The elements and functional significance of Notch-signaling and its potential cross-talk with the TGFb-pathway in the survival of human B-cell non-Hodgkin lymphoma and chronic lymphocytic leukemia cells

Doctoral theses

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1. INTRODUCTION

Non-Hodgkin lymphomas (NHL) are the most common malignant hematologic diseases, 85% of the cases representing the B-cell lineage (B-NHL). The proliferative and apoptotic properties of normal and malignant B-cells are tightly controlled by a number of signaling pathways, which comprise a complex and intricate signaling network. These pathways can be considered as attractive targets for molecular therapy, which – in combination with traditional chemotherapy – may offer several advantages such as less toxicity, lower dosage, more specificity and the circumvention of chemoresistance.

Notch-signaling is an evolutionarily conserved pathway regulating development, differentiation and tumorigenesis. Notch-receptors (Notch 1-4 in mammals) are cell-membrane bound receptors, which, upon binding their ligands (Jagged1,2 and Delta-like 1,3 and 4 [Dll1,3,4]), get cleaved in the membrane by a γ-secretase complex. The Notch intracellular domain is translocated to the nucleus, where it converts the RBP-Jκ transcriptional factor into a transcriptional activator, and regulates the expression of various genes, the best known of which belong to the Hairy/Enhancer of Split (HES) and the HES-related repressor protein (HERP) family.

Activating Notch-mutations have been shown to be present in at least 50% of human T-cell acute lymphoblastic leukemia (T-ALL) cases and have been proved to play a unifying role in the pathogenesis of T-ALL. An important role of Notch has been proposed in cell survival in several B-cell malignancies such as Hodgkin's disease and in two B-NHL entities, chronic lymphocytic leukemia (CLL) and in multiple myeloma. In particular, over-expression of Notch2 in CLL cells was suggested to be involved in the over-expression of CD23 and survival/resistance to apoptosis. However, controversial results have also been published regarding B-cell malignancies, where activated Notch-receptors induced apoptosis and growth arrest in both mature and immature B-cell lines.

Pharmacological inhibitors of the γ-secretase complex (γ-secretase inhibitors; GSI) – which inhibit the cleavage and the activation of all four Notch-receptors – have been suggested to have a therapeutical value in Notch-dependent malignancies, and clinical trials with GSI have been launched in T-ALL and metastatic breast cancer. GSI have also been shown to inhibit proliferation and induce apoptosis in several cell lines of both T- and B-cell origin.

Transforming growth factor-beta (TGFb) is a multifunctional cytokine regulating a wide range of biological processes including cell death, proliferation, epithelial-mesenchymal transition (EMT) and immune function. TGFb induces growth inhibition and apoptosis in

mouse and human lymphocytes and in several lymphoma cell lines; however, malignant lymphoid cells often lose their sensitivity to TGFb, which – in many cases – is not due to the lack of signaling elements in cells. The reversal of impaired sensitivity to apoptotic stimuli may be an important therapeutical tool, however, contributing factors are largely unknown. Notch-signaling has been reported to be necessary for some effects of TGFb, and it is reasonable to hypothesize that its defects may lead to altered TGFb-sensitivity. Cross-talk between the TGFb and Notch pathway has been documented in several contexts, and intact Notch-signaling was required for TGFb-induced EMT and cytostasis. Genes of the HES- and HERP-families have also been shown to be direct transcriptional targets of TGFb in certain cell types.

TGFb-signaling has been thought to be centered around Smad4, a core element of the pathway. Recent results highlight that there is a set of the diverse effects of TGFb which does not require Smad4 and probably employ alternative pathways. Considering the ambivalent role of TGFb in different aspects of tumor growth, this may be important in identifying specific molecular targets for tumor therapy.

