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Notch1 enhances B-cell receptor-induced apoptosis in mature activated B cells without affecting cell cycle progression and surface IgM expression

S Romer[1], U Saunders[1], H-M Jäck*,[1] and BM Jehn[1]

[1] Division of Molecular Immunology, Department of Internal Medicine III, Nikolaus-Fiebiger Center, Friedrich-Alexander-University Erlangen-Nürnberg, Glückstrasse 6, 91054 Erlangen, Germany
* Corresponding author: H-M Jäck. Tel: +49 9131 853; Fax: +49 9131 8539343; E-mail: HJAECK@molmed.uni-erlangen.de

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Abstract

The transmembrane receptor Notch1 plays a crucial role in differentiation and apoptosis of hematopoietic cells. To investigate the influence of Notch1 on apoptosis and cell growth of mature murine B cells, we transduced the murine B-lymphoma line NYC 31.1 with a constitutively active, intracellular form of human Notch1 (Notch1-ICT). NYC cells represent mature activated B cells that can be induced to undergo apoptosis by crosslinking of the B-cell receptor (BCR). In contrast to investigations in immature chicken B-cell lines, transduced Notch1-ICT did not affect cell cycle progression, cell growth or surface IgM levels in NYC cells and resulted only in a slight induction of apoptosis. However, BCR-crosslinking enhanced apoptosis, but did not influence cell cycle progression in Notch1-ICT-positive NYC cells. These data imply a distinct function of Notch1 in mature murine B-cells as compared to immature chicken B cells and provide further evidence for Notch1's involvement in B-cell differentiation and development.
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Keywords: B1 cells; B lymphocytes; hematopoiesis; NYC 31.1; B-cell maturation

Abbreviations: BCR, B-cell receptor; CBF1, core binding factor 1; Ig, immunoglobulin; Notch1-ICT, constitutively active, intracellular domain of human Notch1; Notch1-IC, constitutively active, intracellular domain of Notch1; NP-40, Nonidet P-40; OPA, glutamine-rich region; PAGE, polyacrylamide gel electrophoresis; PEST, proline (P), glutamine (E), serine (S) and threonine (T)-rich region; PCR, polymerase chain reaction; RBP-J, recombination signal binding protein Jkappa; RT, reverse transcription; TAN, translocation-associated human Notch1 homologue; TCR, T-cell receptor.

Introduction

In both murine and human immune systems, B-cell development occurs in the bone marrow and originates from pluripotent hematopoietic stem cells. This development goes through several stages that are characterized by DNA rearrangements, first at the immunoglobulin (Ig) heavy (H) and then at the Ig light (L) chain locus (reviewed in Melchers *et al.*,[1] Osmund,[2] Löffert *et al.*,[3] Winkler and Melchers,[4] Rolink *et al.*,[5] Hardy and Hayakawa[6]). Productive and functional rearrangements of H- and L-chain genes result in the assembly of a membrane anchored IgM molecule that is presented on the cell surface as an antigen or B-cell receptor (BCR). Immature B cells with a functional BCR develop subsequently into mature B cells (reviewed in Hardy and Hayakawa,[6] Osmond *et al.*,[7] Cornall *et al.*[8]), which migrate to secondary peripheral lymphoid organs. Here, if activated by antigen, the B cells differentiate into antibody-secreting plasma cells, a prerequisite for an effective humoral immune response against pathogens (reviewed in Lindhout *et al.*[9]).

The generation of B-lymphoid cells in the bone marrow depends on a delicate balance between proliferation, differentiation and cell death. While proliferation and differentiation ensure the continuous production of B-lymphoid cells from hematopoietic precursors, cell death processes are crucial to eliminate nonfunctional or potentially harmful B-cell clones.[8,10,11] Cell death occurs at various times and stages during the maturation of B-lymphoid cells and one of the best studied processes is the elimination of autoreactive immature B cells.[10,11] Experiments with transgenic mice have shown that the engagement of multivalent autoantigens by immature B cells in the bone marrow triggers their elimination via apoptotic cell death processes.[12] Apoptosis in immature B cells can also experimentally be induced by crosslinking the BCR with antibodies or $F(ab)_2$ fragments against the H-chain of IgM-BCRs.[12–14]

The transmembrane receptor Notch1 and its specific ligands regulate proliferation, differentiation, cell fate decisions and apoptosis of several cell lineages.[15–17] Additionally, Notch1 has been shown to participate in developmental and apoptotic cell death processes during the generation of lymphocytes and other hematopoietic cells (reviewed in Radke *et al.*,[18] Allman *et al.*[19]). The Notch1 transmembrane receptor is synthesized as a large precursor and processed within the Golgi apparatus.[15,20] The mature form of the Notch1 receptor is a heterodimeric complex consisting of an extracellular ligand-accessible (Notch1-EC) and a membrane-anchored intracellular chain (Notch1-IC). Thereafter, receptor–ligand interactions induce proteolytic processing and release of the intracellular portion of Notch1 into the cytoplasm.[21–25] This so-called activated form of Notch1 (Notch1-IC) translocates into the nucleus and, in conjunction with the DNA binding protein CBF-1 (core binding factor 1, also known as RBP-Jκ), modulates the expression of target genes in many vertebrates (reviewed in Artavanis-Tsakonas *et al.*,[15,16]).

Experiments with transgenic mouse models have revealed that signals mediated by Notch1 control the maturation and commitment of thymic T-lymphoid precursors to CD4 and CD8 as well as to $\alpha\beta$ and $\gamma\delta$ TCR lineage.[26–28] Furthermore, the inducible deletion of the Notch1 gene in adult mice results in a block in early T-cell development and in the appearance of a B-lymphoid subpopulation within the thymus of these animals.[29,30] In contrast, reconstitution experiments demonstrated that murine bone marrow cells transduced with an activated form of human Notch1 (Notch1-ICT) give rise to T cells, but are blocked at an early stage of B-cell maturation.[31] Therefore, Notch1 seems to promote early T-cell development and to inhibit maturation of early B-lymphoid progenitor cells.[31]

Anti- as well as proapoptotic effects of Notch1 have been demonstrated in cell lines of hematopoietic origin. For example, a constitutively active form of the intracellular portion of Notch1 (Notch1-ICT) reduces TCR-mediated apoptosis in T-cell hybridoma cells transduced with a Notch1-ICT retrovirus.[32,33] Also, inhibition of Notch1 expression by an antisense approach leads to an increased rate of apoptotic cell death in mouse erythroleukemia (MEL) cells.[34] In contrast, transfected Notch1-ICT induces apoptosis and cell cycle arrest in the chicken B-cell line DT40 in the absence of BCR-signals.[35] In addition, transfected Notch1-ICT downregulates surface IgM on the avian leukosis virus-transformed B-cell line 249L4.[36] Therefore, Notch1 seems to exert different functions in different hematopoietic lineages.

To determine the effect of Notch1 on intracellular signal pathways in B cells representing a more mature stage of development, we stably transduced the murine B-cell line NYC 31.1 (NYC) with Notch1-ICT and investigated the effect of Notch1-ICT on apoptosis, cell growth and surface IgM expression. NYC cells represent mature activated B1-type like B cells[37] that die apoptotically in response to crosslinking of the BCR with anti-IgM antibodies (this study). In contrast to studies investigating the effect of a constitutively active form of Notch1 in immature chicken B-cell lines,[35,36] we found that a constitutive active form of human Notch1 did not affect cell cycle progression or surface IgM levels in NYC cells and resulted only in a slight induction of apoptosis. However, BCR-crosslinking clearly enhanced apoptosis, but did not influence cell cycle progression or IgM expression in Notch1-ICT-positive NYC cells. Based on these findings, we postulate that the effect of Notch1-signals on apoptosis and cell growth depends on the maturation and activation stage of a B cell.

