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Role of Endogenous Brain-Derived Neurotrophic Factor and Sortilin in B Cell Survival

Anne-Laure Fauchais,*† Fabrice Laloué,* Marie-Claude Lise,* Ahmed Boumediene,‡ Jean-Louis Preud’homme,§ Elisabeth Vidal,*† and Marie-Odile Jauberteau2*‡

Brain-derived neurotrophic factor (BDNF), a major neuronal growth factor, is also known to exert an antiapoptotic effect in myeloma cells. Whereas BDNF secretion was described in B lymphocytes, the ability of B cells to produce sortilin, its transport protein, was not previously reported. We studied BDNF production and the expression of its receptors, tyrosine protein kinase receptor B and p75 neurotrophin receptor in the human pre-B, mature, and plasmacytic malignant B cell lines under normal and stress culture conditions (serum deprivation, Fas activation, or their combination). BDNF secretion was enhanced by serum deprivation and exerted an antiapoptotic effect, as demonstrated by neutralization experiments with antagonistic Ab. The precursor form, pro-BDNF, also secreted by B cells, decreases under stress conditions in contrast to BDNF production. Stress conditions induced the membranous expression of p75 neurotrophin receptor and tyrosine protein kinase receptor B, maximal in mature B cells, contrasting with the sequestration of both receptors in normal culture. By blocking Ab and small interfering RNA, we evidenced that BDNF production and its survival function are depending on sortilin, a protein regulating neurotrophin transport in neurons, which was not previously described in B cells. Therefore, in mature B cell lines, an autocrine BDNF production is up-regulated by stress culture conditions and exerts a modulation of apoptosis through the sortilin pathway. This could be of importance to elucidate certain drug resistances of malignant B cells. In addition, primary B lymphocytes contained sortilin and produced BDNF after mitogenic activation, which suggests that sortilin and BDNF might be implicated in the survival and activation of normal B cells also. The Journal of Immunology, 2008, 181: 3027–3038.

Neurotrophins were originally recognized as polypeptides that promote survival and differentiation of specific neuronal populations (1). Brain-derived neurotrophic factor (BDNF),3 a member of the neurotrophin family, plays a critical role in the nervous system because it regulates the differentiation and apoptosis of both neurons and glial cells (1).

BDNF forms noncovalently linked homodimers and binds receptors of two classes, namely p75 neurotrophin receptor (p75NTR), which belongs to TNFR superfamily, and the tyrosine kinase receptor tyrosine protein kinase receptor B (Trk) B, which displays more restricted ligand-binding specificities (2). Besides the gp145TrkB full-length receptor, a truncated gp95TrkB variant lacks the cytoplasmic catalytic tyrosine kinase domain (3), but retains direct signaling activities (4).

Neurotrophins are synthesized in a precursor form (proneurotrophins). Its proteolysis generates the mature neurotrophins (5). Recent work indicates that both the proneurotrophins and the mature forms are secreted and display biological activities (5, 6). Indeed, pro-BDNF is either cleaved by furin proteins to yield C-terminal mature neurotrophin dimers or secreted in its precursor form by neural cells (6). Pro-BDNF and BDNF display opposite effects on neural cell proliferation and apoptosis. The antiapoptotic function of BDNF is mediated by interactions with the high-affinity receptors gp95 and gp145 TrkB (7). P75NTR binds pro-BDNF with a high affinity, and its ability to induce apoptosis requires its interaction with sortilin (a Vps10p-D, vacuolar protein-sorting domain protein) to form a high-affinity dimeric receptor (2, 6). Sortilin was initially described in human neural cells as an intracellular transport protein for neurotrophins and proneurotrophins. In addition, sortilin displays a membrane type I receptor activity, but signal transduction can only be mediated by the association with coreceptors, because the intracytoplasmic tail of sortilin lacks catalytic domain (2, 6, 8, 9).

Sortilin was detected in other cell types, including skeletal muscles, heart, and adrenal gland cells, as well as adipocytes (10). It can also decrease the apoptosis of colorectal adenocarcinoma cell lines (11). No previous report deals with its presence in lymphocytes.

Accumulating evidence suggests that neurotrophins, especially nerve growth factor (NGF), participate in inflammatory responses, including modulation and regulation of immune functions in inflammatory and autoimmune diseases (12). NGF serum levels are increased in systemic lupus erythematosus, rheumatoid arthritis, Kawasaki disease, and systemic sclerosis (12). Previous reports on neurotrophin expression and function in B cells mostly deal with NGF. Autocrine NGF produced by resting and stimulated human B cells appears to be an autocrine survival factor for memory B cells, whereas virgin B cells were not affected by neutralization of endogenous NGF (13). NGF also induced IgG and IgA production in human B cells in a CD40-independent pathway (13, 14) and inhibited IL-4-induced IgE production (14). An antiapoptotic effect of NGF on the apoptosis of B cell lines submitted to anti-IgM Ab was also reported (15).

Abbreviations used in this paper: BDNF, brain-derived neurotrophic growth factor; DRG, dorsal root ganglion; MMP, matrix metalloproteinase; NGF, nerve growth factor; p75NTR, p75 neurotrophin receptor; siRNA, small interfering RNA; Trk, tyrosine protein kinase receptor.