The aim of our study was to characterize and compare the expression of Notch-pathway elements in normal B- and CLL cells, and search for possible correlations with clinical and prognostic factors. The role of the Notch-pathway in apoptosis induction and in the regulation of apoptosis induced by other agents – most importantly, TGFb –, along with a potential cross-talk between the Notch and TGFb pathways in regulating HES-1 gene expression, was also determined in B-NHL cell lines. In addition, Smad4-dependence of TGFb-induced apoptosis was investigated in B-NHL cells.

2. AIMS

The significance of Notch-signaling in B-cell non-Hodgkin lymphomas is not yet clear, and literature data are – at least in part – confusing. We wished to characterize in detail:

- the expression of Notch-pathway elements in circulating normal B-cells and CLL cells, and their correlation with clinical and prognostic parameters of CLL;
- the effect of Notch-ligand and Notch-inhibitor on survival in human B-cell non-Hodgkin cell lines *in vitro*;
- TGFb-dependent regulation of HES-1 gene transcription in B-NHL cell lines;
- potential changes in TGFb-sensitivity in the presence of Notch-ligand and Notch-inhibitor in B-NHL cell lines;
- the Smad4-dependence of TGFb-induced apoptosis in B-NHL cell lines.

3. METHODS

3.1. Cell separation and clinical data

B-cells were isolated with magnetic MACS CD19 microbeads following Histopaque gradient centrifugation from the peripheral blood of 24 CLL patients and normal donors. Clinical data of CLL patients was collected (age, gender, Rai stage, hemoglobin, lymphocyte count, thrombocyte count, therapy and progression).

3.2. Cell cultures and treatments

HT58, Ramos, BL41, BJAB, U266, MED-B1 (B-cell non-Hodgkin lymphoma cell lines), Jurkat (an acute T-cell lymphoid leukemia cell line) cells and peripheral blood mononuclear cells isolated from four B-CLL samples were treated TGFb1 and ALK-inhibitor. The DAPT γ-secretase inhibitor was used for the inhibition of Notch-signaling, and immobilized Delta-like 4 (Dll4) was used for ligand-dependent activation of Notch signaling. HT58 cells were also treated with etoposide, doxorubicine, vincristine, rituximab, staurosporine, bortezomib, PD98059 MEK1-inhibitor, okadaic acid and endothall.

3.3. Flow cytometry

CLL samples were stained with anti-CD5-FITC, anti-CD38-PE and anti-CD19-PerCP for flow cytometric characterization. Apoptosis was detected by flow cytometry in cell cultures. Cells with >2n DNA content were considered apoptotic (subG1 population). Cell morphology was visualized on haematoxylin-eosin stained cytospin preparates.

3.4. Functional inhibition of Smad4

Synthetic siRNA or a dominant negative Smad4 construct cloned into the p-EGFP-N1 vector was used for Smad4 silencing.

3.5. RT-PCR and real-time PCR

Semi-quantitative PCR or Taq-Man based real-time PCR was used to characterize the gene expression of Notch-receptors, Notch-ligands and other genes of the Notch-pathway (HES-1, HERP-1, Deltex, NRARP), C-MYC, TIEG and Smad4. Gene expression was normalized to β-actin or GAPDH.

3.6. Sequence analysis of IgV_H genes

For determining somatic hypermutation status, genomic DNA was isolated from 11 CLL samples, IgV_H gene segments were amplified by PCR with the appropriate V_H primers and consensus J_H primer, and products were sequenced by capillary electrophoresis. Sequences were identified by the IMGT/V-QUEST program; IgV_H gene sequences with <98% identity compared to germ line sequences were considered hypermutated.

3.7. Western-blotting

Cell lysates were resolved by polyacrylamide gel electrophoresis, blotted onto PVDF membranes, incubated by anti-Smad4/DPC4, anti-Notch1, anti-ERK1, anti-JNK2, anti-phospho-MAPK/ERK1/2, anti-phospho-p38MAPK and anti-phospho-SAPK/JNK primary antibodies, followed by the appropriate, HRP-conjugated secondary antibodies. Membranes were stripped and reprobed with anti-β-actin antibody.

