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Differential Expression of Neurotensin and Specific Receptors, NTSR1 and NTSR2, in Normal and Malignant Human B Lymphocytes

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Neurotensin, a 13-aa neuropeptide, was first identified in the central and peripheral nervous system and gastrointestinal tract (1). Initially isolated from bovine hypophalamus, neurotensin is present in several species including human. It is synthesized from a common precursor in mammalian brain and intestine (2). In the brain, neurotensin is exclusively located in nerve terminal fibers and cells (3). Peripheral neurotensin in the endocrine N cells is mostly located in the intestinal mucosa (4).

Three neurotensin receptors (NTSRs) have been identified and cloned from rat, mouse, and human cells (5). They include two G protein–coupled receptors: the high-affinity receptor NTSR1 and the low-affinity receptor NTSR2 (6). NTSR1 expression was recently demonstrated in aggressive malignant solid tumors such as mesothelioma (7), non–small-cell lung (8), breast (9), and head and neck squamous (10) carcinomas. NTSR2 shares 60% homology with NTSR1 (6). Its expression in cancer was rarely reported, except in patients with prostatic cancer (11). The third receptor, NTSR3 or sortilin, common to neurotrophins and neurotensin, is not a G protein–coupled receptor. It belongs to the Vps10p intracellular protein family (12) and was originally described as able to transport immature and mature neurotrophins in the CNS, and to lead to axonal growth and neural cell proliferation (13, 14). NTSR3 is predominantly located in endoplasmic reticulum/Golgi compartment. A minority of NTSR3 is also expressed at the plasma membrane as a coreceptor for p75NTR. It binds pro-neurotrophins, thus activating apoptotic pathways (15, 16), as previously described in human B lymphocytes, as well as in B cell lines independently of their maturation stages (17).

Neurotensin exhibits a wide variety of biologic actions in both peripheral tissues and CNS. Its major action in the brain is the regulation of dopaminergic system (18). In the periphery, it exerts endocrine and paracrine actions, especially in the homeostasis of intestinal functions (19). Neurotensin is also implicated in tumor progression, including proliferation of various types of human cancer lines (colon, prostate, lung, pancreas, breast), especially through its ligation to NTSR1 (20–24).

A few studies reported the involvement of neurotensin in inflammation through mast cell activation (25–28), cytokine (IL-8 and IL-6) production (29–31), neutrophil chemotaxis (32), and NO generation (33).

Neurotensin expression was not previously described in B lymphocytes. However, we have previously characterized NTSR3 (sortilin) in human B lymphocytes. Neurotrophins, especially brain-derived neurotrophic factor (BDNF) and another neuropeptide, neurotensin, share this receptor (17). In B cell lines, sortilin exhibits a dual function: 1) the transport of BDNF that leads to an autocrine B cell survival loop by the activation of its tropomysin-related kinase B receptor; and 2) the function of coreceptor for p75NTR, in the binding of the immature proapoptotic form of BDNF (17). In B cell lines, this proapoptotic function was inhibited by another sortilin ligand, neurotensin, as previously described in neural cells (16). A similar dual function of sortilin was recently reported in NK cells (34).
Some data are available on neurotensin and NTSR expression by dendritic cells. Mouse cutaneous dendritic cell line expressed NTSR1, NTSR3, and neurotensin, which downregulated IL-6, TNF-α, and IL-10 secretions (35). As for T lymphocytes, the binding of neurotensin on the membrane of Sézary cells, several T cell lines and healthy peripheral T lymphocytes, was previously described (36). It leads to the modulation of IL-2–induced proliferation through the binding to membranous NTSR1, which is expressed by T cell lines (37). The presence of another unidentified NTSR was also suggested on both T cell line and peripheral lymphocytes (36). In addition, NTSR receptor was described by ligand-binding analysis on the EBV+ Burkitt cell line RAJI, but RT-PCR analysis did not identify this receptor as NTSR1 (38).

Neurotensin exhibits proliferative or antiproliferative functions on immune cells depending on the cytokine environment. Indeed, neurotensin enhanced proliferation of Molt-4 and Jurkat T cell lines stimulated with PHA (36); by contrast, neurotensin inhibited IL-2–induced proliferation of Sézary cells (37).