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In contrast, rather few data deal with a role of BDNF in B cell homeostasis. In BDNF−/− mice, B cell development is blocked at the pre-BII stage (16). Indeed, BDNF released by stromal cells is able to promote pre-B cell maturation through its interaction with immature B cells expressing TrkB (16). A basal BDNF production in normal mature human B cells was also previously reported, but whether its release depends on sortilin remains unknown (17, 18). BDNF production is enhanced after mitogenic stimulation, but secretion is not affected by IL-6 and TNF-α (19). B cell-derived BDNF is functional and able to promote neuritis outgrowth in neuronal cultures (20). The presence of the BDNF receptors gp95TrkB and p75NTR in human B lymphocytes is well established (13, 14, 21–24). Their membraneous expression is strongly enhanced by mitogenic B cell activation (25). However, B cell responses to BDNF are poorly known. The increase of the intracellular calcium concentration is a direct evidence that B lymphocytes are able to respond to BDNF (16), but BDNF did not appear to exert any significant effects on the proliferation or apoptosis of normal human peripheral B lymphocytes (20). An intracytoplasmic sequestration of TrkB receptor on resting B cells could be deduced from previous studies showing a strong TrkB expression at the protein level (by Western blot analysis) contrasting with a weak (less than 20% of the cells by FACS analysis) membrane location (26). BDNF decreases the production of Th1 cytokines (IFN-γ and IL-12) with no detectable effect on that of Th2 cytokines (IL-4, IL-10) (22). Consequently, BDNF could skew cytokine balance toward a Th2 pattern in inflammatory and/or autoimmune diseases.

Human B cell lines also produce biologically active BDNF (22) and usually express the BDNF high-affinity truncated receptor gp95TrkB (22, 23, 26). In contrast to normal B cells, the presence of p75NTR seems to be erratic in EBV-transformed B cells and depends on both maturation stages and culture conditions (23). Indeed, the protein was detected in human B cell lines in some (13, 14, 24, 25, 27), but not in other studies (23, 26, 28, 29). Interestingly, incubation with exogenous BDNF reduced by 15% the apoptosis induced by 72-h serum deprivation (1% FCS) in mature B cell lines (23). Moreover, exogenous BDNF was shown to promote myeloma cell growth and migration through its interaction with TrkB (28, 30, 31) and to delay dexamethasone- and bortezomib-related apoptosis in plasma cell lines (30).

The concomitant expression of BDNF and TrkB thus suggests an autocrine function of BDNF in B cells. However, the mechanisms underlying the production and the respective functions of BDNF and pro-BDNF in human B cells need to be elucidated, especially in malignant B cells, because they might be involved in the pathogenesis of B cell tumors.

In the present study, we characterize an autocrine production of both forms in human B cell lines. The endogenous BDNF released under stress culture conditions, such as serum deprivation and/or Fas-induced apoptosis, exerts antiapoptotic effects. Furthermore, we demonstrate that such an autocrine regulation is linked to the presence of sortilin, an endogenous protein that was not previously described in B cells. This protein is able to transport and release precursor and mature BDNF forms in the culture medium. Furthermore, our findings show that sortilin, which is produced by normal B cells as well, could exert the function of a cell surface receptor to modulate B cell line apoptosis.

Materials and Methods

**Human B cell lines**

The cell lines under study had a phenotype of either pre-B cells (NalM6, 697 from acute lymphoblastic leukemia), mature B cells (BL2 and BL41, EBV-negative Burkitt lymphoma cell lines), or plasma cells (the myeloma cell lines U266 and RPMI 8226), respectively. They were provided by K. Lassoued (Amiens, France) and I. Feuillard (Limoges, France). Cell cultures were repeatedly free of mycoplastic contamination by the Hoechst staining method (32), and EBV negativity was confirmed by PCR (performed by S. Ranger-Rogeze, Limoges, France).

**Normal B cells**

Blood samples from healthy volunteers were obtained after informed consent. PBMC were obtained by Ficoll gradient centrifugation (Eurobio), and B lymphocytes were isolated by MACS using the CD19 magnetic beads (CD19 Human MicroBeads MACS; Miltenyi Biotec). B cell populations were 96 ± 3.3% pure, as assessed by flow cytometry analysis using PE-conjugated anti-CD19 Ab (Beckman Coulter). B cells from five donors were used for RNA and protein isolations. FACS analysis was done before culture, and in three cases after 1–3 days of culture with PWM. Functional assays aiming at evaluating the effects of anti-BDNF mAb or of exogenous BDNF (100 ng/ml; Promega) were performed after culture of cells from three samples after a 24-h deprivation alone or under combined PWM activation for 1–5 days.

**Cell cultures**

Under basal conditions, B cell lines were cultured at 1–2 × 10⁶ cells/ml with RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 U/ml peni-streptomycin, 1% non essential amino acids, and 1% modified Eagle medium vitamins (Invitrogen) at 37°C in a humidified 5% CO₂/95% air incubator.

Normal B cells were cultured in 96 round-bottom plates (Nunc) at 2 × 10⁵ cells/ml in RPMI 1640 medium supplemented with 20% heat-inacti-vated FCS, 1% MEM vitamins, 1% serum pyruvate, 1% glutamine, and 20 µg/ml PWM (Sigma-Aldrich).

Cultured cells were stressed by 24- to 72-h serum deprivation, 24-h exposure to 100 ng/ml anti-Fas agonistic mAb (clone 7C11, sodium azide free; Beckman Coulter) (33), or both.

In blocking experiments, the mouse antagonistic anti-BDNF mAb clone 35928.11 (10 µg/ml; Calbiochem) or the sortilin receptor-blocking goat antibody antagonistic anti-sortilin Ab (10 µg/ml; R&D Systems) (both sodium azide free) was added to the cultures during 24 h. The specificity of anti-BDNF and anti-sortilin mAb was controlled by Western blotting after preadsorp-tion experiments with either rBDNF (Promega) or sortilin (R&D Systems).

Competitive experiments with a high-affinity ligand for sortilin receptor, neurotensin (40 µM; Calbiochem), which is known to inhibit pro-BDNF binding to sortilin in neural cells (6, 11, 34), were also performed in apo-topsis assays, as previously described (6).

Implication of caspase-8 in the Fas pathway was verified by functional assays performed with a caspase-8 inhibitor II (5 µmol/ml; Calbiochem).