3.8. Phosphatase activity measurements

Protein phosphatase activity was determined by a Non-Radioactive Serine/Threonine Phosphatase Assay System, according to the manufacturer's intructions.

3.9. Statistics

Mann-Whitney U-test, rank corelation test, analysis of co-variance and paired Student's t-test was used for statistics. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. The expression pattern of Notch-signaling elements in CLL and normal B-cells; their relationship with clinial and prognostic characteristics of CLL

Notch1 and Notch2 receptors were detected in both leukemic and normal B-cells on an mRNA level. Notch1 protein was also detected by Western-blotting in CLL cells. Deltex expression was moderate in normal B-cells and variable in CLL cells. Deltex expression was moderate in normal B-cells and variable in CLL cells. Delta-like 1 was not expressed by normal B-cells, however, low expression was detected in some CLL samples. Notch3, Notch4, Jagged1 and Jagged2 mRNA was not present in normal B-cells or CLL. Normal B-cells expressed HES-1; however, HES-1 levels showed large variations among CLL samples: in 19/24 cases HES-1 was low to undetectable (0.1-17.5% of the average of normal B-cells); 4/24 cases showed similar HES-1 expression to normal B-cells (67.6%-115.3%), and only one CLL sample showed higher HES-1 expression (339.8%). Median HES-1 expression in normal B-cells was significantly higher than in CLL samples (95% and 6%, respectively, $p=0.022$).

No correlation was found between the expression pattern of the examined genes, and the clinical and prognostic (CD38 positivity and, in 11 cases, somatic hypermutation status) parameters of CLL patients. CLL samples with intensive HES-1 expression were associated with <10 G/l lymphocyte counts in four out of five cases.

4.2. The effect of Notch-ligand and Notch-inhibition in B-NHL cell lines

All the examined cell lines showed the expression of some Notch-receptor and -ligand by RT-PCR. The effect of Dll4 and DAPT was first tested on the Jurkat T-ALL cell line; Dll4 increased HES-1 mRNA expression, and DAPT decreased both baseline and Dll4-induced HES-1 expression after 48 hours of treatment. Among the B-NHL cell lines tested, only BJAB cells showed substantial changes in HES-1 expression after 48 hours. The level of HERP-1 mRNA was increased both by Dll4 and DAPT in HT58 cells, whereas other cell lines showed no significant changes.

Dll4 or DAPT did not affect the percentage of apoptotic cells in B-NHL cell lines and in Jurkat cells after 72 hours of treatment.

4.3. The effects of TGFb in B-NHL cell lines

TGFb was able to induce apoptosis in HT58, BL41 and Ramos cells. This effect was TGFbR-dependent, as an ALK-inhibitor – which inhibits signals initiated from the receptor – abolished TGFb-induced apoptosis.

TIEG is a known short term transcriptional target gene of TGFb; however, significant TGFb-induced TIEG mRNA expression was observed only in HT58 and Ramos cells after 1h treatment. TGFb-treatment lead to a remarkable increase in HES-1 expression in HT58, Ramos and BL41 cells. TGFb induced a small and nonsignificant increase in HES-1 mRNA in BJAB cells; no change was observed in the other cell lines.

4.4. The effect of Notch-ligand and -inhibitor on TGFb-induced apoptosis in B-NHL cell lines

TGFb-induced apoptosis was significantly decreased – but not completely abolished – by concomitant DAPT treatment in HT58 cells; the effect of DAPT showed a dose-dependent effect up to 1 μM. Dll4 did not modify the rate of TGFb-induced apoptosis significantly. BL41 cells showed similar responses to treatments as HT58 cells. Neither Dll4 ligand, nor DAPT restored apoptotic sensitivity to TGFb in TGFb-resistant BJAB, U266 and MED-B1 cell lines. The effect of Dll4 and DAPT was variable in the TGFb-sensitive Ramos cell line.

4.5. Effect of DAPT on drug-induced apoptosis in HT58 cells

Apoptosis induced by etoposide, doxorubicine, rituximab, vincristine and staurosporine was not affected significantly by DAPT in HT58 cells.

