Expression of Neurotrophins and their Receptors in Human Bone Marrow

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The expression of neurotrophins and their receptors, the low-affinity nerve growth factor receptor (p75^{LN^{GF}R}) and the Trk receptors (TrkA, TrkB, and TrkC), was investigated in human bone marrow from 16 weeks fetal age to adulthood. Using reverse transcription-polymerase chain reaction, all transcripts encoding for catalytic and truncated human TrkB or TrkC receptors were detected together with *trkAI* transcripts, whereas *trkAII* transcripts were found only in control nerve tissues. Transcripts for the homologue of the rat truncated TrkC(ic113) receptor were identified for the first time in human tissue. Stromal adventitial reticular cells were found immunoreactive for all neurotrophin receptors. In contrast, hematopoietic cell types were not immunoreactive for p75^{LN^{GF}R} but showed immunoreactivity for one or several Trk receptors. TrkA immunoreactivity was found in immature erythroblasts. Catalytic TrkB immunoreactivity was observed in eosinophilic metamyelocytes and polymorphonuclear cells. Truncated TrkB immunoreactivity was found in erythroblasts and megacaryocytes. Immunoreactivity for both catalytic and truncated TrkC receptor was observed in promyelocytes, myelocytes, some polymorphonuclear cells and megacaryocytes. Neurotrophin transcript levels appeared higher at fetal than at adult stages, no variation in Trk family transcript levels was observed. The local expression of neurotrophin genes suggests a wide range of paracrine and/or autocrine mode of action through their corresponding receptors within the bone marrow. (*Am J Pathol* 1999, 154:405–415)

Nerve growth factor (NGF)¹ is the prototype of a family of related neurotrophic factors known as neurotrophins (NT), which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4

(NT-4, also called neurotrophin-5 or NT-5 in humans) (reviewed in Lindsay et al²). NT are trophic factors for the growth, differentiation, and survival of specific subsets of neurons in the developing and mature nervous system.² NT can interact with two classes of receptors with distinct ligand affinity and specificity. The low-affinity nerve growth factor receptor, p75^{LN^{GF}R}, binds all known NT.^{3,4} Tyrosine kinase receptors of the Trk family are essential components of NT high-affinity binding sites that trigger neuronal survival, growth, and differentiation.⁵ TrkA is the preferred receptor for NGF^{6,7} but has a lower efficiency for NT-3 or NT-4/5. TrkB is bound by BDNF and NT-4 and, to a lesser extent, by NT-3.^{8,9} TrkC is characterized by a unique ligand, NT-3.¹⁰ In some cell lines, TrkA is sufficient to form high-affinity binding sites through homodimerization,⁷ whereas p75^{LN^{GF}R} potentiates TrkA activation by NGF in the PC12 cell line.¹¹ Variants of tyrosine kinase receptors (TK⁺) with insertions in either the extracellular domain (ECD) or the tyrosine kinase domain have been identified for *trkA*^{12,13} and *trkC*^{13–16} in both human and rat.

Truncated receptors lacking the kinase domain (TK[−]) have been described for TrkB and TrkC but not for TrkA.^{15–18} These receptors may function as dominant negative isoforms or immunoadhesins.^{13,19,20} Both TK⁺ and TK[−] receptors have been detected in neurons while only truncated TrkB and TrkC isoforms have been detected primarily in nonneuronal cells.^{15–18,21}

The expression of functional NGF receptors has been detected in several bone marrow-derived cells such as monocytes,²² mastocytes,^{23,24} and B or T cell clones.^{25–28} Among its pleiotropic effects, NGF induces platelet shape changes,²⁹ triggers monocyte cytotoxic activity,²² and induces basophilic cell differentiation^{30–32} and mast cell development and degranulation.^{24,33} However, NGF receptors have not been consistently detected on bone marrow cells. Although *trkA* and p75^{LN^{GF}R} transcripts have been detected in long-term bone marrow cultures,^{34,35} *trkA* transcripts were not always found within fresh bone marrow aspirates.^{35,36} NGF transcripts have not been detected in human bone marrow.³⁵ The

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local expression of other NT genes has not been investigated.

The expression of NT receptors in human fetal and adult bone marrow was investigated by immunocytochemistry and by reverse transcription-polymerase chain reaction (RT-PCR). Several sets of primers were designed to detect transcripts encoding for catalytic or truncated isoforms of the Trk family receptors. We also studied the local expression of all NT genes. Our data suggest that all NT are synthesized within the bone marrow in a developmentally regulated fashion. Their corresponding transducing or truncated receptors were detected on specific hematopoietic cells and on stromal cells.