**Dorsal root ganglion (DRG) preparation and culture**

DRGs were collected from embryonic day-18 Wistar rat embryos (Depré), following the rules edited by the French National Ethics Committee. DRGs were maintained in 24-well culture plates precoated with collagen (Sigma-Aldrich). Culture medium was DMEM, either with no additive or supplemented with 50% supernatant from U266, BL2, or NalM6 B cells cultured for 3 days without serum (20) or with exogenous BDNF alone (100 ng/ml) as a positive control. Neuritis outgrowth was observed after 13 days. Inhibition experiments systematically performed as controls used anti-BDNF Ab added to B cell line supernatants.

**Flow cytometry analysis**

Expression of TrkB, p75NTR, and BDNF was studied by flow cytometry with or without permeabilization. After washing in PBS, B cells were fixed for 15 min using Intraceint Kit Solution A, washed twice in PBS, and, in some experiments, permeabilized with Intraceint Kit Solution B (Dako-Cytomation). After two further washes in PBS, cells were incubated in PBS containing 1% BSA (PBS-BSA) and with either an anti-TrkB mAb (1/100, clone 72509; R&D Systems), a rabbit anti-p75NTR polyclonal antibody (1/100; Santa Cruz Biotechnology; reactive with the C-terminal portion of p75NTR), a rabbit p75NTR polyclonal antibody (1/100 reactive with the N-terminal portion of p75NTR), or rabbit anti-BDNF Ab (1/100; Santa Cruz Biotechnology) at room temperature for 20 min. After two washes in PBS, mAb were revealed using Alexafluor 488-conjugated goat anti-mouse IgG1 Ab and polyclonal Ab by Alexafluor 488-conjugated goat anti-rabbit IgG Ab (both, 1 µg/ml; Invitrogen) for 30 min at 4°C. Cells stained with either rabbit or mouse isotypic Ab (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 (Beckman Coulter). Each exper-iment was repeated at least thrice.
Briefly, growth medium was collected and centrifuged for 15 min at 20,800 × g. Sortilin release by B cells cultured under serum deprivation conditions. Calbiochem), 1 mM NaF, and 1 mM Na3VO4) and a 15-min centrifugation mouse, or goat IgG (Santa Cruz Biotechnology).

Confocal microscope (Carl Zeiss; LSM 510).

488- or 596-conjugated anti-mouse, anti-rabbit, or anti-goat Ab (Invitrogen) mAb (anti-mannosidase II mAb, 1/200; Chemicon International).

Immunocytochemical staining

After two washes in PBS, the cells were fixed for 15 min in Intrastain Kit Solution A, washed twice in PBS, and, in those experiments that aimed at detecting intracellular proteins such as neurotrophins, sortilin, and sequestered receptors, permeabilized 1 min with a solution of ethanol-acetone (v/v) at 4°C. Then, cells were washed twice in PBS and incubated in PBS-BSA for 2 h at room temperature. Cells were incubated overnight at 4°C with primary Ab diluted in PBS-BSA. The following Ab were used: rabbit anti-BDNF Ab (1/100; Santa Cruz Biotechnology), rabbit anti-pro-BDNF Ab (1/50, Alomone Labs), the mouse anti-TrkB mAb and rabbit anti-p75NTR Ab mentioned above, and goat anti-sortilin Ab (1/50, R&D Systems; 1/100, C-20, Santa Cruz Biotechnology).

Staining aiming at detecting intracellular sequestration used the following Ab: 1) anti-mitochondrial rabbit Ab (Tom20, 1/200; Santa Cruz Biotechnology) and mouse mAb (clone M17, 1/400; Leica); 2) anti-Golgiian mAb (anti-mannosidase II mAb, 1/200; Chemicon International).

Cells were washed twice in PBS and incubated with 1 μg/ml Alexafluor 488- or 596-conjugated anti-mouse, anti-rabbit, or anti-goat Ab (Invitrogen) for 120 min at 4°C. After two further washes in PBS, cells were mounted in glycerol-gelatin medium (Sigma-Aldrich) and studied using a confocal microscope (Carl Zeiss; LSM 510).

Negative controls were cells incubated with irrelevant normal rabbit, mouse, or goat IgG (Santa Cruz Biotechnology).

Western blotting

Proteins were obtained from cell lysates or from supernatants of cells cultured in both normal (containing 10% FCS) and serum-free medium. 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 15-min centrifugation at 20,800 × g.

Supernatants were concentrated to analyze neurotrophins and soluble sortilin released by B cells cultured under serum deprivation conditions. Briefly, growth medium was collected and centrifuged for 15 min at 1600 × g in vivaspin column (Millipore). Equal amounts of proteins from cell lysates and supernatants (20–40 μg/sample) were separated on NUPAGE 4–12% SDS-polyacrylamide gels (Invitrogen) under denaturing conditions and transferred onto nitrocellulose sheets (Hybond; GE Healthcare). Non-specific binding sites were blocked for 2 h with 5% nonfat dry milk in TBS containing 0.1% Tween 20. After overnight incubation at 4°C with specific Ab (dilution 1/200), membranes were incubated with HRP-conjugated secondary Ab to mouse, rabbit, or goat Ig (DakoCytomation; dilution 1/2000) for 60 min at room temperature and revealed by chemiluminescence (ECL reagent; Amersham Life Science). Protein-loading control was performed with anti-GAPDH Ab (Santa Cruz Biotechnology). Western blots were scanned using a bioimaging system (Genesnap; Syngene). The intensity of the cellular specific proteins was expressed as a ratio established in comparison with the intensity of GAPDH in the same sample (Genetool; Syngene), as previously described (35). Band intensities of cell supernatant proteins were analyzed by densitometry and expressed in arbitrary units.