4.6. TGFb-induced gene expression changes and their DAPT-dependence in HT58 cells

Changes in HES-1, HERP-1, TIEG, NRARP and C-MYC gene expression (which may be of importance in the TGFb or Notch pathway), and their DAPT-dependence were determined in TGFb-treated HT58 cells (treated for 1, 2, 4, 6 or 8 h).

TGFb-induced HES-1 expression peaked at 1 hour after treatment, decreased until 4 hours, and slightly increased again at 6 and 8 hours. Concomitant DAPT treatment significantly decreased HES-1 mRNA expression in this later phase only, but did not abolish it completely. TIEG-induction by TGFb was observed only at 1 and 2h, and was not significantly affected by DAPT. A slight and not significant increase in HERP-1 expression at 1 hour was observed, which was not changed by DAPT. NRARP levels slightly increased in TGFb-treated cells as well. C-MYC levels remained unchanged following TGFb and DAPT treatment.

4.7. Gene expression levels in Dll4-treated HT58 cells

No significant changes were detected in the mRNA expression of HES-1, HERP-1, NRARP and C-MYC at different time points (1, 4, 24 and 48h) in Dll4-treated cells. HES-1 was

slightly increased after a 4 hour treatment, nevertheless, its levels remained extremely low [$\Delta CT_{HES\text{-}1}$~35 cycles; ΔCT_{GAPDH}~20 cycles].

4.8. The effect of TGFb and combined treatments in CLL cells

B-cells were separated from peripheral blood samples of four CLL patients, and cells were treated with Dll4, DAPT, TGFb and their combinations in short-term (48h) *in vitro* culture. HES-1 induction was observed in Dll4 treated cells, which was inhibited by DAPT. The rate of apoptosis was not significantly affected by treatments.

4.9. Smad4 gene silencing in HT58 cells

Smad4 was knocked down with siRNA in HT58 cells; the efficiency of siRNA transfection was 95-98%, and cell viability remained intact in siRNA-treated cells. Smad4 mRNA and protein levels were decreased six hours after siRNA treatment. Smad4 mRNA was not detected during the entire period of TGFb treatment. TIEG mRNA was induced by TGFb in control HT58 cells, which was abolished in siRNA-treated cells (6 and 24 hours after siRNA transfection).

4.10. Smad4 knock-down does not affect TGFb-induced apoptosis

The rate of TGFb-induced apoptosis did not change in HT58 cells treated with Smad4 siRNA. Inhibition of Smad4 was also performed by transient transfection of HT58 cells with a dominant negative Smad4 vector (DNSmad4) construct. The efficiency of vector transfection was 65%. Similarly to Smad4 siRNA, DNSmad4 did not affect the rate of TGFb induced apoptosis.

4.11. The role of MAP-kinases and PP2A phosphatase in Smad4-independent TGFb-induced apoptosis

The level of phospho-ERK1/2 and phospho-JNK proteins rapidly decreased in HT58 cells upon TGFb-treatment, but the amount phopho-p38MAPK and total JNK and ERK kinases did not change. To confirm the functional role of ERK/JNK inactivation, cells were co-treated with MEK1 kinase inhibitor and TGFb. This combinational treatment enhanced the apoptotic effect of TGFb: co-treatment induced apoptosis after 48h was as high as that induced by TGFb only at 72 h. Co-treated cell cultures contained mainly apoptotic and necrotic cells at 72h. This suggests that the inhibition of kinases promoted and accelerated the apoptotic program.

TGFb lead to an increase in PP2A activity at an early, signaling initiator phase (20 min and 1h after TGFb-treatment) and also at a later stage, 48h after TGFb treatment, coinciding with the onset of apoptotic effector mechanisms. The role of PP2A activation was assayed by treating cells with phosphatase inhibitors. Okadaic acid and endothall (mainly a PP2A inhibitor in the applied dose range) given in the first four hours of TGFb treatment almost completely abolished TGFb-induced apoptosis in B-lymphoma cells.