Materials and Methods

Patients and Bone Marrow Samples

Four groups of patients were investigated: fetuses ($n = 4$) of 16, 23, 25, and 32 weeks fetal age, adults with reactive bone marrow ($n = 7$), neoplasm-bearing adult patients without bone marrow hematopoietic involvement ($n = 15$) (Hodgkin's disease, $n = 4$; non-Hodgkin's lymphoma, $n = 7$; mycosis fungoides, $n = 1$; treated acute leukemia, $n = 1$; lung carcinoma, $n = 1$; thymoma, $n = 1$), and patients presenting with reactive mastocytosis ($n = 3$). Bone marrow trephine biopsies were obtained with a Jamshidy needle. Fetal necropsy tissues consisted of rib cage samples. In all cases, fresh bone marrow samples were either snap-frozen in liquid nitrogen-precooled isopentane and stored at -80°C or fixed in Bouin's fixative. Bone marrow smears were obtained from fetuses ($n = 4$) and adult patients without hematological disorders ($n = 22$), air-dried, and acetone-fixed for 10 minutes at 20°C . Bone marrow tissues were decalcified by electrolysis in decalcifying buffer (Bayer Diagnostics, Puteaux, France) for 2 hours and embedded in paraffin.

Immunocytochemistry

Antibodies

Two monoclonal anti-human nerve growth factor receptor (p75^{LNGFR}) antibodies, clone 8211 (Boehringer Mannheim, Mannheim, Germany) labeling both ECDs and intracellular domains, and clone NGFR5 (Dako, Copenhagen, Denmark) directed against the ECD of the receptor, were used to study p75^{LNGFR} immunoreactivity (IR). The polyclonal antibodies Trk (C-14), TrkA (763), TrkB (794), TrkB(TK⁻) (C-13), TrkC (798) (all Santa Cruz Biotechnology, Santa Cruz, CA), r-TrkA (gift of Dr. L. F. Reichardt), anti-TrkB In pAb (Promega, Madison, WI), and TrkC NC2 (gift of Dr. F. Lamballe) were used to study Trk family receptors. Trk (C-14) is broadly reactive with an epitope corresponding to amino acids 777–790 of the conserved carboxy terminus of TrkA and cross-reacts with TrkB and TrkC receptors. TrkA (763) reacts against a carboxy terminal epitope of human TrkA (amino acids 763–777). TrkB (794) and TrkC (798) were raised against

carboxy terminal peptides (794–808 and 798–812 residues respectively) of gp145TrkB or gp145TrkC proteins. TrkB(TK⁻) (C-13) reacts with an epitope mapping at the carboxy terminus of the truncated (TK⁻) TrkB protein. r-TrkA is directed against a rat TrkA ectodomain.³⁷ TrkB In pAb is raised in chickens against a mouse TrkB intracellular domain corresponding to amino acids 482–501. The anti-TrkC NC2 is directed against a peptide corresponding to the exon B of the rat TrkC TK⁻ receptors ic158 and ic143.¹⁵

Methods

Bone marrow smears were incubated for 1 hour at room temperature in Tris-buffered saline 0.05 mol/L, pH 7.6, 10% BSA with TrkA (763) (1/100), r-TrkA (1/100), TrkB (794) (1/50), TrkB (C-13) (1/100), TrkB In pAb (1/100), TrkC⁷⁹⁸ (1/100), TrkC NC2 (1/200), or anti-p75^{LNGFR} (clone 8211) (1/150). For monoclonal p75^{LNGFR} antibodies, the slides were incubated directly with a rabbit anti-mouse alkaline anti-alkaline phosphatase (APAAP) complex, then revealed with Nitro blue-bromochloroindolylphosphate phosphatase substrate. For rabbit polyclonal antibodies, an incubation with a mouse anti-rabbit antibody (Dako) was performed before APAAP staining. For the chicken anti-TrkB In pAb, a rabbit anti-chicken IgY HRP conjugate (Promega) was used at a dilution of 1/100 followed by the mouse anti-rabbit step.

For fixed tissues, 5- μm sections were dried overnight at 37°C on SuperFrost/Plus glass slides (Menzel-Glaser, Braunschweig, Germany), deparaffinized in xylol, and brought to water through a graded alcohol series. Sections were then pretreated in a microwave oven in a 0.01 mol/L (pH 6.0) citrate buffer for 15 minutes. Endogenous peroxidase activity was inhibited with 1% H₂O₂-methanol for 5 minutes. Primary antibodies diluted in phosphate-buffered saline (Trk (C-14) (1/300), TrkA (763) (1/1500), TrkB (794) (1/500), TrkB (C-13) (1/300), TrkC (798) (1/300), r-TrkA (1/750), TrkB In pAb (1/300), TrkC NC2 (1/200), p75^{LNGFR} (1/1000)) were applied overnight on tissue sections. Immunodetection was performed with the biotin-streptavidin-peroxidase LSAB kit (Dako) for rabbit anti-Trks antibodies with an additional rabbit anti-chicken antibody for TrkB In pAb. A catalyzed signal amplification system (Dako) was also used with the monoclonal anti-p75^{LNGFR} antibodies. Sections were counterstained by hematein or slow Giemsa.