Results

The avian Bursa of Fabricius-derived B-lymphoid cell line DT40 undergoes somatic gene conversion and represents cells at a developmental stage before the selection against autoreactive B cells occurs. Recent studies showed that transfection of DT40 cells with Notch1-IC results in the enhancement of apoptotic cell death and in a block in cell cycle progression at the G0/G1 phase, even in the absence of a BCR-mediated stimulus.[35] To investigate the influence of activated Notch1 on B cells at a later stage of development, we have chosen the murine B-cell line NYC as a model system. The NYC line was established from a clonal, spontaneous splenic B-cell tumor that originated in a (NZB $\times$ NZW) F1 mouse.[37] NYC cells are characterized by surface expression of CD5, a marker of B1 cells. In addition, NYC cells express surface IgM and secrete soluble IgM; therefore, NYC cells represent mature, activated B1-like B-cell blasts at the transition from the surface IgM-positive B cell to the antibody-secreting plasma cell stage.[37]

Effect of BCR-crosslinking on apoptosis and endogenous murine Notch1 levels in NYC wild-type cells

To determine whether BCR signals can induce apoptosis in NYC cells, NYC cultures were incubated with crosslinking anti-BCR $F(ab)_2$ fragments (i.e., anti-IgM antibodies) for 5 and 28 h; PBS as well as isotype-matched control $F(ab)_2$ fragments were used as negative controls. After each incubation period, cells were costained with AnnexinV-FITC (AnnexinV) and YOPRO-3-Iodide (YOPRO-3), and analyzed by flow cytometry. AnnexinV-FITC staining represents a rather sensitive method to detect apoptotic cells as compared to YOPRO-3-iodide staining, in particular at very early time points of induction of apoptosis. The frequencies of apoptotic and dead cells were determined from the relative numbers of AnnexinV- and YOPRO-3-positive cells, respectively. Representative results from one of four independent experiments are shown in Figure 1a. Flow cytometric analysis revealed a significantly higher frequency of apoptotically dying cells after treatment of NYC cells with crosslinking anti-IgM antibodies as compared to PBS- (Figure 1a) and isotype-matched antibody (data not shown) treated cells.

Since Notch1 has been implicated in the control of apoptosis in lymphocytes,[32,33] we asked whether BCR-induced apoptosis correlates with a change in intracellular Notch1 levels in NYC cells. Western blot analysis of endogenous Notch1 over a stimulation period of 48 h with either PBS or BCR-crosslinking anti-IgM antibodies revealed that NYC cells synthesize endogenous Notch1 at all analyzed time points (Figure 1b). Furthermore, Notch1 levels remained unchanged after treatment of NYC cells with PBS (Figure 1b, lanes 2–5) or with the BCR-crosslinking antibody (Figure 1b, lanes 6–9). However, from this experiment, it is not clear whether membrane-bound Notch1-IC was converted into the active form by proteolysis, since our Western blot analysis did not clearly resolve membrane-bound from clipped, activated Notch-IC. However, even if we would detect the active form of endogenous Notch1 after BCR-crosslinking, we still could not conclude from this correlation that Notch1 participates in the control of BCR-mediated apoptosis. To address this question, we introduced a Notch1-ICT into NYC cells and examined its effect on apoptosis, cell cycle and growth in the presence and absence of a BCR signal.

Generation of stable NYC infectants producing Notch1-ICT

To directly determine the effect of an active form of Notch1 on cellular functions in mature activated murine B cells, we used retrovirus-mediated gene transfer to generate pools and

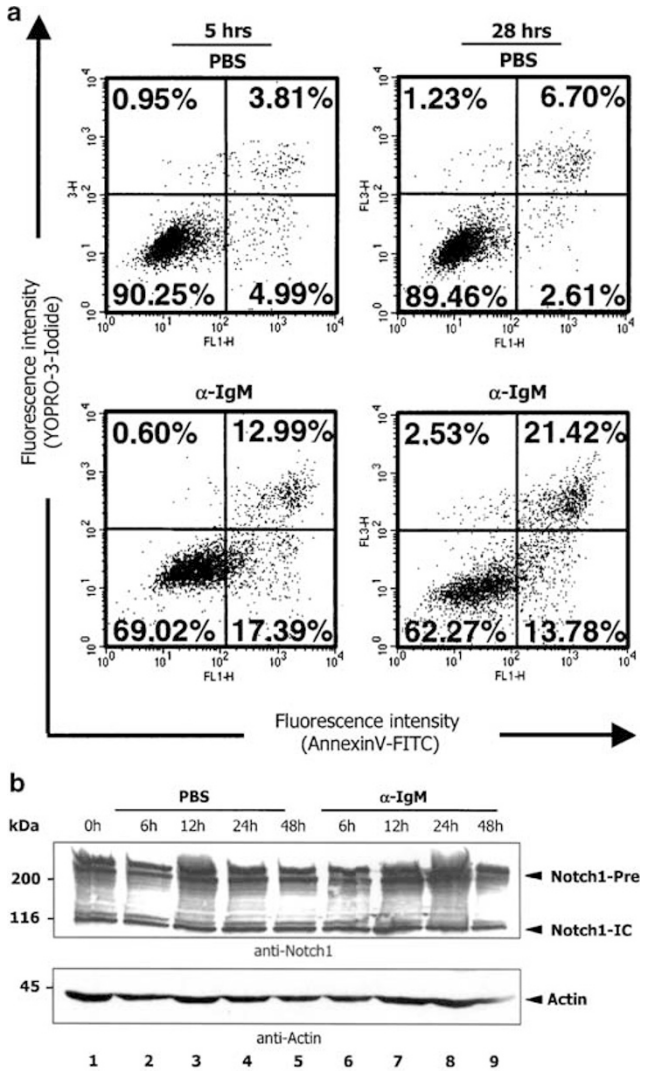

Figure 1 Characterization of wild-type NYC cells. (**a**) Effect of BCR-crosslinking on apoptosis in NYC wild-type cells. Wild-type NYC cells were treated for 5 (left hand panels) or 28 h (right hand panels) with PBS or 10 μg/ml of an anti-IgM-specific antibody. Subsequently, cells were harvested and stained with YOPRO-3-Iodide and AnnexinV-FITC. Apoptotic cell death was assessed by flow cytometric analysis using the CellQuest software. Typical histograms are shown. (**b**) Western blot analysis of endogenous murine Notch1 in NYC cells NYC cells were treated for the indicated time intervals with 10 μg/ml of an anti-IgM-specific antibody or PBS. Proteins from whole cellular lysates (corresponding to 1×10^6 cells/lane) were prepared, separated and transferred to PVDF membranes as described in the Materials and Methods section. Notch1-specific signals were visualized using a goat anti-murine Notch1-specific antibody (M20), which predominantly recognizes the endogenous murine form of Notch1. Equal loading was assessed by reprobing of the blot with a rabbit anti-actin-specific antibody

