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Regulation and function of the CGRP receptor complex in human granulopoiesis

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Objective. **Anatomic studies and animal experiments suggest that neuropeptides such as calcitonin gene-related peptide (CGRP) may be involved in hematopoiesis. Here, we examined the regulation and function of the CGRP receptor in human granulopoiesis.**

Materials and Methods. **Expression of CGRP receptor components on $CD34^+$ cells, peripheral blood granulocytes, and in vitro differentiated $CD34^+$ cells was analyzed at the mRNA level and by measuring the signaling capacity of the receptor. The function of CGRP in human hematopoiesis was investigated by clonal colony formation assays.**

Results. **mRNA transcripts for the cell surface CGRP receptor subunits receptor activity-modifying protein-1 (RAMP-1) and calcitonin receptor-like receptor (CRLR) as well as for the intracellular adapter protein CGRP-receptor component protein (CGRP-RCP) were found in $CD34^+$ cells from 4/4 donors tested. CGRP-RCP mRNA was expressed in peripheral blood granulocytes of 8/15 donors, whereas RAMP-1 and CRLR were not detectable. $CD34^+$ cells, but not granulocytes, exhibited a marked elevation of cellular cAMP after CGRP stimulation, thereby confirming the mRNA expression data. Both RAMP-1 and CRLR mRNA expression and CGRP receptor signaling capacity were lost during in vitro granulocytic differentiation of $CD34^+$ cells. Consistent with a role of CGRP in hematopoiesis, we show that CGRP significantly enhances the formation of granulomonocytic, but not erythroid or mixed, colonies by purified human $CD34^+$ cells.**

Conclusion. **The CGRP receptor is expressed on $CD34^+$ hematopoietic progenitor cells and is downregulated during granulocytic differentiation. CGRP directly acts on $CD34^+$ cells to promote formation of granulomonocytic colonies. Thus, CGRP may have a function in directing hematopoiesis.** **© 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

The human cell surface receptor for calcitonin gene-related peptide (CGRP) has been cloned recently and was found to consist of at least two components. While the seven-transmembrane protein calcitonin receptor-like receptor (CRLR) couples the receptor complex to heterotrimeric G proteins, the receptor activity-modifying protein (RAMP)-1 acts as a chaperone for CRLR and determines the specificity of the heterodimer for CGRP [1]. CRLR may also associate with RAMP-2 or RAMP-3, thereby generating receptors for the neuropeptide adrenomedullin [1]. In addition, recent work has indicated that an intracellular protein termed CGRP-receptor component protein (CGRP-RCP) coimmunoprecipitates with CRLR and is required for both CGRP- and adrenomedullin-mediated signal transduction [2]. Stimulation of cells with the neuropeptide CGRP generally results in increased cellular cAMP levels due to coupling of the CGRP receptor to Gαs proteins [3].

CGRP is a potent vasoactive neuropeptide that has been implicated in vasodilation, migraine, and chronic pain [3]. However, CGRP may also exhibit functions in neuroimmunological communication by mediating distinct immunosuppressive activities. Stimulation with CGRP inhibits the antigen-presenting capacity of dendritic cells by reducing ex-

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pression of major histocompatibility complex (MHC) class II and B7 proteins and by augmenting production of interleukin (IL)-10 [4–7]. Consistent with in vitro experiments showing that CGRP impairs the production of interferon (IFN)-γ by splenocytes and Th1 cells [8], administration of CGRP to mice was shown to inhibit delayed-type and contact hypersensitivity responses [7,9]. Moreover, CGRP may modulate the adhesion and migration of immune cells including T cells, eosinophilic granulocytes, and dendritic cells [10–12].