Protein purification and association assays

Protein extracts (100 μg) from whole-cell lysates and culture supernatants were incubated with either anti-p75NTR or anti-sortilin Ab for 1 h on ice, and then with protein AG-Sepharose beads (Santa Cruz Biotechnology) for 2 h. Beads were washed thrice with lysis buffer. Elution was performed by heating (90°C) for 5 min in SDS-sample buffer; then, proteins were electrophoretically separated on a 4–12% SDS-polyacrylamide gel (Invitrogen) and analyzed by Western blotting with Ab specific for sortilin, BDNF, pro-BDNF, to demonstrate the association of sortilin with p75NTR and with pro-BDNF, respectively, as above. Sepharose beads incubated with the various protein extracts and normal Ig were used as isotypic controls.

Quantification of BDNF and pro-BDNF secretion

BDNF concentrations in cell supernatant were determined by using the BDNF ImmunoAssay System (Promega), according to the manufacturer’s instructions. The results were expressed as pg of BDNF per millions of cells. However, this ELISA measures both pro-BDNF and BDNF proteins, which share at least 80% homology. For specific quantification of BDNF and pro-BDNF, a Western blotting analysis was performed with Ab specific for the pro- and mature forms of BDNF.

RT-PCR analysis

To analyze BDNF, TrkB, p75NTR, and sortilin mRNA expression, B cell lines were cultured in medium containing or not 10% FCS for 24 h. Total RNA was extracted with the 50 total RNA isolation system (Promega), followed by treatment with RNase-free DNase I (Invitrogen). The cDNA synthesis kit (Promega) was used, according to the manufacturer’s instructions, using oligo(dT) and amplification with specific primer sequences

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<tr>
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<th>3′ Location</th>
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*a* F, forward; R, reverse; HT, hybridation temperature.

### Table 1. Primers used in RT-PCR studies

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<td>266 pb</td>
<td>58°C</td>
<td>596</td>
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<tr>
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a F, forward; R, reverse; HT, hybridation temperature.
designed on primer 3 software (Table I). Transcripts of BDNF, TrkB, p75NTR, and sortilin were obtained using GeneAmp 2400 (Applied Biosystems) with Platinium Taq DNA polymerase (Invitrogen). Total RNA isolated from human neuroblastoma cell lines (IMR32, SH-SY5Y) were used as positive controls.

Sequencing

After extraction of PCR products with Wizard SV Gel and PCR Clean-Up System (Promega), according to the manufacturer’s instructions, sequences were analyzed on an automated laser fluorescent DNA sequencer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems) and homologies were checked after blasting with the TrkB, p75NTR, and Sortilin GenBank sequences (NM_001018064, NM_002507, and NM_002959, respectively) (36).

Study of apoptosis

Cells cultured under basal (10% FCS) or 24-h serum deprivation conditions were further incubated with agonistic anti-Fas mAb, and apoptosis was measured after 24 h by the following two methods. 1) 4,6-Diamidino-2-phenylindole dihydrochloride staining: cells were washed twice in PBS and incubated in the dark for 5 min with 1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (Promega) to characterize fragmented nuclear DNA. Apoptotic nuclei scores were based on their appearance, namely chromatin condensation and nuclear fragmentation. In every experiment, five fields...
containing more than 500 cells were counted. All experiments were performed in duplicate. 2) Measurement of cytoplasmic soluble nucleosomes by ELISA (Cell Death Detection ELISAPLUS; Roche Molecular Diagnostic), according to the manufacturer’s instructions. Briefly, cells were seeded in 96-multiwell plate (5 × 10^4 cells/well) and cultured for 24 h. 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 (37). Every experiment was performed thrice independently.

**Sortilin small interfering RNA (siRNA)**

Knockdown of sortilin using RNA oligonucleotides was achieved in U266 cells by transfection of duplex RNA oligonucleotides corresponding to the target sequence of sortilin (NM002959) nt 1466–1486 (AATGTTCCAAT GCCCCACCTC), previously described as interfering sequence (9, 38). The siRNA was purchased from Qiagen. U266 cells were seeded in 24-well plates (2 × 10^3 cells/well) and transfected with HiPerfect transfection reagent (Qiagen), according to the manufacturer’s instructions. siRNA (37.5 ng) was added in 100 μl of medium without serum and antibiotic. After 10 min of incubation to allow the formation of transfection complexes, the cells were incubated for 7 h with RPMI medium containing 10% FCS. The concentrations of both siRNA and HiPerfect reagent and the optimal duration of the transfection process were set up by carrying out a time-course experiment using fluorescein-labeled siRNA (nonsilencing control siRNA AlexaFluor 488 labeled; Qiagen). The level of expression of targeted sortilin mRNA was controlled by semiquantitative RT-PCR. The decrease of sortilin mRNA level only in the plasmacytic cell lines (Fig. 2c) and after stimulation with PWM for 1–3 days (D1–D3). Intracellular BDNF was detected in permeabilized cells. Histograms are means ± SEM of three (D1–D3) to five (D0) independent experiments. Values of p were determined in comparison with D0. Western blot analysis of p75NTR (b) and BDNF (c) in whole-cell lysates from isolated blood B cells of four healthy donors. d, Amplifications by RT-PCR of sortilin mRNA in resting B cells from four healthy donors. The neuroblastoma cell line IMR32 was used as a positive control. Constitutively expressed GAPDH is a control of PCR efficiency. e, Western blot analysis of whole-cell lysates for sortilin expression in primary B cells from five healthy donors. f, Modulation by exogenous BDNF (100 ng/ml) of apoptosis (ratios) of normal B cells cultured without serum. Histograms are means ± SEM of data from three independent experiments. Values of p were determined in comparison with serum-free condition alone (0%).

### Results

**Under basal culture conditions, TrkB and p75NTR are sequestered in B cell lines**

The low (p75NTR)- and high (TrkB)-affinity BDNF receptors were virtually undetectable at the surface of cells cultured in standard (10% FCS) medium, whatever their maturation stage (Fig. 1, a and b), but they were evidenced after permeabilization, as shown by flow cytometry analysis (data not shown). Immunocytochemical analysis showed that sequestration was cytoplasmic without Golgi or mitochondrial location, as assessed by their respective markers (data not shown).