Frozen and paraffin-embedded fixed human and rat forebrains were processed as positive controls. For TrkA (763), TrkB (794), and TrkC (798), specific controls were performed on adjacent slides, either by omitting the primary or secondary antibody or by coincubation with either the corresponding homologous control peptide or the heterologous control peptide (Santa Cruz Biotechnology) as previously reported.^{38,39} Trk (C-14), TrkA (763), TrkB (794), and TrkC (798) IR were not altered by coincubation with the heterologous peptides and the Trk homologous peptide specifically abolished the signal.

To confirm the IR of each Trk, the stainings with antibodies reacting with different regions of each Trk receptor were compared, ie, Trk (C-14), TrkA (763), and r-TrkA

Table 1. Sequence of Primers Used for Analysis of Human *trk* and Neurotrophin Transcripts

Gene	Primer	Sequence (5' to 3')	Forward/Reverse	Position	GenBank
TrkA	ECD 5'	TCT TCA CTG AGT TCC TGG AG	Forward	1103-1122	M23102
	ECD 3'	TTC TCC ACC GGG TCT CCA GA	Reverse	1312-1331	-
TrkB	ECD 5'	TAC ATC TGT ACT AAA ATA CA	Forward	1098-1117	S76473
	ECD 3'	GTG TCC CCG ATG TCA TTC GC	Reverse	1323-1342	-
	Kin 5'	AGG GCA ACC CGC CCA CGG AA	Forward	2035-2054	-
	Kin 3'	GGA TCG GTC TGG GGA AAA GG	Reverse	2586-2605	-
	Trunc 5'	TAA AAC CGG TCG GGA ACA TC	Forward	1385-1404	S76474
	Trunc 3'	ACC CAT CCA GTG GGA TCT TA	Reverse	1526-1545	-
TrkC	ECD 5'	CAT CCA TGT GGA ATA CTA CC	Forward	1056-1075	S76475
	ECD 3'	TGG GTC ACA GTG ATA GGA GG	Reverse	1264-1283	-
	Kin 5'	CAC GCC AGG CCA AGG GTG AG	Forward	1946-1965	-
	Kin 3'	GAA TTC ATG ACC ACC AGC CA	Reverse	2539-2558	-
	Trunc 5'	AGG GAC ACA ACT GCC ACA AG	Forward	1571-1590	S76476
	Trunc 3'	AGC CAT GAC GTC CTT TGC TG	Reverse	1830-1849	-
NGF	NGF 5'	TAA AAA GCG GCG ACT CCG TT	Forward	9363-9382	V01511
	NGF 3'	ATT CGC CCC TGT GGA AGA TG	Reverse	9510-9529	-
BDNF	BDNF 5'	AAC AAT AAG GAC GCA GAC TT	Forward	322-341	M37762
	BDNF 3'	TGC AGT CTT TTT GTC TGC CG	Reverse	524-543	-
NT3	NT3 5'	CCC GAG AGC CGG AGC GGG GA	Forward	321-340	M61180
	NT3 3'	GTG ACT CTT ATG CTC CGC GT	Reverse	531-550	-
NT4	NT4 5'	AGC GAA ACT GCA CCA GCG AG	Forward	721-740	M86528
	NT4 3'	CAC CTT CCT CAG CGT TAT CA	Reverse	903-922	-
ABL	AblA2 5'	GCC GCT CGT TGG AAC TCC	Forward	270-297	X16416
	AblA2 3'	CTT CGA GTT CCA ACG AGC GG	Reverse	392-417	-

for TrkA; Trk (C-14), anti-TrkB In pAb, and TrkB (794) for kinase TrkB; and Trk (C-14) and TrkC (798) for kinase TrkC. Trk-immunoreactive cells were identified on the basis of morphological criteria and, in some instances, immunohistochemistry was performed on adjacent sections with one of the following antibodies: anti-vimentin, BNH9, -PS100, or -CD68 (KP-1) (Dako).

