Jurkat, CEM, MOLT4, HPB-ALL and SIL-ALL, all cultured in RPMI medium containing 10% Fetal Bovine Serum. Retroviral supernatants were used to transduce cells in retronectin-coated tissue culture plates (BioWhittaker, Wokingham, UK). After 48 hrs, GFP + cells were sorted by flow cytometry and cultured in normal growth medium.

Transient transfections

2×10^6 CEM cells were transfected with 10ug plasmid DNA (together with 1ug peGFP transfection marker) using Amaxa Nucleofector kit V (Cologne, Germany) with program G010. Cells were transferred to 1.5 ml growth medium and left for 48 hrs to allow transgene expression prior to treatment with dexamethasone. For siRNA knockdown experiments, control ("Allstars", Qiagen code 1027280) and GIMAP5 siRNA (Qiagen code SI00427364; Qiagen, Crawley, UK) sequences were used as follows: 2×10^6 cells were transfected with siRNA using the Amaxa Nucleofector system (Amaxa, Cologne, Germany) with Solution V and program G-010 according to the manufacturer's instructions. Cells were incubated for 24 hrs in 1 ml of growth medium in a 24 well plate prior to seeding in 48 well plates for apoptosis assays described above.

Apoptosis assays

Cells were seeded at 2×10^5 per ml in 48 well tissue culture plates at 0.5 ml per well and treated with dexamethasone (Sigma, Poole, UK), or ethanol (vehicle control). For inhibition of Notch signalling, cells were pre-treated with gamma secretase inhibitor IX (GSI IX; Calbiochem, Nottingham, UK), or DMSO (vehicle control) for 24 hrs prior to the addition of dexamethasone and GSI IX (or DMSO). Jurkat cells were treated with 100 ng/ml Anti-Fas antibody (R&D Systems, Abingdon, UK) or IgG control for 24 hrs prior to analysis. Apoptosis was analysed by staining with Annexin V-APC conjugate (Caltag, Buckingham, UK) and propidium iodide, followed by flow cytometric analysis. Where GFP + stably transduced cell lines were used or where cells were transiently cotransfected with a GFP marker, analysis was performed by gating on the GFP + population.

RT-PCR for GIMAP gene expression

Total RNA was isolated from cell samples using RNA B (ABgene, Epsom, UK). RNA was reverse transcribed to cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, Warrington, UK) and dilutions of cDNA used for triplicate real-time PCR reactions with PowerSYBR SybrGreen reagents (Applied Biosystems). Primers used were as follows, GAPDH forward: agcacagggatactttattagatg; GAPDH reverse: ccagcaagagcacaagaggaagag; Deltex1 forward aaagcagagtggacagcagagag; Deltex1 reverse atttctccacccacagtgggactgg; GIMAP5 forward: ctgagggagaacgagagtaa; GIMAP5 reverse cttcagggtccagagattta. Fold change in gene expression was determined using the "2^{-ddCT}" method [16].

Western Blotting

Protein extracts from T-ALL cells were concentrated by acetone precipitation and 200ug of protein used for Western blotting with 1:100 rabbit anti-GIMAP5 (anti-IAN4L1) polyclonal antibody (Proteintech, Chicago, USA). Protein samples were analysed for beta-actin as a loading control.

GSI washout assay

Jurkat cells were incubated with 10 uM GSI IX for 48 hrs then cells were washed twice with growth medium and seeded in growth medium plus 10 uM GSI IX (mock washout) or 0.1% DMSO (GSI washout) in the presence or absence of 20 uM cycloheximide to inhibit protein synthesis. After 4 hrs, RNA was isolated and cDNA used for gene expression analysis.

Results

Notch signalling protects T-ALL cells from dexamethasone-induced apoptosis

Inhibition of the Notch signalling pathway using GSIs has been shown to inhibit proliferation of some T-ALL cell lines [4,5,17]). Given previous studies showing a protective role of Notch in T cell apoptosis [6,7], we investigated whether GSI-mediated inhibition of endogenous Notch signalling could increase the susceptibility of T-ALL cells to dexamethasone-mediated apoptosis. Glucocorticoids are an important therapeutic tool in lymphoid malignancies and resistance of malignant cells to glucocorticoids is a significant problem [18,19]. Four dexamethasone-sensitive cell lines known to carry gain of function mutations in Notch1 were analysed for any effect of GSI-treatment on glucocorticoid-induced apoptosis: HPB-ALL and SIL-ALL cells which have previously been shown to undergo cell cycle arrest in response to GSIs, and CEM and MOLT4 cells which were shown not to respond to GSIs in respect of their cell cycle kinetics [4].

Exposure of all four T-ALL cell lines to GSIs led to a significant increase in their responsiveness to dexamethasone (Fig. 1. A-D). The effect of GSIs was dose-dependent; when GSI-treated cells were counted 2 weeks after dexamethasone treatment, it could be seen that GSI-blockade of Notch signalling clearly reduced the numbers of dexamethasone-resistant cells (Fig. 1E).

In order to confirm that the effect of GSIs was specific to Notch signalling, we generated CEM cell lines expressing either GFP alone or a DN-MAML-GFP fusion protein which has been used previously to inhibit Notch-mediated transcriptional activity in T-ALL cells [5]. When exposed to dexamethasone, DN-MAML-transduced cells were more susceptible to apoptosis (as compared to empty vector-transduced cells) in a similar manner to GSI-treated cells (Fig. 1F). Moreover, when a mixed population of GFP- and GFP + cells were exposed to dexamethasone, the cells surviving this treatment from the DN-MAML culture were predominantly GFP-, whereas both GFP + and GFP- cells survived in the empty vector-transduced population (Fig. 1G), confirming the survival disadvantage conferred by DN-MAML to dexamethasone-treated cells. Since GSIs inhibit Notch activation at the cell surface, whereas DN-MAML inhibits the transcriptional activity of Notch, it could be argued from these results that the protective effect of Notch from glucocorticoid-induced apoptosis is primarily mediated by the transcriptional activity of Notch.

Taken together, these data show that although GSI treatment affects the proliferation of only a minority of T-ALL cell lines [4], it has