Several lines of evidence suggest that neuropeptides including CGRP may also be involved in the regulation of hematopoiesis in the bone marrow. CGRP and other neuropeptides have been demonstrated in nerve fibers projecting into both primary lymphoid organs and bone marrow [7,13,14]. Low levels of CGRP receptors have been detected on nonseparated murine bone marrow cells [15]. Mechanical denervation of murine femora or depletion of neuropeptides by treatment of mice with capsaicin was found to severely reduce normal blood cell production and CFU-GM (colony-forming unit-granulocyte/macrophage) activity in bone marrow [16,17]. Moreover, CGRP but not other neuropeptides had a direct stimulatory effect on myeloid progenitor cells [17]. Consistent with these animal studies, a recent clinical investigation has shown that in patients with stable and complete spinal cord injury, long-term colony formation of all hematopoietic cell lineages by decentralized bone marrow was substantially reduced [18].

In the present study, we investigated the role of the CGRP receptor for human granulopoiesis. Our results show that the receptor components RAMP-1 and CRLR are downregulated during myeloid differentiation of $CD34^+$ cells and that CGRP selectively promotes the development of CFU-GM.

Materials and methods

Cells

After informed consent, cord blood (CB) or granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood (PB) cells from patients undergoing autologous stem cell transplantation for lymphoproliferative diseases were obtained. Peripheral blood granulocytes were isolated by Ficoll density centrifugation (Biochrom, Berlin, Germany) followed by lysis of erythrocytes. $CD34^+$ cells were purified using immunomagnetic beads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated $CD34^+$ cells was evaluated by flow cytometry (EPICS XL, Beckman Coulter, Hialeah, FL, USA) and ranged between 87 and 98%.

In vitro granulopoiesis

To induce myeloid differentiation, $CD34^+$ cells were cultured for the indicated time periods in 25 cm^2 culture flasks in presence of 25 ng/mL recombinant human stem cell factor (SCF; Amgen, Thousand Oaks, CA, USA) and 200 ng/mL G-CSF (Amgen). RPMI 1640 medium (Gibco/BRL, Paisley, UK) was supplemented with 10 mM Hepes, nonessential amino acids, 10^{-4} M 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 U/mL penicillin/streptomycin, and 10% fetal calf serum (FCS; Biochrom) with half-medium changes 1 to 2 times per week.

Semiquantitative RT-PCR

Total cellular RNA was extracted using Tri-reagent (Sigma, St. Louis, MO, USA). First strand cDNA was synthesized from 10 μg of total RNA using a mixture of oligo$(dT)_{12–18}$ and random hexamer primers and incubated for 10 minutes at 70°C. Superscript reverse transcriptase II (Gibco/BRL) and dNTPs were added for strand elongation. The reaction was incubated for 60 minutes at 37°C and terminated by heating to 95°C for 5 minutes. Undiluted cDNA or serial 1:3 cDNA dilutions were used as template in PCR reactions. For amplification of cDNA fragments of the CGRP receptor components, primers specific for RAMP-1 (forward: 5′-GAG ACG CTG TGG TGT GAC TG-3′; reverse: 5′-GTA AGT CAA GGT CAC GTC CCT-3′), CRLR (forward: 5′-AGT TCA TTC ATCTTT ACC TGA TGG-3′; reverse: 5′-CTC AGA ATT GCT TGA ACC TCT CC-3′), or CGRP-RCP (forward: 5′-CAG CCA TTT CCT GGA CGT T-3′; reverse: 5′-TAT CTC TGT TGT TCT CGA GG-3′) were used. For control, a 471-bp fragment of human GAPDH was amplified using specific forward (5′-AAC TGC TTA GCA CCC CTG GGC-3′) and reverse (5′-CAC CAC CCT GTT GCT GTA GCC-3′) primers. Primer sequences of GAPDH were separated by introns to control for contaminations with genomic DNA. The amplification reactions were allowed to proceed 30 cycles each, consisting of a 1-minute denaturation step at 94°C, a 1-minute annealing step at 63°C, and a 1-minute extension step at 72°C. For amplification of CGRP-RCP a hot start was performed, after a first denaturation step at 94°C for 5 minutes. After adding Taq polymerase the cycling proceeded using an annealing temperature of 58°C. To normalize mRNA levels, the cDNA titers for RAMP-1, CRLR, and CGRP-RCP were divided by GAPDH titers obtained with the same cDNA template.