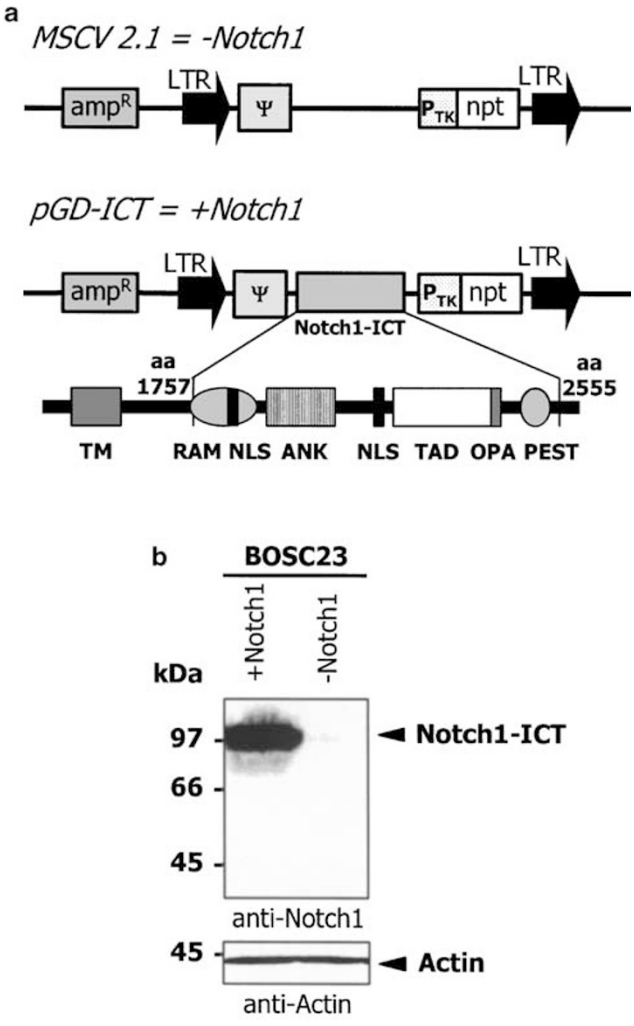

Figure 2 Transient transfection of retroviral vectors into the packaging cell line BOSC 23. (**a**) Retroviral vectors. To introduce the activated form of Notch1 into the packaging cell line BOSC 23[38] and subsequently into the mature activated murine B-cell line NYC 31.1,[37] two different retroviral vectors were used. The MSCV 2.1 plasmid[33,38] represents the control vector with a G418 resistance gene under the control of MoMuLV-LTRs. The pGD-ICT plasmid[33,38] contains the complete intracellular domain of the human Notch1 homologue TAN1 (amino acids 1757–2555), including the RAM domain, Nuclear localization signals (NLS), ankyrin motif repeats, a transcriptional transactivation domain (TAD), and the carboxy-terminal located OPA and PEST domains; the pGD-ICT plasmid is under the control of the MoMuLV-LTRs. The pGD-ICT vector and the MSCV 2.1 vector are identical in regard to the MoMuLV-LTRs and the resistance genes. The MSCV 2.1 vector differs only in the multiple cloning site as compared to the Notch1 containing vector pGD-ICT. Therefore, any unspecific promoter-dependent effects can be excluded. (**b**) Notch1-ICT detection in transfected BOSC 23 cells by Western blot analysis Whole cellular lysates were prepared and extracts corresponding to 1×10^6 cells/lane were separated on denaturing SDS-PAGE gels. Proteins were transferred to PVDF membranes and Notch1-ICT-specific signals (110 kDa) were visualized using a goat anti-Notch1-specific antibody (C20), which predominantly recognizes the retrovirally infected form of human Notch1 (Notch1-ICT). BOSC 23 cells either transfected with pGD-ICT (+Notch1; lane 1) or MSCV 2.1 plasmid DNA (-Notch1; lane 2) were analyzed. Equal loading was assessed by reprobing the blot with an anti-Actin-specific antibody

stable cell clones of NYC cells producing the Notch1-ICT. We then went on to analyze whether constitutive expression of Notch1 modulates apoptosis and proliferation before and after crosslinking of surface IgM. To generate packaging cells capable of producing retroviral particles, we transiently transfected the retroviral packaging cell line BOSC 23[38] with Moloney Murine Leukemia Virus (MoMuLV)-based retroviral vectors (Figure 2a) without (MSCV 2.1=−Notch1) and with the cDNA sequence encoding the complete intracellular portion of the human Notch1 gene (TAN1) including the glutamine-rich region (OPA) and proline(p), glutamine (E),

serine (s) and threonine (T)-rich region (PEST) sequence motifs (pGD-ICT=+Notch1). The efficiency of the transient transfection of BOSC 23 cells was verified by Western blot analysis (Figure 2b). As expected, we obtained a Notch1-ICT-specific signal (110 kDa) only in +Notch1-transfected BOSC 23 cells, but not in BOSC 23 cells transfected with the control vector (−Notch1).

Subsequently, cell culture supernatants of BOSC 23 cells transfected with the Notch1 containing vector (+Notch1) or the control vector (−Notch1) were harvested and used for retroviral infection of NYC cells. After selection of G418-resistant cell pools, single-cell clones were obtained by the limiting dilution method and tested for the expression and synthesis of ectopic Notch1-ICT. We first analyzed pools as well as individual −Notch1- and +Notch1-infected NYC 31.1 clones for the presence of Notch1-ICT-specific mRNA. Human and murine Notch1 show a high degree of identity at the protein as well as the nucleic acid level. However, subtle differences between the two sequences enabled us to design polymerase chain reaction (PCR) primers for the intracellular portion of Notch1 that distinguished between endogenous murine and the transduced human Notch1 mRNA in a reverse transcription (RT)-PCR approach. As shown in Figure 3a, RT-PCR with primers specific for human Notch1 detected signals in NYC pools and clones infected with +Notch1 virus, but not in noninfected

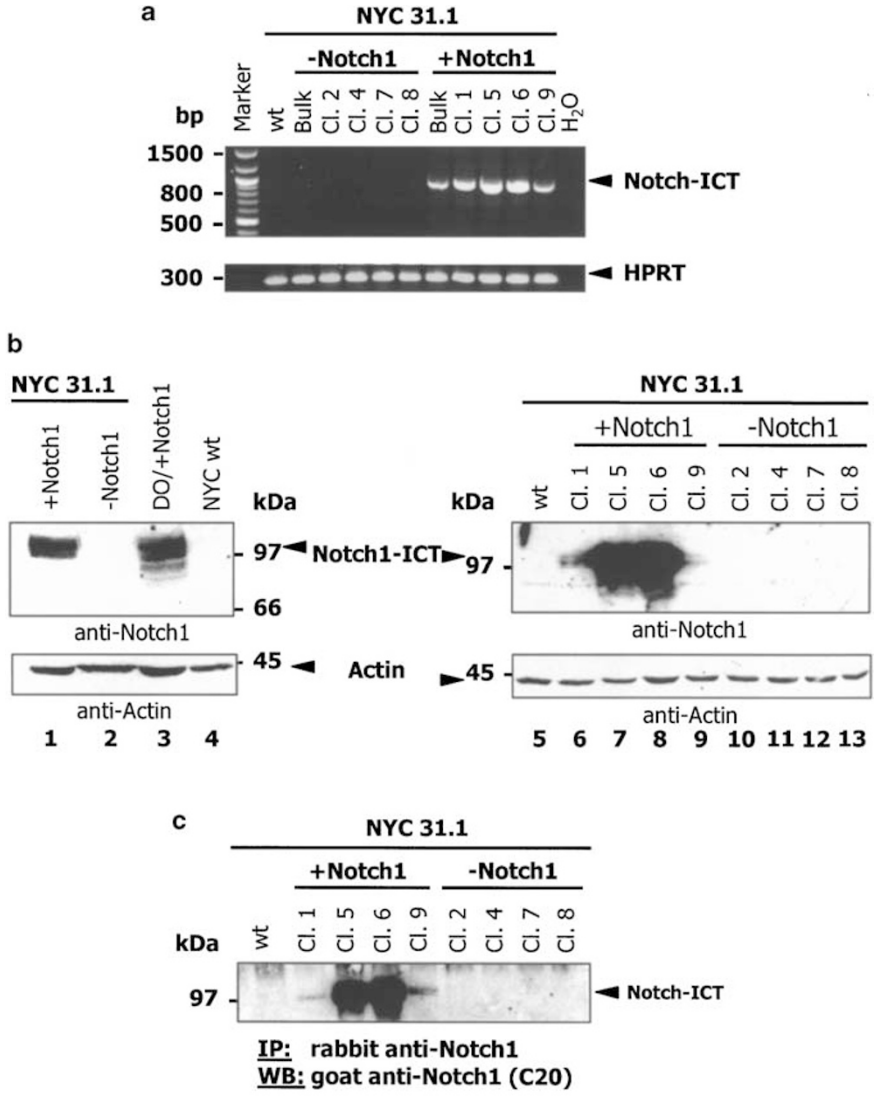

Figure 3 Detection of Notch1-ICT in infected NYC cells. (**a**) RT-PCR analysis. Total cellular RNAs were extracted from the indicated cell lines and reverse transcribed into cDNAs. PCR reactions were performed using either human Notch1-specific primers to detect exogenously expressed Notch1- or HPRT-specific primers for control reasons. One-half of each RT-PCR reaction was loaded per lane. (**b**) Western blot analysis Proteins from whole cellular lysates (corresponding to 1×10^6 cells/ lane) were prepared, separated and transferred to PVDF membranes as described in the Materials and Methods section. Notch1-ICT-specific signals (110 kDa) were visualized using a goat anti-Notch1-specific antibody (C20; predominantly reactive with the retrovirally infected form of human Notch1 (Notch1-ICT)). Wild-type NYC cells (B, lane 4; C, lane 1), +Notch1 cells (B, lane 1; C, Cls. 1, 5, 6 and 9, lanes 2-5) or -Notch1 cells (B, lane 2; C, Cls. 2, 4, 7 and 8, lanes 6-9) were analyzed. Equal loading was assessed by reprobing of the blot with an anti-Actin-specific antibody. (**c**) Immunoprecipitation analysis Whole cellular lysates containing 1×10^7 cells of the indicated cell lines were immunoprecipitated using a rabbit anti-Notch1 polyclonal antiserum and protein G sepharose beads. Precipitated proteins were resolved and visualized as described in the Materials and Methods section

NYC cells and in clones and pools infected with the control virus (-Notch1).