RT-PCR and Western blotting analysis confirmed the expression of both TrkB and p75NTR transcripts and proteins (Fig. 2, a and c) by all cell lines in standard (10% FCS) cultures.

### FIGURE 3.

**Surface and intracytoplasmic expression of TrkB and p75NTR.** a, Confocal microscopy of a U266 cell stained with an anti-TrkB mAb (green) and an anti-BDNF Ab (red) after 3 days of serum deprivation. b, Staining of Nalm6 cells by anti-p75NTR Ab after a 3-day serum deprivation.

### FIGURE 4.

**Expression of BDNF, its receptors, and sortilin in primary B cells.** a, FACS analysis of membranous TrkB and p75NTR expression on unpermeabilized human B lymphocytes from five healthy donors before (D0) and after stimulation with PWM for 1–3 days (D1–D3). Intracellular BDNF was detected in permeabilized cells. Histograms are means ± SEM of three (D1–D3) to five (D0) independent experiments. Values of p were determined in comparison with D0. Western blot analysis of p75NTR (b) and BDNF (c) in whole-cell lysates from isolated blood B cells of four healthy donors. d, Amplifications by RT-PCR of sortilin mRNA in resting B cells from four healthy donors. The neuroblastoma cell line IMR32 was used as a positive control. Constitutively expressed GAPDH is a control of PCR efficiency. e, Western blot analysis of whole-cell lysates for sortilin expression in primary B cells from five healthy donors. f, Modulation by exogenous BDNF (100 ng/ml) of apoptosis (ratios) of normal B cells cultured without serum. Histograms are means ± SEM of data from three independent experiments. Values of p were determined in comparison with serum-free condition alone (0%).

The 145-kDa full-length form of TrkB was detectable at the mRNA level only in the plasmacytic cell lines (Fig. 2a), but not by Western blotting. In contrast, the 95-kDa truncated form was...
evidenced at the protein level in all cell lines (Fig. 2d). P75NTR and 145 TrkB mRNA were isolated and sequenced with the expected results (Fig. 2a). Hence, the two BDNF receptors were detected in the cell cytoplasm under basal culture conditions.

Membranous expression of TrkB and p75NTR is induced by serum deprivation

To search for a membranous expression of both receptors under stress conditions, cells were studied after serum deprivation for 24–72 h. Under such conditions, all cell lines, especially the more mature ones (BL2, U266, and RPMI 8226), displayed a clear-cut membranous expression of TrkB and p75NTR (Fig. 1, a and b). This expression was maximal in BL2 cells, mostly in viable cells (Fig. 1, c and d). Membranous expression was already enhanced after 24 h of serum deprivation (0% H3 to 0% H48). Numbers in gray stand for intensities expressed in arbitrary units. Neuritis outgrowth of embryonic DRGs cultured with (g) or without (control, e) supernatants of U266 cells cultured for 3 days without serum. Neuritis outgrowth in cultures supplemented with 100 ng/ml exogenous BDNF (f) and with U266 supernatant and anti-BDNF neutralizing Ab (h). Magnification ×600.
FIGURE 7. Compared production of BDNF and sortilin by U266 cells. a, Confocal microscopy study. Single staining for BDNF (green) and sortilin (red) and double staining (yellow). b, Sortilin production assessed by Western blotting of extracts of cells cultured with FCS (10%) and after 3–48 h of serum deprivation. Sortilin/GAPDH ratios of band intensities evaluated by densitometry are shown below lanes. c, Time course of sortilin and BDNF secretion (Western blot analysis of cell supernatants) and values of band intensity (expressed in arbitrary units).

BDNF secretion, assessed by ELISA in Nalm6, BL2, and U266 cell lines, was increased by serum deprivation, especially in the plasma cell line (Fig. 6c). However, as ELISA measured both pro- and mature forms of BDNF, we analyzed by Western blotting the kinetics of mature BDNF release in U266 and RPMI 8226 culture supernatants after 3–48 h of serum deprivation. Mature BDNF secretion was negligible with 10% FCS. It was detectable as soon as after 3 h of serum deprivation and peaked after 12 h for U266 cells (Fig. 6d). BDNF release by RPMI 8226 cells was delayed and peaked after 48 h (Fig. 6d). To assess that BDNF secreted by B cell lines under serum-free conditions was functional, we evaluated the effect of supernatants of the lines Nalm6, BL2, and U266 cultured without serum on DRG in vitro. All of them (and not the control medium) induced neuritis outgrowth of embryonic DRG (Fig. 6e, f, and g) that was blocked by anti-BDNF neutralizing mAb (Fig. 6h), confirming that stimulation of neurotogenesis is due to BDNF released from B cells, similarly to those obtained with exogenous BDNF (Fig. 6f).

Sortilin, an interacting BDNF protein, is expressed by normal and malignant B cells

Sortilin transcripts were studied in the B cell lines and peripheral B cells of healthy donors. The primers were designed to specifically recognize the intracellular part of the protein (sortilin IC involved in neurotrophin trafficking) and the extracellular part (sortilin EC involved in its receptor properties). Sortilin transcripts were detected by RT-PCR in all cell lines (Fig. 2a), a result validated by sequencing after elution from agarose gels. Furthermore, the sortilin protein was detected in all cell lines by immunocytochemical analysis and Western blotting (Fig. 2b). Sortilin transcripts were also detected in unstimulated normal B cells from healthy donors (Fig. 4d). Western blot analysis confirmed a strong protein expression by normal cells (Fig. 4e).