For some experiments, PCR products were blotted onto a nitrocellulose membrane and hybridized with cDNA probes spanning the coding regions of RAMP-1, CRLR, or GAPDH using standard protocols. cDNA probes were labeled with [α^{32}P]dCTP (Amersham Pharmacia Biotech, Freiburg, Germany) using the high prime kit (Roche Diagnostics GmbH, Mannheim, Germany).

Determination of cellular cAMP levels

To determine cellular cAMP levels, cells were washed and resuspended in phosphate-buffered saline (PBS) and the endogenous cAMP phosphodiesterase was blocked with 300 μM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO, USA). Cell stimulation was performed with 100 nM CGRP (Bachem, Heidelberg, Germany) or 1 μM prostaglandin E_2 (PGE_2; Sigma) for the indicated time periods. In experiments performed to control for receptor specificity, cells were incubated with 1 μM $CGRP_{8–37}$ (Bachem) and 10 nM CGRP. Thereafter, cells were lysed by 0.1 M HCl and lysates were acetylated to yield a stable cAMP product. Cellular cAMP concentrations were measured by an enzyme immunoassay according to the manufacturer's protocol (Biomol, Plymouth Meeting, PA, USA).

Clonal colony assay

Human $CD34^+$ cells were freshly isolated and 250 cells were plated in 1 mL methylcellulose containing 30% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, and human cytokines (Stem Cell Technologies Inc., Vancouver, Canada) in 35 mm^2 tissue culture dishes in duplicate

and incubated at 37°C and 5% CO_2. Human cytokines were present at the following concentrations: SCF 50 ng/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF) 20 ng/mL, interleukin (IL)-3 20 ng/mL, IL-6 20 ng/mL, G-CSF 20 ng/mL, and erythropoietin (EPO) 3 U/mL. After 14 days of culture, plates were scored by an investigator blinded to the culture conditions for the presence of erythroid (BFU-E), granulomonocytic (CFU-GM), and mixed (CFU-Mix) colonies. Colonies were identified by standard morphological criteria using an inverted microscope.

Statistical analysis

Statistical analysis of data was performed using the Student's *t*-test for paired samples. All data are presented as mean values ± SD. Differences between experimental groups were considered significant for $p < 0.05$.

Results

Expression of the CGRP receptor in CD34$^+$ cells but not in peripheral granulocytes

The present study addressed the question as to whether the neuropeptide CGRP may exhibit a function in human granulopoiesis. To this end, expression of mRNA for the CGRP receptor components RAMP-1, CRLR and CGRP-RCP was determined in freshly isolated CD34$^+$ PB and CB progenitor cells and peripheral blood granulocytes by RT-PCR. We found that mRNA transcripts for RAMP-1, CRLR, and CGRP-RCP were present in CD34$^+$ cells from all donors tested (n = 8). In contrast, RAMP-1 and CRLR mRNAs were not detectable in peripheral blood granulocytes of numerous donors (n = 15). Transcripts for CGRP-RCP were found in granulocytes from 8 of 15 donors tested. RT-PCR results from representative donors are depicted in Figure 1A. Moreover, the lack of RAMP-1 and CRLR expression in granulocytes was confirmed by blotting RT-PCR samples of 4 donors and hybridization with specific probes (Fig. 1B). The results therefore indicate that the CGRP receptor components RAMP-1 and CRLR, but not CGRP-RCP, were differentially expressed by CD34$^+$ cells and granulocytes.