To verify the expression of transduced Notch1, a Western blot analysis was performed with a commercially available Notch1-specific antibody that reacts only with human but not with murine Notch1. As expected, this antibody detected only a Notch1 signal in Notch1-transdcued NYC pools (Figure 3b, lane 1) and clones (lanes 6–9), but not in noninfected wild-type NYC cells (lane 4) and in clones infected with the control virus (−Notch1, lanes 10–13). In addition, this analysis revealed clones with low (Clones (cls.) 1 and 9) and with high levels (Cls. 5 and 6) of human (ectopic) Notch1. For control reasons, the T-cell hybridoma DO11.10-ICT[33] was used, which synthesizes high levels of ectopic Notch1-ICT (Figure 3b, lane 3). The presence and levels of ectopic Notch1 was verified by a combined immunoprecipitation/Western blot analysis (Figure 3c).

Effect of ectopic Notch1 on apoptosis in NYC cells

Initially, we investigated the effect of ectopic Notch1-ICT on apoptotic cell death processes in NYC cells before and after induction of cell death with BCR-crosslinking IgM-specific antibodies. The frequencies of apoptotic and dead cells were determined 5 h after the addition of PBS or anti-IgM antibodies by the AnnexinV/YOPRO-3 method as described earlier. Figure 4 shows the result of a typical flow cytometric analysis of YOPRO-3- and AnnexinV-stained NYC wild-type cells as well as −Notch1- and +Notch1-infected NYC cells. In contrast to experiments with immature chicken B cell lines,[35] +Notch1-infected NYC cells were viable and, when compared to wild-type and −Notch1-infected NYC cells, showed in this experiment a low level of spontaneous apoptosis in the absence of a BCR-signal (Figure 4a). However, repeated experiments confirmed that this increase in the frequency of

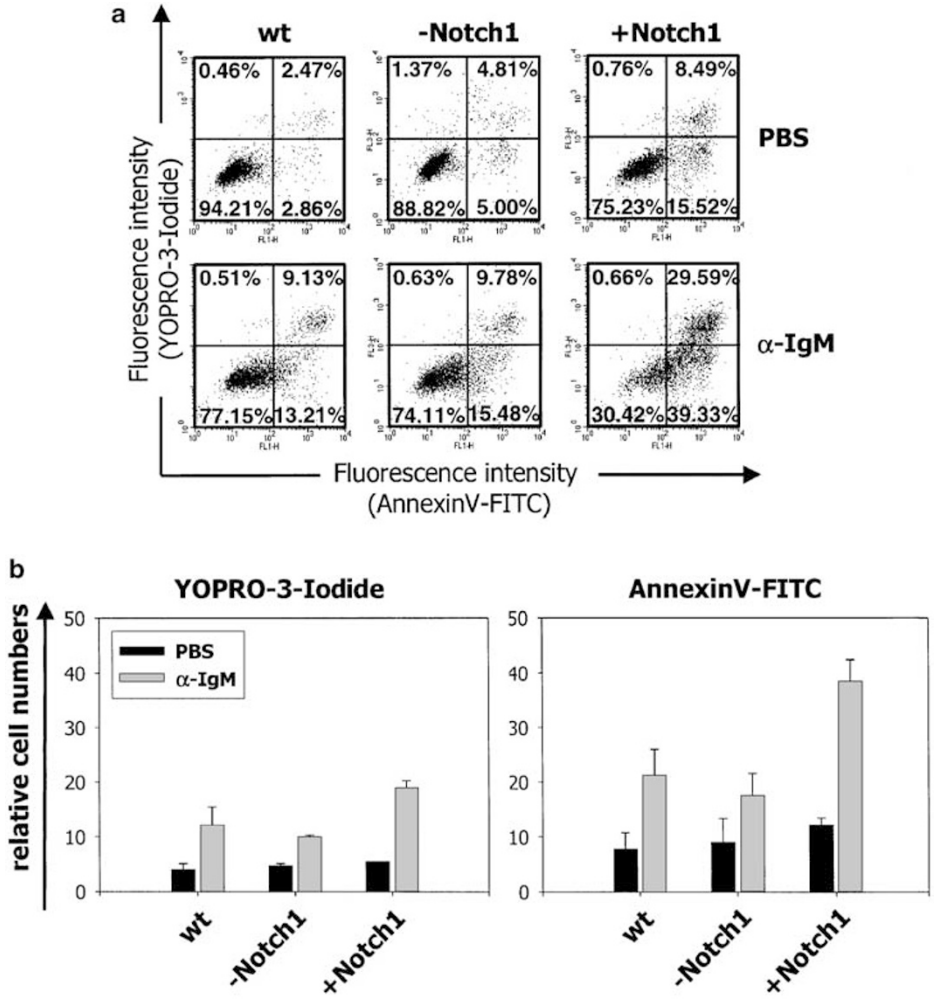

Figure 4 Effect of ectopic Notch1-ICT on apoptosis of NYC cells. Cells 2×10^5 of the indicated cell lines were stimulated for 5 h with 10 μg/ml anti-IgM-specific antibody or an equivalent amount of PBS. Subsequently, cells were harvested and either stained with YOPRO-3-Iodide and/or AnnexinV-FITC. Apoptotic cell death was assessed by flow cytometric analysis using the CellQuest software. (**a**) Analysis of apoptotic cell death via YOPRO-3-Iodide/AnnexinV-FITC staining. Typical histograms are shown. Wild-type (panel 1), -Notch1 (panel 2) and +Notch1 NYC cells (panels 3) were treated for 5 h with 10 μg/ml anti-IgM-specific antibody. The data reflect a representative result of three independent experiments each performed in triplicates. (**b**) Analysis of apoptotic cell death in wild-type, -Notch1 and +Notch1 NYC cells Relative numbers of YOPRO-3-Iodide positive (left panel) or AnnexinV-FITC positive (right panel) NYC cells after stimulation with PBS (black bars) or IgM-antibodies (light gray bars) for 5 h. The data reflect the mean value of three independent experiments each performed in triplicates

spontaneous apoptosis is not statistically relevant (Figure 4b). In contrast, in the presence of anti-IgM-specific antibodies, +Notch1 NYC cells showed a significantly increased percentage of AnnexinV-positive, apoptotically dying cells as compared to uninfected wild-type or −Notch1 NYC cells (Figure 4a, lower diagrams and Figure 4b, right panel).

To verify the effect of BCR-crosslinking in the presence of Notch1 on cell death with a second approach, we determined the frequency of cells with fragmented DNA by the Propidium-Iodide (PI)/Triton method. At 24 h after the addition of PBS (negative control) or anti-IgM antibodies to NYC cells and NYC infectants, cells were first permeabilized with Triton X-100 and stained with PI. The percentage of cells with fragmented DNA was determined from relative cell numbers within the SubG1 gate. Figure 5a, which presents a typical result of an experiment performed 24 h after the addition of anti-IgM antibodies, illustrates a significantly increased number of PI-positive cells in the SubG1 phase (dead cells) of the cell cycle in +Notch1 NYC clones (Cl. 5 and Cl. 9) as compared to a representative −Notch1 NYC clone (Cl. 2) or wild-type NYC cells (wt). This clear increase in the frequency of dead cells occurs only in the presence of anti-IgM antibodies, that is, in the presence of a BCR-signal. PBS-treated +Notch1 NYC cells show only a slight increase in SubG1 phase cells as compared to -Notch1 or wild-type NYC cells. In summary, enhancement of BCR-induced cell death could be demonstrated in +Notch1 NYC cells with both the AnnexinV/YOPRO-3 and the PI/Triton method.

Influence of ectopic Notch1 on the cell cycle progression of NYC cells

Morimura *et al*.[35] have recently demonstrated that inducible expression of a constitutively active form of chicken Notch1 not only causes apoptosis but also a cell cycle arrest at the G1 phase in the chicken B-cell line DT40.[35] To analyze the effect of Notch1 on the cell cycle progression in NYC cells, wild-type NYC cells, a –Notch1 NYC clone and two +Notch1 NYC clones that differ in the level of human Notch1-ICT were stimulated for 24 h with PBS or with BCR-crosslinking anti-IgM antibodies to undergo apoptosis. Subsequently, cells were permeabilized and stained with PI, after which they were subjected to flow cytometric analysis in order to assess their DNA content. Figure 5a shows the PI-staining histograms of a typical experiment and Figure 5b summarizes the ModFit LT analyses of three independent experiments performed in triplicates. Considering cells in the G0/1, S and G2/M phase, the results in Figure 5b revealed no differences in cell cycle distribution of all analyzed cell populations, regardless of the presence and levels of Notch1-ICT.