Data concerning NTSR expression on both B and plasma cells were previously focused on NTSR3 expression. Indeed, an autoimmune regulation linked to sortilin was characterized in human B cell lines, and a production of this endogenous protein was also shown in normal B cells (17). Implication of neurotensin in cell proliferation was clearly established in solid cancer cells, but, to our knowledge, its potential role in B cells was not investigated.

We hypothesized that neurotensin and its two specific receptors, NTSR1 and NTSR2, could also be expressed in B lymphocytes. We therefore studied human B cell lines at different stages of maturation. The functional properties of NTSR1 in B cell lines were studied. Therefore, the expression of neurotensin and its two specific receptors was evaluated by quantitative RT-PCR (qRT-PCR) in malignant human B cell diseases and compared with healthy peripheral resting and activated B cells or other nonhematopoietic malignant or benign lymph nodes.

**Materials and Methods**

**Human B cell lines**

The studied B cell lines at different maturation stages were pro-B cells (RS4; 11 from acute leukemia), pre-B cells (Nalm6 from acute lymphoblastic leukemia), mature B cells (BL2 and BL41, EBV–Burkitt lymphoma cell lines), and plasma cells (the myeloma cell lines RPMI 8226 and U266). They were kindly provided by K. Lassoued (Amiens, France) and EBV negativity was confirmed by PCR (performed by S. Ranger-Courbat, Pont de Claix, France) by Ficoll-Paque density centrifugation (Eurobio, Courtaboeuf, France). In brief, PBMCs were isolated after centrifugation at 40 ml samples of venous blood obtained using heparinized Vacutainer cells preparation tube (Becton Dickinson, Le Pont de Claix, France) by Ficoll–Paque density centrifugation (Eurobio, Courtabouef, France). In brief, PBMCs were isolated after centrifugation at 280 × g for 30 min. B lymphocytes were then isolated by positive selection with either CD19 magnetic beads or B Cell Isolation Kit II Negative selection kit (all MACS; Miltenyi Biotec, Paris, France) according to manufacturer’s protocols. This procedure resulted in >90% CD19+ cells as determined by flow cytometric analyses using PE cyanine 7–conjugated anti-CD19 Ab (Beckman Coulter, Villepinte, France).

**Lymph nodes from patients with diffuse large B cell lymphoma, colonic carcinoma metastases, or nonmalignant disease**

Frozen lymph nodes were obtained from seven patients with diffuse large B cell lymphomas and six patients with metastatic colonic adenocarcinoma. Benign lymph node from three sarcoïdosis patients were used as controls. They were obtained from the Tumor Bank of Limoges University Hospital, under protocols approved by the Institutional Review Board (AC N: 2007-34, DC 2008-604, and 72-2011-18).

**Cell culture**

Cells lines were cultured in a humidified 5% CO2–95% air incubator in 75-cm² flasks (Nunc, Fisher Bioblock Scientific, Illkirch, France) at 37°C in RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 10% FCS and 1% antibiotic-antimycotic mixture, sodium pyruvate, and penicillin-streptomycin (Life Technologies). All cell lines were maintained between 1 × 10⁶ and 2 × 10⁶ cells/ml, and medium was replaced every 2-3 days.

Peripheral normal B cells were cultured in 96 round-bottom plates (Nunc) at 2 × 10⁵ cells/ml in 20% FCS-containing RPMI1640 medium supplemented as described earlier. Normal B cells (2 × 10⁶ cells/ml) were stimulated with affinityPure goat anti-human IgA-IgG-IgM (H+L) Fab(5/7 fragments (10 μg/ml); Jackson Immunoresearch, West Grove, PA), and polyclonal anti-human soluble CD40L (10 ng/ml), B cell–activating factor (10 ng/ml), and IL-4 (0.2 ng/ml; all from PeproTech, Rocky Hill, NJ) for 24 h. Cultured cells were stressed by 24–168 h serum deprivation, a 24-h exposure to anti-Fas agonistic mAb (100 ng/ml; clone 7C11, sodium azide free; Beckman Coulter), or exposed for 24 h to 40 μM exogenous neurotensin (Calbiochem, La Jolla, CA). The optimal neurotensin concentration was determined by colorimetric 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) viability assay (Roche Diagnostic), according to the manufacturer’s instructions. In apoptotic experiments, anti-NTSR1 antagonistic Ab (clone B-N6, sodium azide-free, 50 nM; Diaclon) was added to the cultures during 24 h (39).