To examine whether mRNA expression was correlated with the presence of functional CGRP receptors, the signaling capacity of the CGRP receptor was determined by measuring changes of cellular cAMP levels in response to stimulation with 100 nM CGRP. The results in Figure 2A show that CD34$^+$ cells responded to CGRP stimulation with a rapid and marked elevation of cellular cAMP. To confirm the specificity of cAMP induction by CGRP in CD34$^+$ cells, experiments were performed in the presence of the specific CGRP receptor antagonist $CGRP_{8-37}$ [19]. As is shown in Figure 3, coincubation of CD34$^+$ cells with a 100-fold excess of $CGRP_{8-37}$ completely prevented the increase of cellular cAMP levels induced by CGRP. In contrast, CGRP did not increase cAMP levels in peripheral blood granulocytes (Fig. 2A). Granulocytes were nonresponsive to CGRP stimulation irrespective of whether they did or did not express CGRP-RCP mRNA. Control experiments showed, however, that granulocytes responded to PGE_2 exposure with a marked elevation of cellular cAMP, indicating that the purification methods did not abolish the capacity of these cells to mount a cAMP response (Fig. 2B). Collectively, these results therefore indicate that a functional CGRP receptor is expressed by freshly isolated CD34$^+$ progenitor cells but not by mature granulocytes.

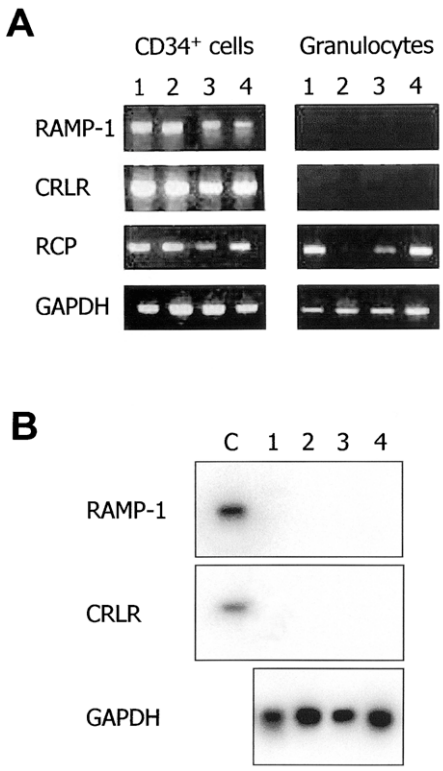

Figure 1. Differential expression of CGRP receptor components by CD34$^+$ cells and granulocytes. **(A)** RNA was isolated from purified CD34$^+$ cells or granulocytes and used for RT-PCR analysis of RAMP-1, CRLR, and CGRP-RCP expression. GAPDH served as a control. Each number indicates an individual donor for whom a representative experiment is shown. **(B)** RT-PCR samples of 4 donors were blotted onto nitrocellulose membranes and hybridized with probes specific for RAMP-1, CRLR, or GAPDH. Each number represents an individual donor. RT-PCR samples from HEK293 cells were used as a positive control for expression of RAMP-1 and CRLR (lane C).

Downregulation of the CGRP receptor during in vitro granulopoiesis

Our results suggested that expression of the CGRP receptor is regulated during human granulopoiesis. To directly address this question CD34$^+$ cells were differentiated in vitro by incubation with SCF and G-CSF and RNA was isolated 2, 5, 13, and 20 days later. RAMP-1 and CRLR mRNA levels were determined by semiquantitative RT-PCR. The results in Figure 4 and Table 1 show that mRNAs for both RAMP-1 and CRLR were downregulated during in vitro