RT-PCR

RNA was extracted from frozen adult ($n = 7$) and fetal ($n = 3$, range, 16–25 weeks fetal age) bone marrow by using Trizol reagent (Gibco BRL, Gaithersburg, MD). Forty units of DNaseI (Boehringer Mannheim) were used per μg of RNA to remove potential genomic DNA contamination. Reverse transcription was performed for 1 hour at 37°C with 10 μg of total RNA using random hexamers (pd(N)6; Boehringer Mannheim), 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl_2 , 500 $\mu\text{mol/L}$ of each deoxynucleotide-5'-triphosphate and 400 U of superscript II RT (Gibco BRL) in a final volume of 40 μl . The reverse transcription step and the relative homogeneity of the cDNA concentration were controlled by amplifying the ubiquitously expressed *Abl* transcript with primers AblA2 5' and 3'. For each PCR, cDNA corresponding to 0.8 μg of total RNA was amplified with primers (Table 1 and Figure 1) designed to investigate the expression of NT and *trk* isoform transcripts. PCR was performed in 10 mmol/L Tris-HCl, pH 9, 50 mmol/L KCl, 1.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ of each deoxynucleotide-5'-triphosphate, 0.1% Triton X-100, with 50 pmol of each primer and 1.5 U of *Taq* polymerase (Promega) in a final volume of 50 μl . A 4-minute denaturation step at 94°C was followed by 36 cycles of 94°C for 1 minute, 64°C (TrkC Kin 5'–3') or 62°C (TrkC ECD 5'–3') or 60°C (TrkA ECD 5'–3', TrkB ECD 5'–3', TrkB Kin 5'–3', TrkB Trunc

Figure 1. Schematic diagram of the human *trk* receptor isoforms from Shelton et al.¹³ The primer sets location and the size of the expected RT-PCR products are summarized. ECD, extracellular domain; Kin, kinase domain; Trunc, truncated isoform.

5'–3', TrkC Trunc 5'–3', NGF 5'–3', BDNF 5'–3', NT3 5'–3', NT4 5'–3', AblA2 5'–3') for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes in an automated thermal cycler (Hybaid, Teddington, UK). To ensure the absence of residual DNA contamination, 0.8 µg of each RNA were amplified using the 5' and 3' BDNF primers without the reverse transcription step. Ten µl of each reaction were run onto a 2.5% agarose gel. Negative (reaction mixture without DNA template) and positive (cDNA from human caudate putamen) controls were included in each PCR set.

Sequencing

For each primer set, RT-PCR products from one adult bone marrow and from the human caudate putamen cDNA were submitted to nucleotide sequencing analysis. Briefly, PCR products were run onto a 10% acrylamide gel and the bands were excised and PCR-amplified using the appropriate set of primers as previously described. PCR products were purified through MicroSpin S300 Columns (Pharmacia Biotechnology, Uppsala, Sweden), then sequenced in both DNA strands using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA) on an automated Applied ABI 377A DNA sequencer (Perkin Elmer). Nucleotide sequence data were analyzed using the Sequence Navigator Software (Perkin Elmer) and a sequence comparison was made with the GenBank database using the Wisconsin Package (Genetics Computer Group, Madison, WI).

Results

Immunocytochemistry

We investigated the immunolocalization of p75^{LNGFR} and Trk receptors *in vivo* in fetal and adult human hematopoietic tissues. Immunostaining of bone marrow smears or sections produced comparable results, allowing precise identification of cells immunoreactive for the different NT receptors according to their distribution and cytomorphology (Figures 2 and 3, Table 2).

In adult bone marrow sections, the two anti-p75^{LNGFR} antibodies revealed a dense network of fibers and adventitial reticular cells (ARC) in the adventitial coat of the bone marrow vessels (Figure 2H), in the endo-osteal surface of bone trabeculae, or in bone marrow parenchyma in contact with hematopoietic cells. The p75^{LNGFR}-positive ARC had an oval nucleus, a triangular cytoplasm, and long, thin, branching dendrites better seen on bone marrow smears (Figures 2H and 3).

Pan-Trk IR, detected with the TrkA (C-14) antibody, overlapped with IR for each TrkA, TrkB, or TrkC catalytic

receptor. In adult bone marrow, IR for a given receptor was observed on specific hematopoietic cell types depending on their maturation stage (Figures 2 and 3). In erythroblastic islets, an intense cytoplasmic or membranous TrkA IR was seen on proerythroblasts and erythroblasts, whereas mature cells with a pycnotic nucleus were stained either weakly or not at all (Figure 2, A-D). A weak cytoplasmic TrkA IR was also seen in some macrophages (data not shown), and in a subset of ARC and plasma cells (Figure 2G). A cytoplasmic staining for the TrkB TK⁺ receptor was found on mature eosinophilic metamyelocytes and polymorphonuclear cells (data not shown), but not on immature eosinophilic cells or on neutrophilic or basophilic cells. Some plasmocytes and a few macrophages also showed a membranous and/or cytoplasmic TrkB TK⁺ IR (data not shown). Membranous and cytoplasmic staining for the truncated TrkB receptor was noted in all erythroblasts and megacaryocytes (Figures 2E and 3). Cytoplasmic staining for the TrkC TK⁺ receptor was found on promyelocytes, myelocytes, metamyelocytes, and polymorphonuclear cells, both in the neutrophilic and eosinophilic lineages and on megacaryocytes (Figure 2F). This staining was also observed on a subset of ARC and macrophages, whereas plasma cells had a membranous staining for TrkC. Interestingly, the staining for truncated TrkC TK⁻ receptor was observed on the same cellular subsets showing TrkC TK⁺ IR on both bone marrow sections and smears. In reactive mastocytosis, for mast cells there was weak staining for TrkA and intense cytoplasmic staining for TrkB TK⁺, TrkC TK⁺, and truncated TrkB and TrkC (data not shown).