The cellular location of sortilin in U266 cells was determined by confocal microscopy. All detectable cells double stained for sortilin and BDNF, with a clear colocalization and a polarization pattern suggestive of a secretion process (Fig. 7a), in agreement with the hypothesis that sortilin might be a transporter protein for BDNF. Sortilin was also detected by Western blotting, with increased amounts after serum deprivation (Fig. 7b). Strikingly, sortilin was secreted after serum deprivation with a kinetic similar to that of BDNF, which suggests a parallel mechanism of production and secretion (Fig. 7c).

contrast to a diffuse spotty staining for BDNF (Fig. 3a). P75\textsuperscript{NTR} membrane staining was more homogenous (Fig. 3b). Staining patterns of Nalm6, BL2, and U266 cell lines were similar (data not shown).

Primary B cells express membranous TrkB and not p75\textsuperscript{NTR} after PWM activation

Flow cytometry studies of peripheral B lymphocytes from five healthy donors showed a basal membranous location of TrkB in 6.2 ± 6% of unpermeabilized purified B cells. This membranous expression reached 71 ± 8% of purified B cells after 3-day PWM stimulation (Fig. 4a). In contrast, membranous p75\textsuperscript{NTR} was not detected in these FACS experiments, contrasting with a strong protein expression by Western blot analysis (Fig. 4, a and b). Altogether, these data suggest an inducible TrkB expression after mitogenic stimulation, contrasting with a persistent intracytoplasmic sequestration of p75\textsuperscript{NTR}. BDNF production was confirmed by Western blotting of cultured primary B cells and was increased after PWM activation (Fig. 4, a and c). Membranous expression of TrkB and p75\textsuperscript{NTR} is hence activated by serum deprivation in B cell lines, whereas only membranous TrkB is enhanced in primary B cells after PWM activation.

B cell lines produce both pro-BDNF and BDNF

Because BDNF receptors were expressed in normal B cells, we investigated the endogenous production of immature and mature forms of BDNF by cell lines. Pro-BDNF was detected by immunocytochemical analysis in the three studied (Nalm6, BL2, and U266) cell lines, whatever the maturation stage. As shown for instance in the line Nalm6, every cell displayed a strong cytoplasmic staining by the relevant Ab (Fig. 5a). The production of pro-BDNF was confirmed by Western blotting analysis of cell lysates. Both the 32- and 34-kDa doublet proteins were found, as described in neurons (6) (Fig. 5b). Interestingly, pro-BDNF was released in culture supernatants, as also detected by Western blots, in decreased amounts after serum deprivation (Fig. 5c).

By flow cytometry, BDNF was detected in the cytoplasm of more than 80% cells under basal culture conditions (with FCS), without significant difference between the studied B cell lines (Fig. 6, a and b). Following serum deprivation for 3 days, this production, evaluated by fluorescence intensity values, was enhanced in both RPMI 8226 and U266 cells (p < 0.05), whereas it was slightly reduced in the other cell lines (Fig. 6b).
Apoptosis induced by serum deprivation is modulated by BDNF and sortilin

To study the role of BDNF in stressed B cell survival, we evaluated the effect of a blocking anti-BDNF mAb known to neutralize the biological activity of human BDNF (39). Used at a 10 μg/ml concentration, it significantly increased the apoptosis of U266 and RPMI 8226 cells cultured for 24 or 48 h under serum deprivation conditions, respectively (in agreement with their respective kinetic of BDNF release) (Fig. 8, a and b, left parts; Table II).

The same effect was observed in the BL2 cell line, but only on the third day of serum withdrawal (Table II). Apoptosis of 697, Nalm6, and BL41 cell lines was unaffected by anti-BDNF and anti-sortilin neutralizing Ab. Thus, the protective effect of BDNF on stress-induced apoptosis is a feature of the most mature B cell, especially plasma cell, lines.

The same experiments with normal B cells failed to point out any antiapoptotic effect of autocrine BDNF release and neutralizing by an antagonist mAb. In contrast, exogenous BDNF rescued normal B cells isolated from three donors from a 24-h serum deprivation-induced apoptosis ($p < 0.002$) (Fig. 4f).

To define a potential function of receptor for sortilin in B cells, we evaluated the functional effect of blocking Ab. In contrast to BDNF, blocking of sortilin with Ab known to inhibit its receptor function significantly decreased the apoptotic index in U266 and RPMI 8226 cells (Fig. 8, a and b, left parts), and not in the other B cell lines. A similar protective effect was observed with a competitive ligand of sortilin, neurotensin, which was previously shown to inhibit pro-BDNF fixation on sortilin receptor in neuronal cells (6) (Fig. 8, a and b, left parts; Table II).

The latter results suggested that sortilin had a proapoptotic effect in U266 and RPMI 8226 cells, through its binding to pro-BDNF, as described in neurons (6). To verify that sortilin binds pro-BDNF, we performed coimmunopurification experiments of sortilin and pro-BDNF secreted by U266 cells after a 24-h serum withdrawal. The proteins isolated using anti-sortilin Ab and protein AG Sepharose beads contained pro-BDNF, as shown by Western blotting (Fig. 8c). In contrast, no association of sortilin with the mature form of BDNF could be detected by immunopurification in the supernatants of any cell line (Fig. 8d). Sortilin was described as the coreceptor of p75NTR in neurons, its high-affinity domain binding pro-BDNF (2, 6). Similarly, sortilin was associated with p75NTR in the pre-B (Nalm6) as well as in the plasmacytic U266 cell line, as shown by immunopurification of whole-cell lysates using anti-sortilin Ab and revelation by anti-anti-p75NTR Ab (Fig. 8e).