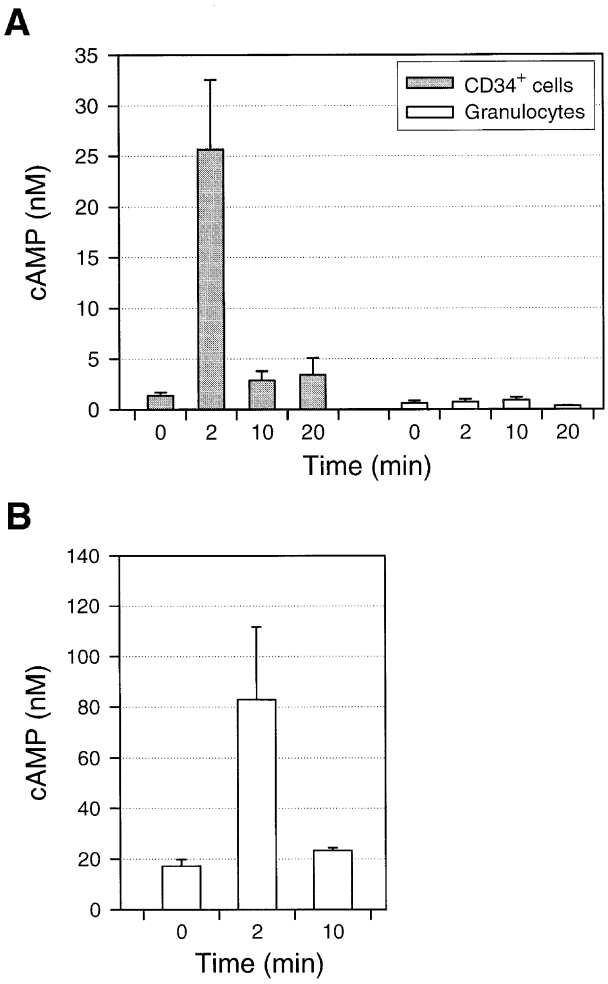

Figure 2. CGRP induces elevated cAMP levels in $CD34^+$ cells but not granulocytes. **(A)** Purified $CD34^+$ cells and peripheral blood granulocytes were stimulated with 100 nM CGRP for the indicated time periods and cellular cAMP levels were determined. Results are derived from 3–4 independent donors each. **(B)** Peripheral blood granulocytes were isolated from 3 independent donors, stimulated with 1 μM PGE_2 for the indicated time periods, and cellular cAMP levels were measured.

granulopoiesis. Although both receptor subunits were expressed at day 2 and day 5 of culture, mRNA levels continuously declined during in vitro differentiation and were not detectable at late time points (day 13 or day 20). To further corroborate these findings, CGRP receptor function and responsiveness of in vitro differentiated cells to stimulation with CGRP was analyzed. The results in Figure 5A demonstrate that $CD34^+$ cells that were incubated with G-CSF und SCF for 2 days exhibited a marked increase in cellular cAMP levels after CGRP stimulation. In contrast, $CD34^+$ cells stimulated for 14 days with G-CSF and SCF did not reveal any CGRP responsiveness (Fig. 5B). These results therefore indicate that human granulopoiesis is associated with a structural and functional loss of CGRP receptor expression.