At early fetal stages (16, 23, and 25 weeks), the p75^{LNGFR}-positive cells also formed a network spreading from the vessel adventitial layer into the bone marrow parenchyma (data not shown). Trk IR was weak or absent in a few hematopoietic cells with a similar pattern to adult bone marrow consisting in TrkA IR in erythroblasts, TrkB TK⁺ IR in eosinophils, TrkB TK⁻ IR in erythroblasts and megacaryocytes and TrkC TK⁺ and TK⁻ IR in megacaryocytes but not in myelocytes (data not shown). On these fetal bone marrow sections, osteoblasts were intensely stained for TrkB TK⁺ and osteoclasts were sometimes stained for TrkA and TrkC (data not shown). At 32 weeks fetal age, the bone marrow was hypercellular and the IR for each receptor was similar to the IR in adult samples.

RT-PCR Detection of Neurotrophins and their Receptor Transcripts

The RT-PCR study was performed to identify transcripts encoding for the different isoforms of the Trk family receptors, as summarized in Figure 1 according to Shelton

Figure 2. Immunohistochemical detection of p75^{LNGFR} and Trk in normal adult bone marrow: In the erythroblastic islets, a similar cytoplasmic and/or membranous TrkA IR was seen after immunostaining with both intracytoplasmic kinase TrkA (763) (A and C) and extracellular anti r-TrkA (B and D) antibodies. A truncated TrkB (C-13) IR was noted in erythroblasts and megaryocytes (E). In megacaryocytes, a strong TrkC IR was observed as a diffuse or focal cytoplasmic granular staining (arrowhead) (F). An intense cytoplasmic granular TrkC IR was also observed on immature promyelocytes and myelocytes (arrows) and in a few mature myeloid cells (F). A TrkA IR was observed in endothelial cells, in plasmocytes (arrowhead) and in adventitial reticular cells (ARC) (G). p75^{LNGFR} antibody also stained fibers in the adventitial coat of the larger capillaries (arrowhead) and ARC (arrows). Original magnifications, ×400 (A, B, E) and ×1000 (C, D, F-H).

Figure 3. Immunocytochemical detection of p75^{NGFR} and Trk in normal adult smears (APAAP method). Immunocytochemistry showed concordant results with IHC. Megacaryocytes (column 1) showed a diffuse intracytoplasmic truncated TrkB and an intense intracytoplasmic granular TrkC IR. In the granulocytic lineage (column 2), neutrophilic myelocytes, metamyelocytes and polymorphonuclear cells were TrkC immunoreactive. Erythroblasts (column 3) showed an intense TrkA IR (arrows) and, to a lesser degree, a truncated TrkB IR. p75^{NGFR} IR was restricted to adventitial reticular cells (arrowheads). Original magnification, $\times 1000$

et al.¹³ The specificity of each fragment was checked both by electrophoresis and by sequencing. The expression pattern of Trk receptor transcripts was found to be similar in adult and fetal bone marrow. All transcripts identified in human central nervous system¹³ were also detected in human bone marrow, except for the TrkAll isoform. Interestingly, the TrkA ECD primer set amplified a 229-bp fragment corresponding to the TrkAI isoform in human bone marrow, whereas the 247-bp fragment cor-

responding to the TrkAll-spliced variant was detected only in the human caudate putamen control (Figures 4 and 5). The TrkB primer sets amplified a 571-bp fragment coding for the kinase domain, a 245-bp fragment for the ECD, and a 161-bp fragment corresponding to the truncated TrkB T1 isoform.

TrkC ECD primers amplified two fragments of 204 and 228 bp corresponding to two alternatively spliced *trkC* transcripts. Although these fragments were amplified

Figure 4. Trk transcripts expression in adult bone marrow. Amplification of regions of TrkA, TrkB and TrkC containing potential insert coding for extra-cellular or tyrosine kinase domain. The size of the RT-PCR product is indicated on the right. Each lane corresponds to the same bone marrow sample analyzed by RT-PCR. CP, caudate putamen RNA. MW: 100-bp ladder (Gibco BRL).