The slight increase in numbers of cells in the G1 phase (Figure 5b, compare panels b and f) as well as the slight decrease of cells in the S phase (compare panels c and g) in a +Notch1/Cl. 9 culture treated with anti-IgM antibodies was not reproducible. Repetition of this experiment (+6 times) with +Notch1/Cl. 9 (low level of ectopic Notch1-ICT) and comparison with +Notch1/Cl. 1 (also low level of ectopic Notch1–ICT) did not confirm the observations for Cl. 9 (data not shown); therefore, we conclude that these variations are not statistically relevant and that a constitutively active Notch1 does not affect cell cycle progression.

To determine the rate of cell death, wild-type and infected NYC cells were cultured over a period from 12 to 96 h in the presence and absence of anti-IgM antibodies. Cells were removed at indicated time points and subjected to a PI/Triton staining. The percentage of dead/apoptotic cells in the SubG1 phase was determined as described earlier by flow cytometry. Figure 5c exemplifies the ratios of the analyzed NYC cells in the apoptotic SubG1 phase. Most of cell death occurred within 24 h after the addition of anti-IgM antibodies, and prolonged stimulation periods of up to 96 h caused only a limited additional increase of apoptosis. In addition, +Notch1 cells show a higher maximum in the frequency of dead cells than wild-type and –Notch1 NYC cells, suggesting that Notch1-ICT-positive NYC cells are more susceptible to apoptosis. Finally, when compared to wild-type and −Notch1 NYC control cultures, we observed an increased rate of cell death in anti-IgM-treated NYC cells expressing human Notch1-ICT; for example, 24 h after administration of the BCR-signal up to 70 % of +Notch1 NYC cells (Cls. 5 and 9) died, whereas 70% of cells were still viable in anti-IgM-treated control NYC cultures. These data indicate that a constitutively active Notch1 not only increases the rate of but also the susceptibility to BCR-induced apoptosis.

Influence of ectopic Notch1 on the growth of NYC cells

Apoptotic cell death is often associated with a cell cycle arrest and, thus, an inhibition of proliferation. In addition, the inducible expression of a constitutive active form of chicken Notch1 in avian Bursa of Fabricius derived cell lines has been shown to cause a cell cycle inhibition at the G0/G1 boundary of the cell cycle, which is accompanied by a significant growth suppression of the cells.[35] Since we did not detect an arrest of the cell cycle and found only a slight increase of spontaneous apoptosis in the absence of a BCR-signals in +Notch1 NYC cells, we would predict that −Notch1 and +Notch1 NYC cells show the same growth behavior. To determine the proliferation capacity of independent –Notch1 and +Notch1 NYC cell clones, we measured the number of cells over a period of 4 days. Cells were plated initially at low density (1×10^4) in triplicate. Cells were mixed with a defined number of fluorescence-labeled counting-beads, and absolute numbers were determined flow cytometrically. Figure 6a summarizes the cell counts of three independently performed experiments obtained at day 4. Although the average growth rate of the individual +Notch1 clones appears to be different, after comparing the mean of the cell counts of −Notch1 and +Notch1 NYC cell clones, it is apparent that there is no significant difference in the increase of cell numbers for all analyzed cell populations (Figure 6a).

Similar results were obtained when we measured the ^{3}H-thymidine incorporation rate of independent −Notch1 and +Notch1 NYC cell clones (Figure 6b). Again, no significant difference between the mean values of the ^{3}H-thymidine

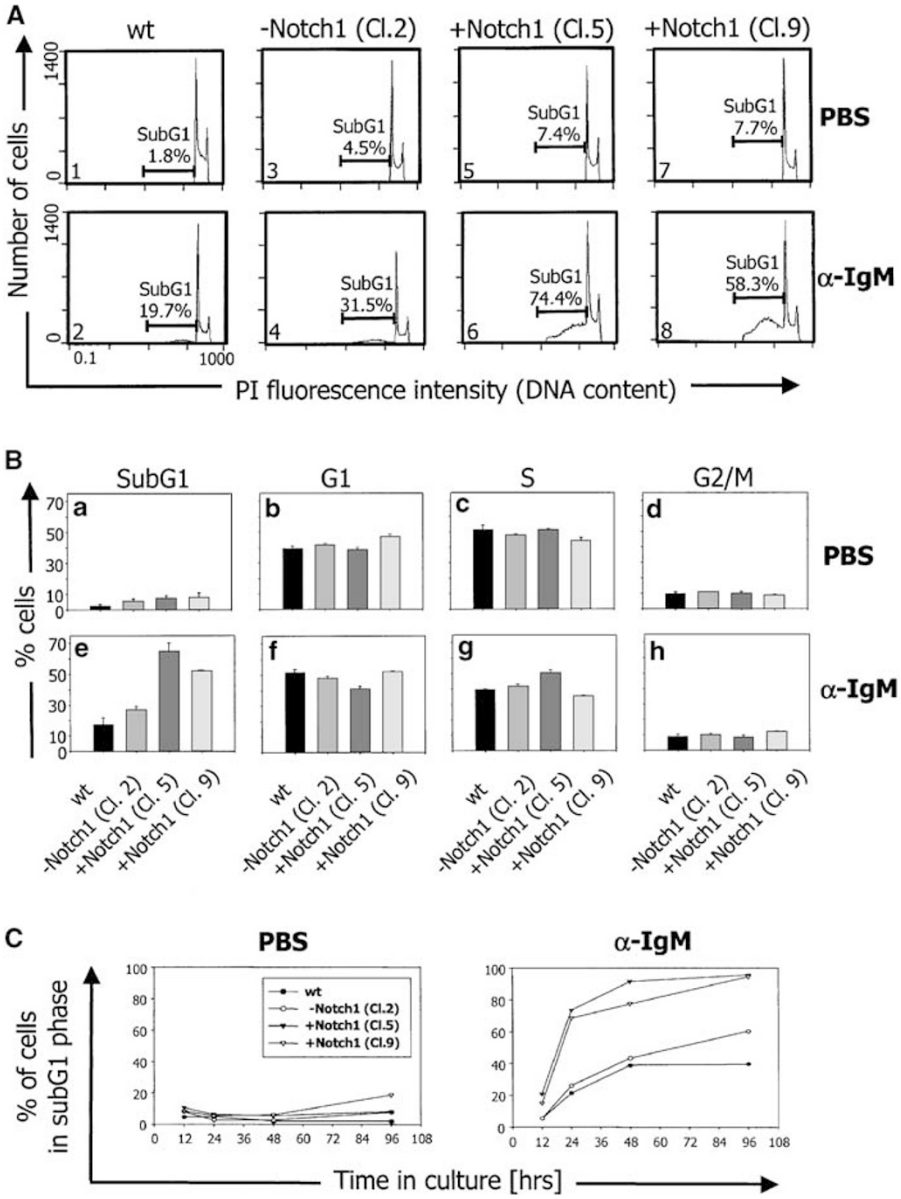

Figure 5 Effect of ectopic Notch1-ICT on cell cycle progression (A) Analysis of cell death via PI staining in wild-type, -Notch1 and +Notch1 NYC cells. Cells 2×10^5 of the indicated cell lines were stimulated for 24 h with 10 μg/ml anti-IgM-specific antibody or an equivalent amount of PBS. Subsequently, cells were harvested, permeabilized and stained with PI (50 μg/ml). Apoptotic cell death was assessed by flow cytometric analysis using the CellQuest software. Typical histograms are shown. Wild-type (panels 1 and 2), -Notch1 (Cl. 2) (panels 3 and 4) and +Notch1 cells (Cls. 5 and 9) (panels 5 and 6, and 7 and 8, respectively) were either treated for 24 h with PBS (panels 1, 3, 5 and 7) or with 10 μg/ml anti-IgM-specific antibody (panels 2, 4, 6 and 8). Dead cells are represented within the SubG1 gate. (**b**) Cell cycle distribution of NYC 31.1 cell lines after induction of apoptotic cell death. Cells 2×10^5 of the indicated cell lines were stimulated for 24 h with 10 μg/ml anti-IgM-specific antibody or an equivalent amount of PBS. Subsequently, cells were harvested, permeabilized and stained with PI (50 μg/ml). Cell cycle distribution was measured by flow cytometric analysis using the CellQuest and ModFit software. Panels a-d show the distribution of wild-type, −Notch1 and +Notch1 infected cell clones in the SubG1 (panel a), the G1 (panel b), the S (panel c) and the G2/M phase (panel d) of the cell cycle after stimulation for 24 h with PBS. Panels e-h show the distribution of wild-type, −Notch1 and +Notch1 infected cell clones in the SubG1 (panel e), the G1 (panel f), the S (panel g) and the G2/M phase (panel h) of the cell cycle after stimulation with a BCR-crosslinking antibody for 24 h. Black bars represent wt NYC cells, gray bars represent −Notch1 NYC cells (Cl. 2) and dark and light gray bars represent +Notch1 NYC cells (Cls. 5 and 9, respectively). (**c**) Kinetics of the SubG1 phase after induction of apoptotic cell death in NYC 31.1 cell lines. Cells 2×10^5 of the indicated cell lines were stimulated for different time periods (12, 24, 48, 60, 72, 84 and 96 h) with 10 μg/ml anti-IgM-specific antibody or an equivalent amount of PBS. Subsequently, cells were harvested, permeabilized and stained with PI (50 μg/ml). Apoptotic cell death was assessed as described in (a)