**FIGURE 8.** Relationship between BDNF, sortilin, and apoptosis. **Left part,** Apoptosis (ratios) of U266 cells (a) or RPMI 8226 (b) induced by a 24-h serum deprivation alone (0%) or associated either with a neutralizing anti-BDNF mAb (0% anti-BDNF), with blocking anti-sortilin Ab (0% anti-sortilin), or recombinant neurotensin (NT), a competitive ligand. Values of $p$ were determined in comparison with serum-free condition alone (0%). **Right part,** Apoptosis of U266 cells (a) or RPMI 8226 (b) cultured for 24 h without serum after Fas activation by an agonistic mAb (Fas), an anti-BDNF mAb (Fas anti-BDNF), or with anti-sortilin Ab (Fas anti-sortilin) (right). Values of $p$ were determined in comparison with apoptotic ratios under serum deprivation and Fas activation (Fas). c, Copurification of sortilin and pro-BDNF. Western blots of proteins isolated with anti-sortilin mAb and Sepharose beads from U266 cell lysates and supernatants under a 3-day serum deprivation. Revelation by anti-pro-BDNF Ab. d, Similar experiment: immunopurification of supernatants from Nalm6, U266, and BL2, with anti-BDNF mAb, and revelation with anti-sortilin Ab. e, Immunoprecipitates of Nalm6 and U266 cell lysates with anti-sortilin Ab revealed by anti-p75NTR Ab. f, Effect of caspase-8 inhibitor. Apoptosis of U266 cells submitted to combined 24-h serum deprivation and Fas activation (0% Fas) and in the presence of neutralizing anti-BDNF mAb (0% Fas anti-BDNF) alone or with a caspase-8 inhibitor (0% Fas anti-BDNF C8I, $p < 0.001$). Same experiment with neutralizing anti-sortilin Ab (0% Fas anti-sortilin) and a caspase-8 inhibitor (0% Fas anti-sortilin C8I).
Table II. Effect of neutralizing anti-BDNF or blocking anti-sortilin Abs on apoptosis in B cell lines under serum deprivation alone or with Fas activation

<table>
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<tr>
<th>Cell Line</th>
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<tr>
<td>U266 D1</td>
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</tr>
<tr>
<td>RPMI 8226 D2</td>
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<tr>
<td>BL41 D1</td>
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<tr>
<td>697 D1</td>
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</tr>
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</tr>
<tr>
<td>Anti-sortilin</td>
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</tr>
<tr>
<td>Fas 0%</td>
<td>0.03</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Fas-induced apoptosis is down-regulated by endogenous BDNF

To further evaluate the protective effect of BDNF on Fas-induced apoptosis, we studied the exposure to an agonistic anti-Fas mAb. The apoptosis induced by this mAb was lower (mean 1.5-fold, p < 0.03) in standard cultures with FCS than after serum deprivation for 24 h. Therefore, all subsequent experiments were conducted in serum-free cultures. The incubation of 697, Nalm6, BL2, BL41, U266, and RPMI 8226 cells cultured without serum during 24 h with the agonistic anti-Fas mAb 7C11 significantly increased apoptosis (Table II). This strong apoptosis was further increased only in the two plasmacytic cell lines in the presence of blocking anti-BDNF Ab, in terms both of apoptotic ratios (Fig. 8, a and b, right parts) and percentages of apoptotic cells (Fig. 8e) (Table II).

The ability of BDNF to down-regulate Fas activation was confirmed by similar experiments performed with a caspase-8 inhibitor, which suppressed the apoptotic effect of neutralizing anti-BDNF Ab (Fig. 8f).

Sortilin regulates Fas-dependent apoptosis in B cell lines

In contrast to the effect of BDNF, a strong decrease of Fas-induced apoptotic indexes was observed in the presence of neutralizing anti-sortilin Ab. This effect was observed with U266 and RPMI 8226 (Fig. 8, a and b), and not with the other lines (data not shown), and was not affected by a caspase-8 inhibitor.

Interestingly, after Fas receptor activation by agonistic mAb during 24 h, the pattern of sortilin expression in U266 cells was modified, with a membranous relocation (data not shown). Hence, sortilin might act as a membranous receptor under stress conditions.

Sortilin inactivation by siRNA in U266 cells decreases BDNF secretion and its protective effect on apoptosis induced by serum deprivation

The effect of sortilin on the antiapoptotic function of BDNF might relate to a putative control of BDNF synthesis and/or secretion. We hence inactivated sortilin in U266 cells and assessed it by using RT-PCR and Western blotting. The sequence previously described as a sortilin-interfering sequence in a Cos-7 cell model (38, 40) inhibited the expression of sortilin at the mRNA (detected by RT-PCR at different time points as soon as after an 8-h incubation; data not shown) and the protein levels. Immunoblots of proteins extracted from cell lysates showed a reduced sortilin synthesis by transfected cells (60% lower than in control cells 14 and 18 h after transfection; Fig. 9a). The inhibition of sortilin synthesis was confirmed by immunofluorescent study. Transfected U266 cells showed a strong decrease of fluorescence intensity in all cells 24 h after transfection, with a complete disappearance of detectable immunostaining in 25 ± 1.22% of the cells (p = 0.01 in comparison with control cells).

Twenty-four hours after transfection, sortilin protein levels were decreased by only 12% in cell lysates (data not shown). In contrast, the amounts of sortilin in culture supernatants were still markedly low (minus 60%) (Fig. 9b).

Strikingly, by confocal microscopy, the whole cytoplasmic area of siRNA-transfected cells stained for BDNF (Fig. 9c) instead of the cell polarization observed in nontransfected cells (Fig. 7a). This might suggest that sortilin is implicated in the cell release of BDNF. Accordingly, BDNF secretion by transfected U266 cells was decreased by Western blotting (48% diminution) (Fig. 9b).

Finally, we studied the apoptosis induced by a 24-h serum deprivation in siRNA-transfected and control U266 cells. Transfection resulted in increased apoptotic ratios, a phenomenon further
amplified by the addition of neutralizing anti-BDNF Ab in the culture medium (Fig. 9d). Similarly, apoptotic ratios tended to be higher in Fas-activated transfected cells maintained in serum-free medium, with or without anti-BDNF Ab, than in control cells (Fig. 9d), but differences were not significant.