5. DISCUSSION

Deciphering signaling pathways has revolutionized cancer research and therapy and lead to the identification of potential molecular therapeutical targets. Notch-signaling is considered such a therapeutical target in acute T-cell leukemias. If activation the Notch pathway was proved to be a survival factor in B-cell malignancies as well, then GSI-s (inhibitors of Notch-signaling) could be used as alternative drugs also in these disease entities. Nevertheless, the detailed characterization of the elements, activity and biological effects of the pathway in different types of B-cell non-Hodgkin lymphomas is required before farmacological inhibitors of Notch can be introduced in clinical practice.

Here we show that the gene expression pattern of Notch-signaling elements is similar in human circulating CLL cells and normal B-cells. We assessed the activity of the Notch pathway by determining the abundance of the HES-1 target gene: the majority of CLL samples showed much lower HES-1 expression than normal B-cells. HES-1 mRNA-expression comparable to that of normal B-cells was detected only in five out of 24 CLL samples. No correlation was found between the mRNA expression of the examined genes and the clinical and prognostic data of CLL patients. Thus, our results do not support support the abnormal accumulation and activation of Notch2-receptor in peripheral blood CLL cells. We are aware of the importance of parallel, mRNA and protein level studies, but such studies may be hindered by the limited amount of protein isolated from the cells, and the varying quality of commercially available antibodies for different applications. Cells bearing Notch-ligands in tissue microenvironments, such as the lymph node, may be abundant and may activate Notch-signaling, but their biological importance in CLL remains to be determined

B-NHL cell lines do express Notch-receptors, which provide the basis for the inhibition or ligand-dependent activation of the pathway. We set out to examine the apoptotic effects of the Dll4 Notch-ligand and the DAPT γ-secretase inhibitor in B-cell lymphoma cell lines *in vitro*. The rate of apoptosis was not changed by Dll4 or DAPT only. Thus it seems that Notch-signaling is not a central, key regulating factor of apoptosis in B-cell non-Hodgkin lymphomas. Notch-sensitivity may of course be cell line specific, and literature data shows that the inhibition of the pathway induces apoptosis in certain B-cell lines, whereas in other cell lines it is the activation of the pathway which causes cell cycle arrest and apoptosis.

Recent studies have shown that Notch- and TGFb-signaling may interact, however, cross-talk between the two pathways has not been studied in B-cell lymphomas. In our experiments, the addition of Dll4 or DAPT did not restore TGFb-sensitivity in TGFb-resistant B-NHL cell lines. However, DAPT did decrease TGFb-induced apoptosis significantly in the

TGFb-sensitive HT58 and BL41 cell lines, which does not support the pro-survival role of Notch in lymphoma cells. Indeed, the Notch pathway may be in part necessary for the apoptotic effects of TGFb in these cell lines. The apoptotic effect of TGFb, Dll4, DAPT and their combination was also examined in CLL cells isolated from peripheral blood.

An increasing amount of evidence show cross-talk between Notch- and TGFb-signaling, but the interaction between the two pathways in B-cell lymphomas has not been examined yet. TGFb-sensitivity was not restored by Dll4 or DAPT in TGFb-resistant B-NHL cell lines in our experiments. However, DAPT significantly decreased TGFb-induced apoptosis in the TGFb-sensitive HT58 and BL41 cell line – this observation does not support the pro-survival role of Notch in B-lymphoma cells, either. Thus, the apoptotic effect of TGFb may in part require Notch-signaling in these cell lines. The apoptotic effect of TGFb, Dll4 and DAPT was also examined in CLL cells isolated from peripheral blood. In short term (48h) *in vitro* cultures, none of the treatments and co-treatments changed the rate or apotposis. Our results contradict those recently published results, according to which GSI-treatment increased and Jagged1 ligand treatment decreased apoptosis in CLL cells *in vitro*. Differents in results obtained with GSI-treatment may be explained by the different types and dosages of GSI-s, but further studies are needed to clarify these contradictions.