incorporation rate of the –Notch1 and +Notch1 NYC cell clones was observed. Curiously, the replication capacity of the individual clones does not correlate with their growth rate, regardless of ectopic Notch1-ICT expression. Since BCR-crosslinking causes apoptosis in NYC cells, it was not feasible to measure cellular growth rates in NYC cells after administration of the BCR-signal. Nevertheless, these data show that, in contrast to similar experiments performed in chicken DT40 cells, a constitutively active Notch1 does not affect cellular growth in the absence of a BCR-signal.

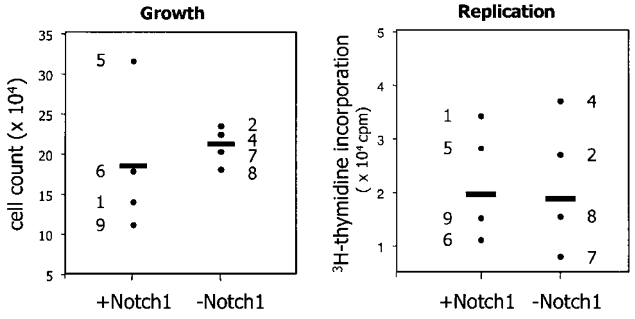

Figure 6 Effect of ectopic Notch1-ICT on cell growth. (**a**) Proliferation capacity of wild-type, −Notch1 and +Notch1 infected NYC 31.1 cells. Cells 1×10^4 per ml of four independent −Notch1 and +Notch1 NYC clones were plated in 24-well plates and cultured for 4 days. The number of cells was determined on days 0–4 as described in the Materials and Methods section. Measurements were performed in triplicates in a series of three independent experiments and a representative result of the analysis on day 4 is shown. The bold black bars represent the mean value of the 4 independently analyzed −Notch1 and +Notch1 NYC 31.1 clones. The numbers adjacent to the dots indicate the nomenclature of the analyzed –Notch1 or +Notch1 clones. (**b**) Measurement of the ^{3}H-thymidine incorporation rate of wild-type, −Notch1 and +Notch1 infected NYC 31.1 cells. Cells 1×10^4 per 200 μl of four independent −Notch1 and +Notch1 NYC clones were plated in 96-well plates and cultured for 4 days. At day 4, 1 μCi of ^{3}H-thymidine was added to each well and incubated for another 6 h. The incorporation of ^{3}H-thymidine was measured after fixation of the cells on filters in a β-counter with scintillation fluid. The measurement of the cell number was performed in triplicates and the mean values of this analysis are shown. The bold black bars represent the mean value of independently analyzed –Notch1 and +Notch1 NYC clones. The numbers adjacent to the dots indicate the nomenclature of the analyzed −Notch1 or +Notch1 clones

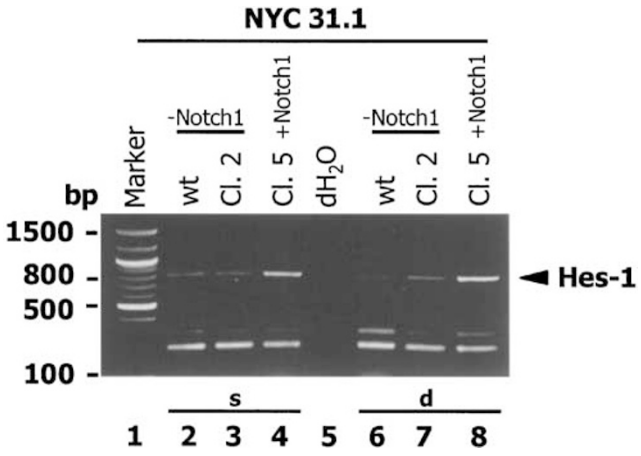

Figure 7 Effect of ectopic Notch1-ICT on Hes1 gene expression. At 24 h before RNA isolation, the indicated cell lines were seeded at a density of 2×10^5/ml (**s**) or 5×10^5/ml (**d**). Total cellular RNAs were extracted and reverse transcribed into cDNAs. PCR reactions were performed using Hes1-specific primers. One-half of each reaction was loaded on agarose gels

Influence of ectopically expressed Notch1-ICT on intracellular Notch signaling pathways

To investigate whether ectopically expressed Notch1-ICT activates Notch signaling pathways in NYC cells, we continued our investigations with the analysis of the expression pattern of Hes1, a Notch1-specific target gene. Hes1 encodes a basic helix–loop–helix transcription factor functioning downstream of the Notch1 receptor.[39,40] Using a RT-PCR approach, we analyzed the expression profile of the Hes1 gene in wild-type, −Notch1 and +Notch1 NYC cells. Since parental NYC cells synthesize endogenous Notch1 (Figure 1b), we expected to detect Hes1 gene expression in NYC cells, but only if the endogenous murine Notch1 is activated. As revealed by RT-PCR and illustrated in Figure 7, a low signal of a Hes1-specific band with the expected size of 850 bp was detected in wild-type (lanes 2 and 6) and –Notch1 infected NYC cells (Cl. 2; lanes 3 and 7), indicating that some of the endogenous murine Notch1 is active. In contrast, a much stronger signal for Hes1 mRNA was detected in +Notch1 NYC cells (Cl. 5; lanes 4 and 8), indicating that ectopically expressed Notch1-ICT is able to induce the expression of the Hes1 target gene and potentially activates intracellular Notch1 signaling pathways.

Influence of ectopic Notch1 on the IgM surface expression of NYC cells

Recently, it has been shown that stable expression of a constitutively active form of chicken Notch1 or Notch2 in the avian leukosis virus transformed B-cell line 249L4 resulted in a downregulation of surface IgM expression. This was also accompanied by the reduction of IgH transcripts, most probably because of a Notch1-mediated inhibition of the IgH enhancer activity.[36] Additionally, it was shown that Notch1–ICT negatively regulates IgM expression in Burkitt's lymphoma cell lines,[41] indicating that activated Notch1 is capable of participating in gene regulation in B-lymphoid cells. We therefore investigated if activated Notch1 participates in the regulation of the levels of surface IgM on NYC cells; hence, wildtype, -Notch1 and +Notch1 NYC cells were analyzed for surface IgM levels. Flow cytometric analysis with a FITC-conjugated anti-IgM antibody revealed equal surface IgM expression levels in NYC cells regardless whether activated Notch1-ICT was present (data not shown). This finding implies that an activated form of Notch1 is involved in the regulation of neither the IgH gene expression nor the transport of IgM to the surface of NYC cells, which further strengthens the point for distinct functions of Notch1 during B-cell differentiation.