Phenotypical assays were performed with SRA6692, a synthetic inhibitor, also known as meclinchetin (Axon Medchem, Groningen, The Netherlands), with a high specificity for NTSR1 (40) at the optimal concentration of 8 μM. A pseudopeptide analog of neurotensin VlM449 (8-13; Toecris Biosciences, Bristol, U.K.) was also evaluated in same experiments at the optimal concentration of 40 μM for plasmacytic cell lines and 60 μM for B mature cell lines (41).

**RNA extraction and reverse transcription**

Total RNA was extracted with Qiagen RNeasy isolation system (Qiagen, Hilden, Germany), treated with RNA-free DNase I (Qiagen), and quantified by NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies, Wilmington, DE) at the absorbance ratio of 260 and 280 nm. The RNA quality was evaluated on Agilent 2100 bioanalyzer using the RNA 6000 Labchip kit (Agilent Technologies, Palo Alto, CA). cDNA synthesis was performed with high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), as recommended by the manufacturer.

**RT-PCR, real-time PCR analyses, and sequencing**

cDNA of neurotensin, NTSR1, NTSR2, and sortilin, was used for conventional RT-PCR. cDNA were amplified for PCR with Platinum TaqDNA polymerase (Invitrogen, Cergy Pontoise, France) using the primers shown in Table I in UnoCycler (VWR, Fontenay-sous-Bois, France). Total RNA isolated from human colon adenocarcinoma cell line (HT29) was used as positive control for neurotensin (42), NTSR1 (43), and NTSR2 expressions (44), and verified by sequencing. Thirty-five PCR cycles were performed (94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and an extension step of 72°C for 7 min).

Real-time PCR was performed with cDNA by means of TaqMan probes, labeled with 3′ reporter dye 6FAM and the 3′ Minor Groove Binder-Non-Complementary Quencher (Applied Biosystems). The quantitative PCR efficiency was assessed using regression curve performed with cDNA serial dilutions samples (400, 200, 100, 50, and 25 ng cDNA) (45).

Experiments were performed in duplicate in at least three independent experiments using 400 nM of the forward and reverse primers, 200 nM labeled probe, and 100 ng cDNA template (RNA-cDNA equivalent) per reaction mixed with TaqMan Fast-Univ Master Mix (Applied Biosystems). PCR runs were analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with a two-step PCR protocol (95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s). The data were normalized to HPRT gene as an internal control (Human HPRT Primer/Probe Mix; Applied Biosystems). The comparative ΔCt method was used for relative quantification of gene expression on duplicate of each reaction.

After extraction and purification of PCR products with QIAquick Gel Extraction kit (Qiagen), 700 ng of the manufacturer’s instructions for sequencing PCR was performed with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and sequences were analyzed on an automated laser fluorescent DNA sequencer (ABI Prism 3130xl Genetic Engineering System).
Analyzer: Applied Biosystems). Homologies were checked after blasting with human neurotensin: NTSR-1, NTSR-2 GenBank sequences (NM_006183; NM_002531.2 and NM_012344.3).

**Western blot analysis**

Proteins were obtained from cell lysates of B cell lines cultured in both normal (containing 10% FCS) and serum-free medium, and from resting circulating normal human B cells. After two washes in PBS, cell lysates were prepared using lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitors mixture set III [2 μg/ml; Calbiochem], 1 mM NaF, and 1 mM Na3VO4) and a 10-min centrifugation at 14,000 × g.

Equal amounts of proteins from cell lysates (50 μg/lane) were separated on 10–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) under denaturing conditions and transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France). Nonspecific binding sites were blocked for 2 h with 5% nonfat dry milk in TBS. After overnight incubation at 4°C with specific Ab (dilution 1/200), membranes were incubated...

**FIGURE 1.** (A) Transcription of neurotensin (NT), its two G protein receptors (NTSR1, NTSR2), and sortilin by B cell lines cultured with 10% FCS (basal conditions) and 0% FCS (stress condition). The colic adenocarcinoma cell line HT29 was used as a positive control. Constitutively expressed 18S is a control of PCR efficiency. (B and C) qRT-PCR analysis of neurotensin (NT), NTSR1, and NTSR2 expression in RS4, Nalm6, BL2 (B) BL41, U266, and RPMI (C) B cell lines cultured in 72-h serum starvation (0% FCS, white histograms) compared with basal condition (10% FCS, black histograms), normalized with HPRT gene expression. The p values were determined in comparison with standard condition (10% FCS). Only significant results are indicated.
with HRP-conjugated secondary Ab to mouse, rabbit, or goat Ig (Santa Cruz; dilution 1/1000) for 60 min at room temperature and revealed by chemiluminescence (ECL reagent; Amersham Life Science). Protein-loading control was performed with anti-GAPDH or anti-actin Ab (Santa Cruz Biotechnology, Heidelberg, Germany). Western blots were scanned using a bioimaging system (Genesnap; Syngene).