GSI-s may be used in combination with other chemotherapeutical drugs if they could be shown to synergize with them. In our experiments, DAPT did not modify the rate of apoptosis induced by different drugs in HT58 cells. Thus, our results warn that different types of GSI-s must be extensively tested (including *in vivo* models) in different lymphoma entities before clinical applications can be initiated.

The best known target genes of Notch-signaling are HES-1 and other transcription factors belonging to the HES and HERP family. HES-1 expression changes due to Dll4 and DAPT were observed only in the BJAB cell line in our experiments. (in addition the Jurkat T-ALL cell line, which was used for testing Dll4 and DAPT). The reason for this is not yet clear; it is possible that the affinity of Notch-receptors to Dll4 ligands is variable, or different receptors may activate alternative pathways not involving HES-1.

The transcription of HES and HERP genes may be regulated by signaling pathways other than Notch. Our data indicate that HES-1 mRNA-expression is regulated by TGFb in certain types of B-cell non-Hodgkin lymphomas, probably at a direct, transcriptional level. HES-1 induction by TGFb was biphasic, and DAPT partially inhibited HES-1 expression in the second phase. Thus, TGFb-induced HES-1 may be in part Notch-dependent in the later phase in selected B-cell lymphomas.

An increase in HEY-2/HERP-1 and HEY-1/HERP-2 gene transcription was also observed during TGFb-induced epithelial-mesenchymal transition, in addition to HES-1. We could not observe a significant increase in HERP-1 mRNA-expression upon TGFb-treatment in HT58 cells, and it seems that HERP-1 is not a TGFb target gene in this cell line. Interestingly, HERP-1 expression was slightly increased by Dll4 in HT58 cells, and also by DAPT. The reason for this remains to be determined. However, it has to be taken into account that Notch target genes may vary in a cell type dependent manner.

The role of the HES-1 transcription factor in the biology of B-cell lymphomas is not yet known. TGFb increased HES-1 transcription cell lines sensitive to the apoptotic effects of TGFb in our experiments, and it cannot be excluded that HES-1 is necessary for apoptosis induction. Our observation that DAPT decreased TGFb-induced HES-1 expression 6-8 hours after treatment and it partially inhibited TGFb-induced apoptosis in HT58 cells supports this hypothesis.

In addition to HES-1 and HERP-1, the expression of three other genes were characterized, which may be under the regulation of TGFb, Notch or both pathways (TIEG, NRARP and C-MYC, respectively). Our data indicate that TIEG belongs to TGFb-signaling, and – in contrary to HES-1 – does not represent a connection between the two pathways. NRARP has been described as a Notch target gene, but its mRNA level was not changed by Dll4 or DAPT in HT58 cells; a slight increase was observed in TGFb-treated cells, which was too small to consider NRARP a TGFb target. NRARP has been shown a Notch-target in T-cells among hematolymphoid cells, and its Notch-dependent regulation may be characteristic for these cell types only.

C-MYC may be a direct target gene of Notch1 in acute T-cell leukemias, and its increased level may promote the survival of neoplastic cells. C-MYC may also be the target ot TGFb-signaling, and its down-regulation may be required for the cytostatic properties of TGFb. Based on these literature data, we presumed that these two pathways might regulate C-MYC gene transcription in B-cell non-Hodgkin lymphomas as well. However, no changes were seen in C-MYC gene expression upon Notch-ligand, Notch- inhibitor or TGFb treatment in the B-NHL cell lines included in our study.

The importance of TGFb in the apoptotic regulation of B-NHL cells is widely known, and Smad4 was considered for a long time to be indispensable for the diverse roles of TGFb. However, recent studies showed that some of the regulatory functions are independent of Smad4. In our work we characterized the Smad4-dependence of TGFb-induced apoptosis in B-NHL cell lines. Transient silencing of Smad4 was carried out by the transfection of synthetic siRNA, and Smad4 mRNA and protein levels were shown to be down-regulated in

siSmad4 transfected cells. TGFb was able to induce apoptosis also in the absence of Smad4. We described for the first time the existence of a TGFb-induced, Smad4-independent apoptotic pathway.