Discussion

Notch1 appears to be a key regulator of lineage determination in the hematopoietic system, particularly in regard to the T- and B-cell lineages. Several recent publications have shown that Notch1 is involved in the regulation of cell fate decisions in the T-cell lineage,[26–31,42] but only little is known about the role of Notch1 during B-cell development as well as the differentiation processes and apoptotic cell death processes occurring therein. Interestingly, it was recently reported that in late B-cell stages and in some B-cell lymphoma, such as Hodgkin's lymphoma, Notch1 might exert antiapoptotic and proproliferative functions.[43] This survival promoting function of Notch1 appears to be also important for specialized B-cell subsets, such as marginal zone B cells.[44]

However, in immature and autoreactive B cells, Notch1 seems to impose a proapoptotic function. Morimura *et al*.[35] have shown that conditional expression of a constitutive active form of chicken Notch1 in the immature avian Bursa of Fabricius derived cell line DT40 not only induces apoptosis in these cells but also an arrest at the G0/G1 boundary of the cell cycle. Importantly, Notch1-induced apoptosis in DT40 cells is independent from any additional stimuli, such as BCR-crosslinking, which explains why DT40 clones with a stably expressed active form of Notch1-IC could not be obtained. Therefore, depending on the cell line, the constitutive synthesis of activated Notch1 can be disadvantageous or even toxic to cells.[35] In addition, Notch1 downregulates surface IgM expression, most probably because of a suppression of IgH gene expression.[36]

The toxic effects of a constitutively active Notch1 in immature chicken B-cell lines is also in accordance with our observation that repeated attempts to generate stable Notch1-ICT-positive clones by retroviral infection of the immature, murine B–lymphoid cell line WEHI 231[45] failed (S Romer, H-M Jäck and BM Jehn, unpublished data). Ectopic Notch1-ICT appears to be lethal for immature murine B–lymphoid WEHI 231 cells, most probably because of a Notch1-induced severe growth disadvantage or continuous spontaneous apoptosis. WEHI 231 and NYC 31.1 differ in the regard that WEHI 231 cells represent a classical immature B-cell line characterized by surface IgM expression, whereas NYC 31.1 cells represent mature activated B1-type-like cells, characterized by surface IgM and CD5 expression and IgM secretion.[37] Furthermore, the BCR in WEHI 231 cells does not translocate into lipid rafts following BCR-crosslinking, whereas the BCR of mature B cells can be found in these cholesterol- and sphingolipid-enriched membrane microdomains.[46] Therefore, the BCR of immature B-lymphoid cells signals outside from rafts, which results in a dramatic difference in the signaling in immature and mature B cells.[46] These differences between WEHI 231 and NYC 31.1 may account for the differential responses to ectopic Notch1.

To test the hypothesis that Notch1 regulates apoptosis in mature activated murine B cells, we infected the murine B-cell line NYC 31.1 with Notch1-ICT. In contrast to the studies performed by Morimura co-workers[35,36] with the chicken DT40 line, we observed that activated Notch1 clearly increases the susceptibility to and enhances the rate of cell death in the mature activated murine B-cell line NYC 31.1, but only after a BCR-signal has been initiated by crosslinking with anti-IgM antibodies. In the absence of BCR-crosslinking, only a low increase in spontaneous apoptosis of +Notch1 NYC cells was detected. Furthermore, it was evident that the enhancement of cell death by activated Notch1 is not associated with either a cell cycle arrest, a block in proliferation or a downregulation of surface IgM. Hence, it appears likely that the differential effects exerted by Notch1 in chicken and murine B cells are highly dependent on the cell type and the cellular microenvironment in which Notch1 is functioning.

Bursa of Fabricius derived B-lymphoid cells in birds undergo a distinct developmental pathway as compared to B-lymphoid cells derived from the bone marrow of mice and man. The bone marrow of mammals represents a site of B-cell lymphopoiesis, which is free of exogenous antigens. Murine immature B cells are eliminated in response to autoantigens to ensure the removal of potentially harmful autoreactive B-cell clones before migration to secondary peripheral lymphoid organs occurs.[10,11] Avian B–cell development proceeds differently: B-lymphoid precursor cells with a completely assembled BCR migrate into the Bursa of Fabricius, a gut-associated lymphoid tissue, where they are exposed to gut-derived exogenous antigens and change their antigen specificity by gene conversion.[47] B-lymphoid cells that either lose the expression of surface Ig complexes or create a self-reactive BCR as a result of the gene conversion process are eliminated via apoptotic cell death in the bursa.[48] It is believed that the surface Ig complex is responsible for basal signaling levels in the absence of exogenous receptor ligation.[47] Taking these differences in murine and avian B-lymphopoiesis into account, it is highly likely that Notch1 exerts different functions in different hematopoietic lineages.

Additional evidence that Notch1 functions in a cell type and developmental stage-specific way can be drawn from studies investigating the role of Notch1 in apoptotic cell death processes in T-cell hybridoma lines. Constitutive active Notch1 was demonstrated to inhibit apoptotic cell death in T-lymphoid cell lines only in response to TCR crosslinking.[32,33] TCR-independent stimuli to induce apoptosis did not result in a Notch1-specific inhibition of apoptosis.[33] For the T-cell hybridoma cell line DO11.10, activated Notch1 was shown to inhibit specifically Nur77-dependent, TCR-mediated forms of cell death. Also, ectopic Notch1 results in reduced activity of a Nur77 reporter gene, raising the possibility that Notch1 inhibits Nur77-dependent apoptosis through direct binding to Nur77 and a subsequent inhibition of Nur77 transcriptional activity.[33] In the context of T-cell-specific apoptosis, Notch1 exerts inhibitory effects most probably because of the interaction with Nur77, a transcription factor shown to be essential for thymocyte apoptosis.[49–51] Nur77, however, could not be detected in any B-lymphoid cell line or in *ex vivo* isolated B-lymphoid cell populations (U Saunders, H-M Jäck and BM Jehn, unpublished data), implying that Notch1 may interact with other and as yet unidentified B-lymphoid-specific transcription factors. Depending on the intra- as well as extracellular environment of a developing B cell, this hypothesized interaction may cause either an enhancement or inhibition of death in B-lymphoid cells. The finding that Notch1-signaling is required for the generation of marginal zone B cells, another B-cell subset, supports the contradicting actions of Notch1.[44]

It is tempting to speculate about the physiological role of Notch1 during B-cell development and the apoptotic cell death processes, which occur naturally during B-cell development and antigen-driven differentiation into effector cells, such as memory B and plasma cells.[10,11] Analysis of the mRNA and protein levels of Notch1 in different B-cell lines, representing different developmental stages of B-lymphoid cells and different *ex vivo* derived B-lymphoid cell populations, revealed that Notch1 mRNA and protein is detectable in all, so far analyzed cell lines and cell populations; thus, indicating a role for Notch1 during B-lymphopoiesis (Bertrand and co-workers[52,53] and U Saunders, H-M Jäck and BM Jehn, unpublished data). However, the sole presence of Notch1 does not

necessarily indicate that it is biologically active. Endogenous Notch1 might render cells susceptible to apoptotic stimuli provided by surrounding cells and tissues in murine bone marrow and peripheral lymphoid organs. External signals from the microenvironment together with intracellular signals from the BCR might also dictate the outcome of Notch1 signaling, that is, death or survival.

We also can provide evidence that Notch1 is capable of inducing Notch1-specific target gene expression in B cells. Analysis of the expression of Hes1, a basic helix–loop–helix transcription factor functioning downstream of the Notch1 receptor,[39,40] revealed that ectopically expressed Notch1-ICT is able to induce the expression of the Hes1 target gene and potentially activate intracellular Notch1 signaling pathways. In addition, low levels of Hes1 transcripts could be detected in −Notch1 or wild-type NYC cells, indicating that a small amount of the endogenous murine Notch1 might be present in a active from. This further supports the idea that Notch1 signaling occurs in mature B cells. Further analysis will provide a detailed insight into Notch1-dependent signaling networks in B cells.

Besides the direct influence of Notch1 on gene transcription via Hes1, we also have to consider that some of our observation are mediated by an interaction of Notch1 with other intracellular factors like NF-κB. Three recent publications have studied the role of Notch1 and its regulatory capacity on the transcription factor NF-κB. Wang *et al*.[54] demonstrated in transient transfections assays that overexpressed Notch1 and NF-κB are tightly interconnected, since an N-terminal portion of the intracellular domain of Notch1 is capable of inhibiting NF-κB-dependent gene expression. Furthermore, NF-κB regulates Notch1 signaling through inducing the expression of the Notch1-specific ligand, Jagged1.[55]. In contrast, however, using a Notch1 antisense transgenic mouse model (Notch1-AS-Tg), Cheng *et al*.[56] reported that DNA binding of NF-κB and its ability to induce gene expression was strongly impaired in Notch1-AS-Tg mice as compared to wild-type mice,[56] indicating that physiological amounts of Notch1 have a positive effect on NF-κB activity. Accordingly, precise physiological levels of active Notch1 are critical for exerting its specific functional effects. Even subtle changes in the balance between Notch1 and cognate transcription factors seem to be sufficient to cause severe cellular responses, including impaired proliferative capacity, diminished differentiation and also increased apoptotic cell death.