equally in fetal bone marrow, the 204-bp fragment was repeatedly found to be less abundant than the 228-bp fragment in adult bone marrow (Figures 4 and 5). The sequence of the 204-bp *trkC* transcript was slightly different from that previously reported for human tissue.¹³ In both bone marrow and central nervous system samples, the splicing event led a 24-nucleotide in-frame deletion and an additional valine encoded by a GTT codon (Figure 6). TrkC kinase primers allowed the amplification of two 571- and 613-bp fragments corresponding to the two alternatively spliced transcripts.¹³ The shortest transcript was dominant in both fetal and adult bone marrow specimens, whereas the largest fragment was dominant within brain samples (Figures 4 and 5). The TrkC truncated primer set amplified a 279-bp fragment corresponding to a human truncated isoform.¹³ In addition, a 144-bp fragment was amplified in both bone marrow and brain samples. Sequencing analysis of this TrkC TK⁻ 144-bp frag-

Figure 5. Trk transcript expression in fetal bone marrow. Amplification of regions of TrkA, TrkB, and TrkC containing potential insert of extracellular domain or tyrosine kinase domain. The size of the RT-PCR product is indicated on the right. Each lane corresponds to the same bone marrow sample analyzed by RT-PCR. CP; caudate putamen RNA. MW: 100-bp ladder.

	ATCCATGTGGAACTACTACCAAGAGGGAGAGATTTCGAGGGCTGCCTCTTCAACAAG
TrkCII	I H V E Y Y Q E G E I S E G C L L F N K
TrkCI	I H V E Y Y Q E G E I S E G C L L F N K
	CCCACCCACTACAACTGGCACTATACCTTCATTGCCAAAAACCCACTGGGCACAGCC
TrkCII	P T H Y N N G N Y T L I A K N P L G T A
TrkCI	P T H Y N N G N Y T L I A K N P L G T A
	AACCAGACCATCAATGGCCACTTCTCAAGGAGCCCTTCCACAGGAGCAGGATACTTT
TrkCII	N Q T I N G H F L K E P F P E S T D N F
TrkCI	N Q T I N G H F L K E P F P P V
	ATCTGTGTGACGAAGTGAGTCCCAACCTCTATCAGTGAC
TrkCII	I L F D E V S P T P P I T V T
TrkCI	I L F D E V S P T P P I T V T

Figure 6. Nucleotide sequence and deduced amino acid sequence of the human *trkC* extracellular RT-PCR-generated fragments. The boxed nucleotide sequence (24 bp) is missing on the shorter isoform (TrkCI). Nucleotides corresponding to the primers TrkC ECD 5' and 3' are underlined.

Table 2. Immunological Detection of Neurotrophin Receptors in Human Normal Bone Marrow

Lineage/Cell type	p75 ^{LN^{GR}}	TrkA	TrkBk	TrkBt	TrkCk	TrkCt
Erythroblasts						
Proerythroblasts	—	+++	—	++	—	—
Erythroblasts	—	++	—	++	—	—
Granulocytes						
Promyelocytes	—	—	—	—	++	+++
Myelocytes	—	—	—	—	++	+++
Metamyelocytes	—	—	—	—	+	++
PMN	—	—	—	—	+	++
PMN eosinophils	—	—	++	—	+	++
Mastocytes	—	+	++	++	++	+
Megacaryocytes						
Megacaryocytes	—	—	—	+++	+++	++
Stroma						
ARC	+++	++	+	+	++	++
Macrophages	—	+	+	+	+	+
Osteoclasts	—	+/-	—	—	+	ND
Osteoblasts	—	—	++	ND	—	ND
Endothelial cells	—	++	—	—	+	++

PMN, polymorphonuclears; ARC, adventitial reticular cells; ND, not done.

—, negative; +, weak immunoreactivity (IR); ++, moderate IR; +++, strong IR, TrkBk, TrkB kinase; TrkBt, TrkB truncated; TrkCk, TrkC kinase; TrkCt, TrkC truncated.

Antibodies used for immunostaining: p75^{LN^{GR}}; p75^{LN^{GR}} clone 8211 (Boehringer Mannheim) and clone NGFR5 (Dako). TrkA: TrkA (763) (Santa Cruz Biotechnology), r-TrkA (gift of D.O. Clary). Kinase TrkB: TrkB (794) (Santa Cruz Biotechnology), trkB (Transduction Laboratories). Truncated TrkB: TrkB(TK-) (C-13), trkB (Transduction Laboratories). Kinase TrkC: TrkC (798) (Santa Cruz Biotechnology, Santa Cruz, CA). Truncated TrkC: TrkC NC2 (gift of F. Lamballe).

ment showed a 135-nucleotide in-frame deletion (Figure 7), homologous to the truncated rat TrkCic113,¹⁵ not previously reported in human tissue.¹³ The GenBank accession number for the human TrkC sequence (Figure 6)

Figure 7. A: Schematic diagram of the rat TrkC truncated splice isoforms as reported by Valenzuela et al.¹⁵ Four alternative spliced exons (exons A, B, C, and D) encode for four different isoforms. TrkC ic158 is the only truncated isoform previously reported in human tissue. B: Human TrkC truncated isoforms. Upper lane: nucleotide sequence of the two RT-PCR products (279 and 144 bp) generated with primers TrkC trunc by amplification of human bone marrow RNA. The boxed nucleotide sequence (135 bp) is missing on the shorter PCR product and is homologous to the rat exon B. Nucleotides corresponding to the primers TrkC trunc 5' and 3' are underlined. Predicted amino acid sequence is indicated for the two human TrkC ic158 and TrkC ic113 isoforms.

is AF058389 and for the human TrkC ic113 isoform sequence is AF058390.