Discussion

In the present study, we demonstrate that endogenous BDNF is an important survival factor for B cells. It is secreted under stress conditions, especially in the most mature cell lines, the plasmacytic U266 and RPMI 8226 cell lines. Interestingly, this growth factor is secreted in association with sortilin, a protein not previously described in B lymphocytes that regulates neurotrophin transport and also the function of BDNF receptors in neurons (6, 9). Sortilin was also detected in blood B lymphocytes from healthy donors. The BDNF receptors (TrkB and p75NTR) were present in all B cell lines, with an intracytoplasmic sequestration in standard cultures containing 10% FCS, but with a membranous relocation when the cells were stressed by serum deprivation alone or associated with Fas activation. The membrane relocation of TrkB was previously described in human activated T lymphocytes (22). The prominent form of TrkB detected at the mRNA and protein levels in the studied cell lines was the truncated form, as previously reported in normal B cells.

BDNF production by normal B after mitogenic activation cells was already reported in another study (20). The high (TrkB)- and low (p75NTR)-affinity receptors for BDNF were constitutively present in the six B cell lines, with a cytoplasmic sequestration in standard cultures containing 10% FCS, but with a membranous relocation when the cells were stressed by serum deprivation alone or associated with Fas activation. The membrane relocation of TrkB was previously described in human activated T lymphocytes (22). The prominent form of TrkB detected at the mRNA and protein levels in the studied cell lines was the truncated form, as previously reported in normal B cells (18). Such a truncated TrkB receptor exerts a functional activity in neurons. It allows cell activation and proliferation of neuroblasts in the presence of BDNF (4, 42). As shown in the present study, under stress culture conditions, B cell TrkB overexpression and BDNF production increase.

The presence of p75NTR demonstrated in our study at the mRNA and protein levels (including sequencing) in all studied B cell lines is not a constant finding in the literature. Indeed, p75NTR transcripts and proteins were detected in human normal B cells or in B cell lines in some (24, 43), but not all studies (23, 28–30). These controversies might point out differences in culture conditions and serum supplementation that could influence neurotrophin receptors, gene transcription, and membrane location (18).

BDNF is synthesized by neurons as a proneurotrophin, pro-BDNF that is cleaved to produce the mature protein. This process was not previously reported in normal B cells. However, pro-BDNF and BDNF were detected at the protein level in primary myeloma cells in a study (30). The present data showed that the studied B cell lines, whatever their maturation stage, produced and released both BDNF and pro-BDNF, and that the increase of secreted BDNF paralleled the disappearance of pro-BDNF after serum deprivation. Pro-BDNF is secreted by neurons as a dimer that is cleaved in mature BDNF by matrix metalloproteases (MMP), especially MMP-3, MMP-7, and MMP-9 (5, 44). Interestingly, these MMPs are produced by myeloma cell lines, especially U266 and RPMI 8226 (45).

The previously unreported presence in B cells of endogenous sortilin (evidenced at the transcriptional and protein levels) correlated with the ability of B cells to transport and release BDNF and pro-BDNF. Moreover, the present results point for a key role of
sortilin in the BDNF traffic in B cell lines, whatever their maturation stage. Sortilin was secreted by all studied lines, especially under stress conditions and with a kinetic parallel to that of BDNF secretion. Sortilin secretion by neurons could result from a specific shedding process by metalloproteases (11). Knockdown of sortilin in U266 cells using specific siRNA resulted in the decrease of autocrine BDNF secretion and consequently to that of BDNF antiapoptotic abilities. By contrast to its survival role due to its transport function, sortilin can also enhance apoptosis, as shown by the decrease of apoptosis following sortilin blocking by specific Ab in U266 and RPMI 8226 cells. This proapoptotic effect of sortilin could be related to its stable binding to pro-BDNF. Indeed, binding of soluble sortilin secreted by neurons to pro-BDNF appears to protect the proneurotrophins from proteolytic cleavage (6, 11). Similarly, in the present study, sortilin associated with p75NTR appears to act as a receptor for pro-BDNF, as shown by coimmunopurification and Western blotting experiments. The proapoptotic function of pro-BDNF through its high-affinity interaction with heterodimeric receptor formed by p75NTR and sortilin was previously evidenced in neurons (6, 9, 46–48). In contrast, mature BDNF preferentially binds to TrkB, leading to cell survival (49). Therefore, the role of sortilin appears to be a complex balance between that of a receptor leading to cell death through the binding of pro-BDNF and its antiapoptotic properties through the secretion of mature BDNF. Shedding of sortilin could be another way for myeloma cells to avoid the counterbalancing effects of pro-BDNF and BDNF. A metalloprotease TNF-α converting enzyme (TACE) or a disintegrin and metalloprotease (ADAM)-17 plays a role in sortilin secretion, and is also responsible for shedding p75NTR. Removal from the cell surface of the complex formed by sortilin, p75NTR, and pro-BDNF might protect from its proapoptotic action (11, 50).

In conclusion, our results point out the induction of BDNF secretion by stress conditions in both mature B cell and plasma cell lines. It suggests a suppression of the physiological down-regulation of TrkB by the binding of its ligand, BDNF, leading to TrkB endocytosis, as described in neural cells (51). Interestingly, this regulation pathway was blocked by proteasome inhibitors (52). Furthermore, it is worth noting that dexamethasone fails to reduce BDNF production in myeloma cell line (30), and that BDNF delays the onset of apoptosis induced by bortezomib, a proteasome inhibitor (30). Altogether, the secretion of endogenous BDNF is able to protect mature B cells and plasma cells from the apoptosis induced by deprivation, and it could be implicated in dexamethasone and bortezomib therapeutic escape. Sortilin, as the sorting protein as well as a potential pro-BDNF receptor, appears to be the key actor of this autocrine loop. These data could lead to hypothesize that BDNF could act as an autocrine growth factor that is not influenced by chemotherapies commonly used in the treatment of multiple myeloma.

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Disclosures
The authors have no financial conflict of interest.

References