Flow cytometry analysis

Expression of the three NTSRs by B cell lines was studied by flow cytometry with or without permeabilization. After a 30-min incubation at room temperature with rabbit anti-NTSR1, anti-NTSR2, and goat anti-NTSR3 Ab (all 1/200; Santa Cruz Biotechnology) in PBS containing 1% BSA followed by two washes in PBS, Abs were revealed using Alexa Fluor 488–conjugated goat anti-rabbit IgG Ab (10 mg/ml; Invitrogen) for 30 min at 4˚C. Cells stained with rabbit isotypic Ig (Santa Cruz Biotechnology) were used as controls to determine background and positivity thresholds. After washing twice in PBS, cells were suspended in PBS and analyzed with a flow cytometer (FACSCanto II).

Immunocytochemical staining

After two washes in PBS, the cells were fixed with frozen 90% acetone, washed twice in PBS, and incubated for 1 h in PBS containing 5% goat serum. Cells were then incubated overnight at 4˚C with primary Ab diluted in PBS-BSA. The following Abs were used: rabbit polyclonal anti-NTSR1, anti-NTSR2 (1/200; Alomone Labs, Jerusalem, Israel), goat anti-NTSR3/Sortilin (1/100; R&D Systems, Lille, France), and rabbit anti-neurotensin (1/200; Santa Cruz Biotechnology). Cells were washed twice in PBS and incubated with 1 μg/ml Alexa Fluor 488– or 596–conjugated anti-rabbit or anti-goat IgG Ab (Invitrogen) for 30 min at room temperature. After two washes in PBS, cells were mounted in glycerol-gelatin medium (Sigma-Aldrich) and studied using a fluorescence microscope (Leica). Negative controls were cells incubated with irrelevant normal rabbit or goat IgG (Santa Cruz Biotechnology).

Proliferation assay

Proliferation was determined by the method of click-it EDU flow cytometry assay kits (Invitrogen), which used a modified nucleoside, 5-ethynyl-2'-deoxyuridine (EdU), that is incorporated during DNA synthesis. Detection is based on a copper-catalyzed covalent reaction revealed with Alexa Fluor 488 dye; then flow cytometry analysis was performed to identify percentage of cells in S-phase (FACSCanto II). Each experiment was repeated at least three times.

Apoptosis assay

Apoptosis was evaluated by measurement of cytoplasmic soluble nucleosomes determined following the manufacturer’s instructions of ELISA Cell

Table I. Forward primers, reverse primers, and probe used in qRT-PCR studies for the expression of human neurotensin and NTSR

<table>
<thead>
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<th>Names</th>
<th>Sequences</th>
<th>Length (bp)</th>
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<td>113</td>
<td>NM_002531.2</td>
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<tr>
<td>R</td>
<td>TACGTCCGCAAGCATTCAA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Probe</td>
<td>ACAGGCGTTGCCTGTCTGCTG</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>114</td>
<td>NM_012344.3</td>
</tr>
<tr>
<td>R</td>
<td>CCAAGGTGGTGCTGCTGCTA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Probe</td>
<td>ACTAACTGCTTCTGCAATAGCTGCACTG</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>—</td>
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<tr>
<td>Probe</td>
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<tr>
<td>R</td>
<td>CGCTTACCCACATTCAGGAGG</td>
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F, Forward; NT, neurotensin; R, reverse.

FIGURE 2. Western blot analysis of whole-cell lysates for NTSR1, NTSR2, and NTSR3 in all studied cell lines in basal (10% FCS) (A) and in serum deprivation (0% FCS) (B) conditions. Isoforms of NTSR1 receptor depend on culture conditions in all studied cell lines (C).
Death kit (Roche) (17). In brief, cells were seeded in 96-multiwell plate (5 × 10^4 cells/well) and cultured for 24 to 168 h in serum deprivation alone or with recombinant neurotensin (40 μM). Absorbance values were measured at 405 nm with an ELISA reader (Labsystems). The absorbance obtained in controls was normalized to a value of 1, as previously described (46). Every experiment was performed three times independently.