TGFb-induced, Smad4-independent apoptosis involves alternative pathways: it requires the activation of PP2A phosphatase and the inactivation of ERK and JNK kinases. We showed that TGFb-induced apoptosis is accompanied by the activation of PP2A phosphatase and the inactivation of ERK1/2 and JNK kinases in B-lymphoma cells. Functional importance of these pathways were supported by the fact that the inhibition of MAP-kinases increased the rate and accelerated the onset of TGFb-induced apoptosis, whereas the inhibition of PP2A phosphatase virtually abolished TGFb-induced apoptosis. We suggest that TGFb activates PP2A phosphatase, which in turn dephosphorylates MAP-kinases, and thus shifts the balance of survival signals towards apoptosis – however, the direct relationship of these pathways during TGFb-induced apoptosis is yet to be confirmed.

Based on our results, the Notch pathway is not a central regulatory factor of apoptosis in B-NHL cells, however, it may modify the effects of TGFb. TGFb-induced apoptosis may require Notch-signaling (at least in part), and perhaps the fine-tuning of HES-1 regulation by the two pathways. In addition, the characterization of Smad4-dependent and -independent effects of TGFb may promote the identification of more efficient and specific targets in lymphoma therapy.

Signaling pathways offering attractive molecular targets for personalized therapies are extremely complex. One signal may involve various, alternative pathways, each resulting in different biological outcomes, and may interact with several other pathways in a cell type and context dependent manner. Thus, we have to aim at deciphering the events of this complex network precisely in the system that we plan to manipulate. We hope that our work has contributed to this goal.

6. CONCLUSIONS

- The expression of Notch-receptors and -ligands are similar in normal B cells and CLL cells. The expression of Dll1 ligand the Deltex regulatory molecule are slightly different.
- HES-1 mRNA is expressed to a higher extent in normal B-cells than in the majority of CLL samples.
- Gene expression intensity of Notch-receptors, -ligands, HES-1 and Deltex does not correlate with the clinical and prognostic parameters of CLL.
- The inhibition or ligand-dependent activation of the Notch-pathway itself does not influence apoptosis in B-cell non-Hodgkin lymphoma cell lines *in vitro*. Thus, our results do not support a central, decisive role of the Notch pathway in the regulation of apoptosis in B-NHL cells.
- DAPT decreases the rate of TGFb-induced apoptosis in TGFb-sensitive B-NHL cell lines. Neither DAPT, nor Dll4 restores TGFb-sensitivity in TGFb-resistent cell lines.
- Drug-induced apoptosis was not influenced by DAPT in HT58 B-lymphoma cells.
- HES-1 is a transcriptional target gene of TGFb in selected B-NHL cell lines. TGFb-induced HES-1 expression is biphasic: it is Notch-independent in the early phase, whereas it may be partly Notch-dependent in the later phase.
- HERP-1, C-MYC and NRARP gene expression was not majorly affected by neither Notch- nor TGFb-signaling in B-NHL cells. TIEG is the target gene of TGFb only; its expression was not influenced by Notch.
- TGFb is able to induce apoptosis in the absence of Smad4 in HT58 B-lymphoma cells. We were first to describe the existence of a TGFb-induced, Smad4-independent apoptotic pathway.
- TGFb-induced, Smad4-independent apoptosis requires the inactivation of ERK1/2 and JNK MAP-kinases and the activation of PP2A phosphatase.