All NT transcripts were amplified by RT-PCR. Complete removal of potential genomic DNA contamination was strictly checked for all samples (Figure 8). The sequences of NT cDNA fragments were found to be identical with the related GenBank human sequences. All cDNA corresponding to NGF, BDNF, NT-3, and NT-4 transcripts were found to be more abundant in fetal samples than in adult samples (Figure 8). These differences were found in at least two separate experiments. Constant low levels of NGF and BDNF transcripts were found in adult bone marrow, whereas inconstant NT-3 and NT-4 transcripts were barely detected.

Discussion

The multiple functions of NGF on immune or bone marrow-derived cells have been studied mainly at the peripheral blood level.²⁷ NGF induces proliferation of both human B and T lymphocytes and is a survival factor for memory B cells.^{26,28} NGF also triggers several biological functions on mature myeloid cells such as monocytes,²² basophils^{32,42} and mast cells,^{23,24} which accordingly express TrkA receptors. NGF promotes the growth and differentiation of myeloid and erythroid progenitors,^{30,43} but the distribution of NT receptors (except for p75^{LN^{GR}}) has not been investigated in the bone marrow.³⁴

Our study concentrated on the identification of cells expressing NT receptors, which may represent targets for locally synthesized NT. Certain hematopoietic cells were found to be immunoreactive for only one member of the Trk family receptors. For example, neutrophil precursors expressed both TK⁺ and TK- TrkC but not TrkA or

Figure 8. Neurotrophin transcript distribution in human bone marrow. Amplification of NGF, BDNF, NT-3, and NT-4 transcripts in four adult and three fetal bone marrow samples which have been studied for Trk receptor expression (see Figures 4 and 5). Each lane corresponds to the same sample analyzed for the presence of the different transcripts. The size of the RT-PCR product is indicated on the right. Neurotrophin RT-PCR fragments are located on one exon. To ensure complete removal of potential DNA contamination, the amplification reaction was performed without the reverse transcription step (BDNF PCR without RT panel) for all samples except the caudate putamen RNA. The constitutively expressed Abelson (Abl) transcript was amplified by RT-PCR to ensure homogeneity within our sample. Adult bone marrow Abl amplification product was slightly less abundant than fetal and caudate putamen products. CP, caudate putamen RNA. MW: 100-bp ladder.

TrkB. Among the NT, NGF is unique in priming human blood basophils, which express only TrkA receptors.⁴² However, most cells were immunoreactive for several Trk receptors and in turn may respond to several NT. For example, an intense TrkB TK⁺ IR was found on mature eosinophils that were also immunoreactive for TK⁺ TrkC. Other cells were immunoreactive for a given catalytic TK⁺ receptor and for another truncated TK- receptor, like erythroblasts expressing TrkA and TK- TrkB IR. Some cells, like mastocytes or ARC, were immunoreactive for all Trk receptors. In macrophages, the weak cytoplasmic staining for all Trk receptors could result from phagocytosis and should be compared with the *in situ* detection of the corresponding transcripts.

A direct link between the distribution of NT receptors in the bone marrow and the peripheral function of NT is difficult to establish for several reasons. First, these functions have not yet been defined for NT other than NGF. The IR for both TK⁺ and TK- TrkC receptors suggests that NT3 could promote megacaryocyte differentiation, but only NGF has been studied and shown to induce platelet shape changes.²⁹ Second, a development-related switch in the pattern of NT receptor expression may occur, as observed in embryonic sensory neurons.⁴⁴ Both in the erythroid and the neutrophilic lineage, the IR for TrkA or TrkC respectively decreased in mature cells, suggesting

that NT support specific stages of hematopoiesis. Indeed, NGF, in synergy with stem cell factor, supports the short-term maintenance of CD34⁺/CD38⁺ erythroid progenitors.⁴³ Similarly, a decline in TrkB expression was observed during thymocyte differentiation,³⁸ whereas TrkA expression was detected in activated peripheral CD4⁺ T cells.²⁵ Third, the pattern of NT receptor expression may also change after cellular isolation or stimulation, thus accounting for some discrepancies. Whereas NGF promotes survival and functional properties of murine neutrophils and eosinophils,^{45,46} NT receptor transcripts were not detected in human peripheral blood granulocytes.³⁷ However, the myeloid (TF1 and KG1) cells and some leukemia cell lines have been shown to express *trkA* transcripts.^{35,36,43,47} In human blood monocytes, *trkA* expression has been shown to decrease during macrophage differentiation²² and was absent in monocytic (U937 and THP1) cell lines.^{35,36} However, NGF triggers phagocytosis, parasite killing, and interleukin-1 β production by macrophages through activation of the TrkA receptor,⁴⁸ whose expression can be enhanced by several inflammatory molecules.^{22,26} Finally, the expression of TrkA and p75^{LNGFR} in PC12 cells was recently shown to alternate in a cell phase-specific manner.⁴⁹