Data analysis
Data are presented as mean values ± SEM of at least three independent experiments. To determine statistical significance of difference versus control, one-way ANOVA was used. The p values <0.05 were considered statistically significant.

Results
Neurotensin and NTSR transcripts and proteins are expressed in human B cell lines
Expression of neurotensin and NTSR in human B cells lines was performed by RT-PCR in comparison with HT29 line (Fig. 1A) (Table I). Under basal (10% FCS-containing culture) or starvation conditions, NTSR1, NTSR2, and NTSR3 transcripts were detected in all B cell lines whatever their maturation stages (Fig. 1A). By qRT-PCR, a significant decrease of NTSR1 transcription was detected after a 72-h serum deprivation in Nalm6 and RPMI cell lines (p = 0.0002 and p < 0.0001, respectively; Fig. 1B, 1C), whereas it was enhanced in U266 cells (p = 0.02; Fig. 1C). NTSR2 transcription levels were also diminished by a 3-d serum starvation in RS4, Nalm6, and BL2 cell lines (p = 0.0002, p = 0.03, and p = 0.02, respectively; Fig. 1B) but increased in U266 (p = 0.02; Fig. 1C). By contrast, neurotensin transcription, detected in 10% FCS-culture condition in all cell lines, was unchanged by serum deprivation (Fig. 1B, 1C).

Sequencing of B cell line transcripts eluted from agarose gels showed the expected sequences of human neurotensin, NTSR1 and NTSR2, thus confirming their expression in human B cell lines.

Western blotting analysis identified two NTSR1 protein isoforms depending on culture conditions. Under basal condition, a 56-kDa form was present in the control HT-29 cell line, whereas a lower 54-kDa form was observed in all B cell lines (Fig. 2A, 2C). However, in deprivation condition, only the 56-kDa NTSR1 protein was identified in the B cell lines and control (Fig. 2B, 2C). A NTSR2 protein was also identified in all B cell lines whatever the culture conditions (Fig. 2A, 2B). We confirmed the presence of NTSR3 protein in all B cell lines independently of culture conditions, as previously described (17) (Fig. 2A, 2B).

FIGURE 3. (A) Intracytoplasmic expression of NTSR1 and NTSR2 in mature (BL41) and pre-B (Nalm6) cell lines. Similar staining of NTSR1 and NTSR2 were obtained with the four other cell lines (data not shown). (B) Neurotensin expression in plasma cells (U266) cultured with (10%; left panel) and without (0%) FCS. Rabbit IgG isotype controls staining with goat anti-rabbit Alexa Fluor 488–conjugated Ab (right panels). (C and D) FACS analysis of membranous NTSR1 (white histograms), NTSR2 (gray histograms), and NTSR3 (spotted histograms) expression on unpermeabilized pre-B (Nalm6) (C), mature B cells (BL41) (C) and plasmocytes (RPMI) (D) in standard conditions (10% FCS) and after 24 h of serum deprivation (0% FCS) conditions. Histograms are means ± SEM of three independent experiments. The p values were determined in comparison with standard condition (10% FCS). Only significant results are indicated. (E) Flow cytograms of membranous NTSR1 expression in BL41 cell line cultured under standard conditions (10% FCS) or after a 24-h serum starvation (0% FCS). Cells are stained with isotypic controls (left graphs) and anti-NTSR1 Ab (Alexa Fluor 488; right graphs). Cytograms are representative of three independent experiments. Similar stainings are obtained for other cell lines and NTSR2 and NTSR3 expression.
Immunofluorescence studies showed NTSR1 and NTSR2 expression in all B cell lines under both culture conditions (Fig. 3A). In contrast, neurtensin was detected only in mature B and plasma cells (Fig. 3B) with a polarized staining enhanced after a 3-d serum deprivation (Fig. 3B).

Membranous expression of NTSR1 and NTSR3 is induced by serum deprivation in human B cell lines

The membranous expression of the three NTSRs was analyzed by flow cytometry. NTSR3 membranous expression was restricted to proapoptotic conditions (serum starvation) as previously described (17). Similarly, deprivation culture induced a clear-cut membranous expression of NTSR1 on pre-B, mature B, and in plasmacytic cell lines (all \( p < 0.05 \); Fig. 3C–E). In contrast, NTSR2 expression was expressed on the membrane of 40–50% of the mature and plasmacytic cells cultured with 10% FCS, with no significant modification by proapoptotic conditions (Fig. 3C, 3D).