Ectopic Notch1 might disturb a tightly regulated balance of endogenous Notch1 and associated pro- and antiapoptotic factors, resulting finally in increased levels of cell death in B-lymphoid cells. However, the ability of Notch1 to render NYC cells more susceptible to apoptotic cell death seems not to be dependent on levels of activated Notch1. For example, NYC cells with low levels of ectopic Notch1-ICT synthesis (Cls. 1 and 9) show similar enhanced rates of apoptotic cell death as compared to NYC clones with high levels of ectopic Notch1-ICT synthesis (Cls. 5 and 6). Therefore, it seems that the effects of Notch1 we observed in NYC cells are a consequence of rather the differentiation stage of NYC cells than that of Notch1 levels. Further support comes from the observation that endogenous Notch1 levels do not change in NYC cells after a BCR signal has been delivered (Figure 1b) and that stable Notch1 infectants of WEHI 231 cells could never be obtained (data not shown). If the amount of Notch1 in WEHI cells would be critical to induce 'toxic' effects, we would have expected to find at least some WEHI clones with a low expression of transduced Notch1. In this context, it will be of particular interest to identify all relevant transcriptional regulator proteins mediating Notch1-specific effects in B-lymphoid cells. Investigations to identify these transcriptional partners of Notch1 in B cells are currently underway.

Materials and Methods

Cell culture and subcloning

The lymphoid cell line NYC 31.1[37] was cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (Gibco), 2% L-glutamine (Gibco), 1% sodium-pyruvate (GIBCO), 2% penicillin/streptomycin (GIBCO) and 0.1% β-mercaptoethanol (GIBCO). Infected cells were maintained in medium additionally supplemented with 1 mg/ml G418 (Pan Biotech). Single-cell clones were generated out of cell pools using the limiting dilution method. A total of 150 cells were diluted in 100 ml medium and plated on 96-well plates. After 10 days in culture, the plates were screened for clones clearly deriving from single cells. These clones were marked and further expanded.

Transient transfection of BOSC 23 cells

The BOSC 23[38] cell line was cultured in DMEM medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2% L-glutamine (GIBCO), 1% sodium-pyruvate (GIBCO) and 2% penicillin/streptomycin (GIBCO). The retroviral construct pGD-ICT[33,38] and the control retroviral construct MSCV 2.1[33,38] were transiently transfected into BOSC 23 packaging cells. The transfections were performed in six-well plates using the SuperFect reagent (Qiagen) with a total amount of 5 μg DNA/well. Supernatants 24 h post-transfection were discarded and cells were transferred to RPMI medium. The 48 and 72 h supernatants were finally collected for retroviral infection. At 72 h after transfection, whole-cell lysates were prepared and analyzed on Western blots for Notch1-ICT synthesis.

Retroviral infection of NYC 31.1 cells

Retroviral infections of NYC 31.1 cells were performed using the centrifugation method. In brief, 1×10^6 cells were harvested and resuspended in 4 ml retroviral supernatant. After adding 1 ml fresh medium plus 10 μg/ml heat-inactivated polybrene (Sigma), the mixture was centrifuged for 3.5 h at 1671 $\times$ *g* at 33°C in order to achieve close contact between the retroviral particles and the cells. Finally, cells were cultured in fresh medium and 24 h postinfection, G418 selection (1 mg/ml) was initiated.

Western blot analysis and immunoprecipitations

For preparation of whole-cell lysates, 1×10^6 cells were lysed in NP-40 lysis buffer containing 1 $\times$ PBS, 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS and the protease inhibitors aprotinin (15 μg/ml) (Sigma), leupeptin (5 μg/ml) (Sigma), pepstatin (10 μg/ml) (Sigma), Pefabloc (500 μg/ml) (Boehringer-Roche) and sodium-orthovanadate (1 mM) (Sigma). Lysates were separated on 8% denaturing SDS-PAGE gels

and the proteins were transferred to PVDF membranes (Schleicher & Schuell). To detect ectopically expressed or endogenous murine Notch1, the membranes were probed with a goat anti-human or –murine Notch1 antibody (C-20 and M-20) (Santa Cruz), respectively. Signals were visualized by a horseradish-peroxidase (HRP) coupled donkey anti-goat secondary antibody (Santa Cruz) followed by enhanced chemiluminescence (ECL) and autoradiography (Amersham Pharmacia). To assess loading, a rabbit anti-Actin antibody (Sigma) in combination with a HRP-coupled donkey anti-rabbit antibody (BioRad) were used. Again, signals were visualized using an ECL detection system (Amersham Pharmacia).

For immunoprecipitations, 1×10^7 cells were harvested and lysed in 750 μl lysis buffer. Lysates were cleared by centrifugation and mixed with 5 μg of an precipitating, polyclonal Notch1 antibody, generated by immunization of rabbits with a peptide corresponding to amino acids 1766–1780 of the mouse Notch1 protein (Jehn *et al.*, unpublished data). After incubation for 2 h on ice, 50 μl of protein G sepharose beads (Pierce) were added to each sample and incubated over night at 4°C with gentle agitation. Beads were washed four times, resuspended in $1 \times$ SDS-loading buffer and loaded immediately on denaturing 8% SDS-PAGE gels. Proteins were transferred and specific signals were visualized as described above.

RT-PCR analysis

Cells 1×10^7 were harvested and total cellular RNAs were isolated using the RNeasy-Mini protocol (Qiagen). RNAs were reverse transcribed into cDNA with the SuperScriptII-Polymerase (GIBCO). Resulting cDNAs were amplified using the Deep-Vent DNA Polymerase (Biolabs) and specific Notch1-ICT primers (5′-CTGAGGGCTTCAAAGTGTCTG-3′ and 5′-CAT-ATCTTTGTTAGCCCCGTTC-3′) (MWG Biotech) or for control reasons, with specific hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) primers (5′-GCTGTTGAAAAGGACCTCT-3′ and 5′-CACAGGACTAGAA-CACCTGC-3′) (MWG Biotech). The Notch1-ICT primers specifically recognize the ectopically expressed form of human Notch1 and not the endogenous murine form of Notch1. PCR products were separated on 0.8% 1X TAE agarose gels and visualized by ethidium bromide staining. To analyze Hes1 expression, cDNAs were amplified using the *Taq* DNA Polymerase (Biolabs) along with Hes1 primers specific for the endogenous murine form (5′-CGAAAATGCCAGCTG-3′ and 5′-GATATCGAGGCTCT-CAGTTCCG-3′) (MWG Biotech). PCR products were separated on 1.0% 1X TAE agarose gels and visualized by ethidium bromide staining.

Induction and assessment of apoptosis

In 1 ml R10 medium, 2×10^5 cells were plated on 24-well plates in triplicate and induced to die apoptotically with 10 μg anti-mouse-IgM $F(ab)_2$-fragment (i.e., anti-IgM antibodies) (Jackson Dianova). PBS as well as isotype-matched control $F(ab)_2$ fragments were used as negative controls. Apoptosis was evaluated in the flow cytometer (EPICS XL3/ Coulter) 24 h postinduction by staining cellular DNA contents with hypotonic PI solution, containing 50 μg/ml PI (Merck), 0.1 % sodium-citrate (Sigma) and 0.1% Triton X-100 (Sigma). To calculate the percentages of cells in the individual cell cycle phases, the results were analyzed with the ModFit LT software (Becton-Dickinson). The cell death rate was determined as percentage of cells in the SubG1 phase.

Alternatively, apoptosis was assessed 5 h postinduction by a combined AnnexinV-FITC/YOPRO–3-Iodide staining according to the manufacturer's instructions (Becton-Dickinson). The measurement was performed in the flow cytometer (FACS-Calibur/ Becton-Dickinson) using the CellQuest software (Becton-Dickinson).

Analysis of cell growth

In 1 ml R10 medium, 1×10^4 cells were plated on 24-well plates in triplicate and cultured for 4 days. Every 24 h, cells were collected and resuspended in 200 μl PBS. An equivalent amount of fluorescence-labeled beads (Coulter) was added and the mixture was analyzed in the flow cytometer (EPICS XL3/ Coulter). The absolute number of cells was determined by calculating the number of cells in relation to the number of counted beads.

^{3}H-thymidine incorporation assay

In 200 μl R10 medium, 1×10^4 cells were plated in triplicate and were cultured for 4 days. On day 4, 1 μCi ^{3}H-thymidine was added to each well and incubated for 6 h. Cells were finally harvested and fixed on filters. The incorporation of ^{3}H-thymidine was determined by measuring the filters with the fixed cells in a β-Counter in the presence of scintillation fluid.

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