Whatever their differentiation stage, hematopoietic cells were not found to be immunoreactive for p75^{LNGFR}, suggesting that NT signaling may occur through Trk receptor homodimers in such cells.¹⁹ Indeed, activation of TrkA receptors in the absence of p75^{LNGFR} has been shown to mediate NGF biological effects in several hematopoietic cell types including the erythromyeloid human cell line K562,³⁶ human basophils,⁴² rat and human mast cells, and cell lines.^{23,24,50} The absence of p75^{LNGFR} is a common feature of immune or hematopoietic cells.²⁷ However, the coexpression of p75^{LNGFR} and TrkA has been detected in certain immune cells such as human B lymphocytes^{28,51} and bone marrow-derived mouse mast cells.⁵² In addition, bone marrow stromal cells were found to be immunoreactive for all NT receptors including p75^{LNGFR}. As for specific sets of neurons,⁵³ p75^{LNGFR} may modulate the interaction of a defined NT to its specific Trk receptor on bone marrow stromal cells. p75^{LNGFR} also signals on its own⁵⁴ and triggers neuronal apoptosis in the absence of NGF-induced TrkA activation.^{55,56}

The generation of TK⁺ or TK- receptor isoforms, which retain NT high-affinity binding sites, may also modulate the local function of NT.⁵ For example, variants with insertion in the TK⁺ domain of TrkC have been shown to retain autophosphorylation on NT3 binding, but could no longer phosphorylate specific cellular substrates.¹⁴ NT also bind to truncated TK- receptors, identified for TrkB and TrkC,¹⁵⁻¹⁸ which may function as dominant negative isoforms,²⁰ or immunoadhesins.¹³ Using RT-PCR, we detected transcripts encoding for all TrkA1, TrkB, and TrkC human catalytic TK⁺ receptors except those encoding for the TrkAII isoform. Their absence within the fetal and adult bone marrow, as opposed to their exclusive expression in the human caudate putamen, suggests a tissue-specific regulation of the splicing event in humans that has not been observed in the rat.¹² Although both TrkA

isoforms are comparably activated by NGF,¹² TrkA is not activated by NT3,⁵⁷ which might exclude cross-talk between NT3 and TrkA at the bone marrow level. Two analogous spliced TrkC isoforms identified in the human nervous system¹³ were also detected in the bone marrow. The nucleotide sequence of the shortest transcript was further characterized by showing that the splicing event results in the deletion of 9 amino acids in the ECD but with an additional valine. Two transcripts differing by a nucleotide sequence encoding for a 14-amino acid insertion in the TrkC TK⁺ domain were detected in the bone marrow as they were in human central nervous system.¹³ The detection of transcripts encoding for the homologues of rat TrkB1 TK- receptor¹⁸ and rat TrkC158 TK- and TrkC113 TK- receptors¹⁵ shows that the expression pattern of *trk* family genes is as complex in the bone marrow as it is in the nervous system.

Similar levels of transcripts encoding for TK⁺ and TK- receptors of the Trk family were detected from fetal stages to adulthood. The higher abundance of NT transcripts at fetal stages paralleled a higher content of stromal cells, arguing for their role in the local biosynthesis of NT. During development, NGF may be synthesized by mesenchymal target cells of sensory and sympathetic neurons.⁵⁸ NGF or *trkA* expression may also be supported by osteoblasts or osteoclasts.⁵⁹ Interestingly, the murine bone marrow stromal cell line MS-5 has been shown to produce both NGF and stem cell factor.⁴³ The constitutive expression of several NT in certain leukemia cell lines and in human cord blood-derived mast cells also suggests an autocrine role for NT on hematopoietic cells.^{24,47} The cellular source of NT within the bone marrow needs to be further characterized by cellular separation or *in situ* hybridization studies. The wide distribution of NT receptors in the bone marrow suggests that NT may play an important role during the onset of hematopoiesis and on the differentiation of specific hematopoietic progenitors and precursors throughout life. The comparative study of hematopoietic progenitors isolated from normal mice and from knockout animals for either *trk* or NT genes may help to investigate these interactions. Finally, the presence of NT receptors on bone marrow stromal and hematopoietic cells highlights the potential side effects of systemic administration of NT in neurodegenerative diseases.⁶⁰

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