Exogenous neurotensin promotes proliferation and prevents apoptosis of human B cell lines

Because neurotensin and specific receptors were detected in human malignant B cell lines, we searched for their potential functions under the different culture conditions. Although NTSRs were expressed under basal culture condition, a 24- to 48-h incubation of the lines with exogenous neurotensin did not modify cell proliferation, whatever the maturation stage (pre-B, B mature, or plasmacytic lines; Fig. 4A).

**FIGURE 4.** Neurotensin induced B cell proliferation under deprivation conditions. (A) Percentage of proliferative cells determined with EdU incorporation by flow cytometry on pre-B (Nalm6), mature B (BL41), and plasma (RPMI) cells cultured 24 (D1) to 48 (D2) h with (gray histograms) or without (white histograms) neurotensin and 10% FCS. (B) Percentage of proliferative cells determined with EdU incorporation by flow cytometry on pre-B (Nalm6), mature B (BL41), and plasma (RPMI) cells cultured 24 (D1) to 72 (D3) h with (gray histograms) or without (white histograms) 40 \( \mu \)M neurotensin and 0% FCS. The \( p \) values were determined in comparison with serum-free condition alone (0%).
In contrast, neurotensin increased the proliferation of pre-B, B mature, and plasmacytic cells cultured under deprivation conditions (Fig. 4B). The most significant effects of neurotensin were observed after 24 h for pre-B (Nalm6) and B mature (BL41), and after 72 h for plasma cells (RPMI; Fig. 4B). Therefore, we hypothesized that neurotensin could be involved in a mechanism of escape to cell death.

We thus performed apoptosis assays in the presence of exogenous neurotensin (40 μM) under stressed conditions (1–7 d serum deprivation). Apoptosis was inhibited by neurotensin after 6 d of serum deprivation of the pro-B cell line RS4, 3 d for the pre-B cell line Nalm6 and the two B mature cell lines (BL41 and BL2), 2 d for the U266 plasma cell line (all p < 0.05; Fig. 5A). The antiapoptotic effect of neurotensin was significant after 24 h of serum starvation of the RPMI cell line and maximum after 2 d (all p < 0.05; Fig. 5B).

**FIGURE 5.** Relationship between neurotensin (NT) and serum deprivation-induced apoptosis. (A) Apoptosis (ratios) of RS4, Nalm6, BL41, BL2, and U266 B cell lines induced by a 24- (D1) to 168-h (D7) serum deprivation alone (white histograms) or associated with 40 μM recombinant NT (gray histograms). The p values were determined in comparison with serum-free condition alone (0% FCS). (B) Apoptosis (ratios) of RPMI plasma cell line induced by a 24- (D1) to 36-h (D3) serum deprivation alone (white histograms) or associated with 40 μM recombinant NT (gray histograms). The p values were determined in comparison with serum-free condition alone (0% FCS).

**Fas-induced apoptosis is downregulated by exogenous neurotensin**

The effect of neurotensin on another apoptotic pathway was performed by the activation of the cell surface death receptor, Fas (CD95) in pro-B (Nalm6), mature B (BL2), and plasmacytic (RPMI) cell lines under both standard and deprivation conditions. Incubation with the agonistic anti-Fas Ab 7C11 induced a significant decrease of the apoptotic ratio in the presence of exogenous neurotensin in 10% FCS cultures of pro-B (Nalm6) and plasma (RPMI) cell lines (p = 0.04 and p = 0.01, respectively), but not in the BL2 mature B cell line (Fig. 6A). After serum starvation, the protective effect of neurotensin was clear-cut in all studied cell lines. Indeed, neurotensin inhibited Fas-induced apoptosis in pre-B (Nalm6; p = 0.01) and mature B (BL2; p = 0.005) cell lines, as well as in plasmacytic U266 (p = 0.004) and RPMI (p = 0.002) cells as previously reported (Fig. 6B) (17).
JMV 449, a pseudopeptide analog of neurotensin, also decreased cell death ratios in both U266 and RPMI cells cultured without serum, in accordance with the protective effect of natural neurotensin \( (p < 0.05; \text{Fig. 6C}) \). These results led us to study the involvement of NTSR1 in the antiapoptotic function of neurotensin.