7. PUBLICATIONS

7.1. Publications in the subject of the theses:

1. **Hajdu M**, Sebestyén A, Barna G, Reiniger L, Jánosi J, Sréter L, Várkonyi J, Demeter J, Kopper L. Activity of the notch-signalling pathway in circulating human chronic lymphocytic leukaemia cells. *Scand J Immunol.* 2007;65:271-5. **IF: 1.928**

2. *Sebestyén A, ***Hajdu M**, Kis L, Barna G, Kopper L. Smad4-independent, PP2A-dependent apoptotic effect of exogenous Transforming Growth Factor beta1 in lymphoma cells. *Exp Cell Res.* 2007;313:3167-74. (*Sebestyén A. and Hajdu M. contributed equally to this work.) **IF: 3.695**

3. **Hajdu M**, Kopper L, Sebestyén A. Notch-regulation of TGFb-induced apoptosis and gene expression in human B-cell non-Hodgkin lymphomas. *Cytokine* – submitted for publication.

7.2. Independent publications:

1. Kohut E, **Hajdu M,** Gergely P, Gopcsa L, Kilián K, Pálóczi K, Kopper L, Sebestyén A. Expression of TGFβ1 and its signaling components by peripheral lymphocytes in SLE. *Pathol Oncol Res.* 2008. Epub ahead of print. **IF: 1.272**

2. **Hajdu M,** Luttun A, Pelacho B, Burns CT, Chase L, Gutiérrez-Pérez M, Jiang Y, Lenvik T, Vas V, Uher F, Sebestyén A, Verfaillie C. Transcriptional characterization of the notch signaling pathway in rodent multipotent adult progenitor cells. *Pathol Oncol Res.* 2007;13:302-10. **IF: 1.272**

3. Végső G, Sebestyén A, Paku S, Barna G, **Hajdu M**, Tóth M, Járay J, Kopper L. Antiproliferative and apoptotic effects of mycophenolic acid in human B-cell non-Hodgkin lymphomas. *Leuk Res.* 2007;37:1003-8. **IF: 2.561**

4. Sebestyén A, **Hajdu M,** Kopper L. TGFb – the Janus-faced cytokine: the dual role of TGFb as a tumor suppressor and a tumor promoter. *Orvosképzés.* 2006;3:169-183.

5. Kopper L, **Hajdu M**. Tumor stem cells. *Pathol Oncol Res.* 2004;10:69-73. Review.

6. **Hajdu M**, Puskás É, Sipos A, Barta A, Pálóczi K, Uher F. Homogeneous immunoglobulins following allogeneic bone marrow transplantation. *Acta Haematol.* 2003;109:124-8. **IF: 1.874**

7. Uher F, **Hajdu M**, Vas V. Self-renewal and differentiation of hematopoietic stem cells: a molecular approach (a review). *Acta Microbiol Immunol Hung.* 2003;50:3-21.

8**.** Vas V, **Hajdu M**, Pálóczi K, Uher F. Alternative views of tissue stem cell plasticity. *Haematologia.* 2002;32:175-90. Review. **IF: 0.293**

7.3. Presentations and posters

1. **Melinda Hajdu**, Anna Sebestyén, László Kopper. Characterization of Notch and TGFb induced early HES-1 and TIEG expression in B-cell lymphomas. EMBO Meeting – Cellular Signaling and Molecular Medicine; May 29 – June 4, 2008; Dubrovnik, Croatia. Poster abstract: P65.

2. **Melinda Hajdu**, Anna Sebestyén, László Kopper. Interacting effect of TGFb and Notch signaling in B-cell lymphomas. ECCO 14 – the European Cancer Conference; September 23-27, 2007; Barcelona, Spain. Poster abstract: P-382.

3. **Hajdu Melinda**. Cross-talk of the TGFb and Notch signaling pathways in B-cell lymphomas. Semmelweis University School of PhD Studies, PhD Scientific Days; Budapest, April 12-13, 2007. Abstract no.: E-IV/3.

4. **Melinda Hajdu**, Anna Sebestyén, Gábor Barna, Lilla Kis, László Kopper. Potential cross-talk of TGFb and Notch-signaling in B-cell lymphomas. International Conference of Immunogenomics and Immunomics; October 8-12, 2006; Budapest. Poster abstract: P-1-58.

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