**NTSR1 inhibition suppresses the antiapoptotic function of neurotensin in mature and plasma cells after serum starvation**

We evaluated the effect of an NTSR1 inhibitor (SR48692 or meclinertant) on neurotensin-induced cell survival. The inhibitor suppressed the protective effect of neurotensin on RPMI and U266 \( (p < 0.001) \) cells maintained in 24- to 72-h serum-free cultures (Fig. 6C). Similar results were obtained with antagonistic anti-NTSR1 Abs (B-N6) in RPMI cell line after a 24-h serum starvation (Fig. 6C).

In view of the effects of neurotensin and NTSR1 in malignant B cell lines, especially in mature and plasmacytic cells, we looked for their expression in normal human peripheral B lymphocytes.

**NTSR and neurotensin expression in healthy peripheral human B lymphocytes**

Peripheral B cells isolated from 6 healthy donors were studied by qRT-PCR under basal conditions (10% FCS; \( n = 6 \)) or after a 24-h

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**FIGURE 6.** Relationship between neurotensin (NT) and Fas-induced apoptosis. Effect of NTSR1 antagonist and neurotensin analog on 0% FCS-induced apoptosis.  
(A) Apoptosis (ratios) of Nal6, BL2, and RPMI B cell lines induced after 24 h of Fas activation by an agonistic mAb (Fas; black histograms) associated or not with 40 \( \mu \)M recombinant NT (Fas+NT; black hatched histograms). *Significant \( p \) values determined in comparison with standard condition (10%) are indicated.  
(B) Apoptosis (ratios) of Nal6, BL2, U266, and RPMI B cell lines induced after a combination of serum deprivation (0%) and a 24-h Fas activation by an agonistic mAb (Fas; black histograms) associated or not with 40 \( \mu \)M recombinant NT (Fas+NT; black hatched histograms). *Significant \( p \) values determined in comparison with serum deprivation condition (0%) are indicated.  
(C) Apoptosis (ratios) of U266 and RPMI B cell lines induced in serum deprivation (0%) by NT (light gray histograms), NT analog JMV449 (JMV; gray hatched histograms), pharmacological inhibitor of NTSR1 meclinertant (M; dark gray histograms) combined or not with NT (M+NT; gray spotted histograms) and anti-NTSR1 antagonistic Ab with NT (BN6+NT; gray hatched histograms). *Significant \( p \) values determined in comparison with serum deprivation condition (0%) are indicated.
stimulation with sCD40L, anti-Ig, BAFF, and IL-4 (n = 5). Analysis revealed the presence of NTSR1, NTSR2, and neurotensin transcripts in resting B lymphocytes. However, their activation induced a significant downregulation of NTSR1 (p = 0.04) and NTSR2 (p = 0.03; Fig. 7A). Western blot analysis confirmed the expression of both NTSR1 and NTSR2 by circulating B cells at the protein level (Fig. 7B).

Quantification of NTSR1, NTSR2, and neurotensin gene expression in CLL B cells and in lymph nodes from diffuse large B cell lymphoma

NTSR2 expression was significantly higher in purified B cells from the six CLL patients than in human normal resting (n = 6; p = 0.0002, Fig. 7A) or activated B cells (n = 5; p < 0.0001; Fig. 7A). In contrast, neurotensin expression by CLL cells was undetectable.

Levels of NTSR1 and NTSR2 gene expression were variable in lymph nodes from seven diffuse large B cell lymphoma patients and (as controls) from six metastatic colic adenocarcinoma patients, and benign lymph nodes from three patients with sarcoidosis. A significant difference of NTSR2 expression was observed between metastatic and normal lymph nodes (p = 0.03; Fig. 8). Neurotensin expression was lower in B cell lymphoma lymph nodes than in nonmalignant nodes (p = 0.0005; Fig. 8).

Discussion

The neurotrophin BDNF was recently identified as endogenous growth and survival factor in B lymphocytes (17, 47). Whereas BDNF acts through its high-affinity receptor, we identified sortilin in human B lymphocytes. This protein displays dual functions—transport of neurotrophins and coreceptor for immature form of neurotrophins—that activating death signaling through sortilin/NTSR2 receptor complex (17). Sortilin is also known as a receptor for another neuropeptide, neurotensin (12). Because sortilin is detected in human B cells whatever the maturation stage (17), we hypothesized that neurotensin and other NTSRs could also be expressed by B cells.

We detected in this study for the first time, to our knowledge, the expression of neurotensin and its high (NTSR1) and low (NTSR2)-affinity receptors in human B cell lines, primary malignant B cells from CLL patients and healthy peripheral B cells with variable mRNA expression levels by the lines, depending on culture conditions. Although NTSR2 mRNA levels decreased in RS4 and Nalm6 cells maintained in a 72-h serum-free culture, NTSR1 or both NTSR1 and NTSR2 levels increased in BL2 and U266 cells. However, NTSR1 and NTSR2 protein levels appeared to be unchanged. Such a discrepancy between mRNA and protein levels in cell lines was previously reported for NTSR expression (11). In addition, we also observed m.w. variations of NTSR1, depending on culture conditions. It was particularly high under serum starvation conditions. These findings are in accordance with a recent study that demonstrated the occurrence of a posttranscriptional maturation of the receptor, depending on glycosylation, after serum starvation. This mature NTSR1 is able to recruit small G proteins in membranous microdomains, especially under serum starvation (48). These data suggested that stress-culture conditions of B cell lines induced the maturation of NTSR1 as detected by higher molecular form of this receptor (48).

In addition, serum starvation enhanced the membranous location of NTSR1 in all human B cell lines. We have previously observed such heterogeneity of tropomyosin-related kinase B receptor cell expression under serum starvation in these cell lines (17). Similar findings concerning plasmacytoid cell lines, especially U266, were previously reported (49). Moreover, neurotensin enhanced B cell line proliferation in stressed cultures.

The involvement of neurotensin and specific receptors was previously examined in nonhematological cancer models (50). They stimulate cancer cell growth (21), mainly through binding to NTSR1, that is overexpressed in many cancer cell lines (51). NTSR1 expression is also associated with a poor prognosis in human lung cancer (8, 9). In contrast, neurotensin is able to prevent serum starvation or IL-induced apoptosis in pancreatic cells after binding to NTSR2 or NTSR3 (52).

Recently, another role of neurotensin was established. Coppola and colleagues (53) demonstrated its implication in antiapoptotic pathways. Similarly, we studied the effects of neurotensin on B cell line apoptosis, and we showed a protective effect in human B cell lines cultured under two stress conditions: serum starvation combined or not with Fas activation.

We thus could hypothesize that neurotensin enhanced B cell line survival through the NTSR1 activation pathway. Indeed, inhibition of NTSR1 using the pharmacological inhibitor SR-48692, also named meclinertant, or antagonistic anti-NTSR1 mAb suppressed protective effect of neurotensin on stress-induced apoptosis. In plasma B cell lines, the mechanism involved the high-affinity receptor. Interestingly, SR-48692 inhibitor is a potential antican-
FIGURE 8. qRT-PCR analysis of neurotensin (NT), its two specific receptors (NTSR1, NTSR2) in lymph nodes (LNs) from sarcoidosis (n = 3, black histograms), colonic adenocarcinoma (n = 6, white histograms), and diffuse large B cell lymphoma (DLBCL; n = 7, gray histograms). Gene expressions are normalized with the housekeeping gene HPRT expression. Data are expressed as means of fold expression (relative quantification) values ± SEM of each gene in comparison with benign lymph nodes normalized to an arbitrary value of 1. Significant p values are indicated in the graphic and calculated from ΔCt values with ANOVA tests.

Levocabastine is a histamine-H1 receptor antagonist, also known to inhibit the neuropeptide and its two specific receptors (NTSR1, NTSR2) in lymphocytes (56), and its inhibition is involved in B cell apoptosis (57). To our knowledge, the function of NTSR2 was not established in other cancer cells, although its expression in cancer was recently described in prostatic cancer. It was found in the most differentiated malignant cell lines and patient’s primary cells, whereas NTSR1 was prominently expressed in undifferentiated neoplastic cells (11). The antiapoptotic effect of neurotensin on pancreatic β-TC3 cells (54). To evaluate the effect of NTSR2 inhibition on B cell lines, levocabastine, a pharmacological histamine-H1 receptor antagonist, also known to inhibit the neurotensin binding on NTSR2, was evaluated (55). We observed that levocabastine at 2–8 mM either induced or diminished apoptosis in combination or not with neurotensin depending on concentrations and cell lines (data not shown). This could be because of another effect of levocabastine in B cell lines. Indeed, levocabastine was previously described as a ligand to VLA-4 integrin (56), and its inhibition is involved in B cell apoptosis (57).

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