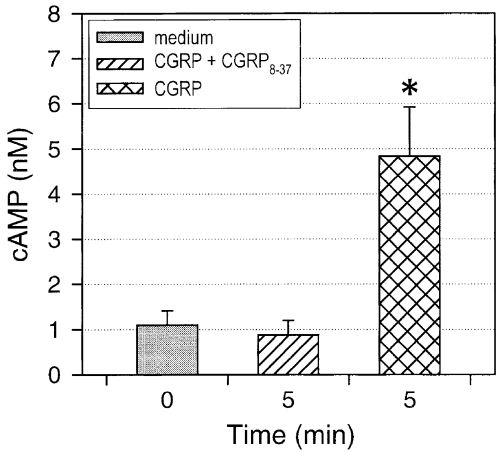

Figure 3. Specificity of cAMP induction in $CD34^+$ cells by CGRP. For competitive inhibition of CGRP receptors, purified $CD34^+$ cells were incubated with 1 μM $CGRP_{8–37}$ and subsequently stimulated with only 10 nM CGRP. $CD34^+$ cells were stimulated with 10 instead of 100 nM CGRP to avoid addition of excessive amounts of $CGRP_{8–37}$. Due to its low affinity for the CGRP receptor, $CGRP_{8–37}$ has to be present in 100-fold or greater excess over CGRP to inhibit signal transduction [19]. Cellular cAMP concentrations were measured after 5 minutes, because in most experiments maximal cAMP levels were observed between 2 and 10 minutes of stimulation. Results are derived from 4 independent experiments. * $p < 0.05$ (CGRP vs unstimulated or CGRP + $CGRP_{8–37}$).

CGRP supports in vitro myelopoiesis

Previous studies in the mouse system have shown that in the presence of IL-3, exposure of hematopoietic progenitor cells to CGRP directly promotes myelopoiesis [17]. We therefore examined whether CGRP might also stimulate myeloid differentiation of human hematopoietic progenitors. Purified $CD34^+$ cells were incubated with SCF, GM-CSF, IL-3, IL-6, and EPO in the presence or absence of CGRP and formation of granulomonocytic, erythroid, or mixed colonies was quantified. Absolute numbers of colonies in the absence of neuropeptide were 33.4 ± 5.4 (CFU-GM), 24.8 ± 5.2 (BFU-E), and 4.7 ± 1.7 (CFU-Mix). Consistent with the proposed role of CGRP in myelopoiesis, the results in Figure 6 show that CGRP increased the formation of CFU-GM by about 1.2-fold. This CGRP-induced increase in CFU-GM numbers was significantly and completely blocked by the addition of excess CGRP receptor antagonist $CGRP_{8–37}$. In contrast, CGRP did not affect the development of erythroid (BFU-E) or mixed (CFU-Mix) colonies (Fig. 6). Collectively, these findings therefore indicate that CGRP preferentially promotes granulopoiesis of human $CD34^+$ cells.

Discussion

Results of the present report reveal that human $CD34^+$ hematopoietic progenitor cells express functional receptors for the neuropeptide CGRP. Binding of CGRP to $CD34^+$ cells resulted in a rapid and strong increase of intracellular cAMP levels. The receptor specificity of this signaling event was

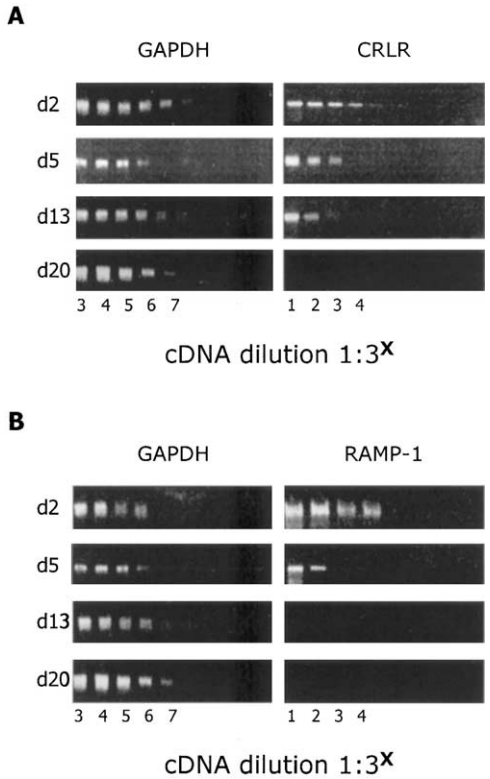

Figure 4. Downregulation of RAMP-1 and CRLR mRNA during in vitro granulopoiesis. RNA was isolated from $CD34^+$ cells after the indicated time periods of in vitro stimulation with SCF and G-CSF. Serial 1:3 cDNA dilutions were used as template for RT-PCR analysis to detect RAMP-1, CRLR, or GAPDH transcripts. Results from a representative experiment are shown.

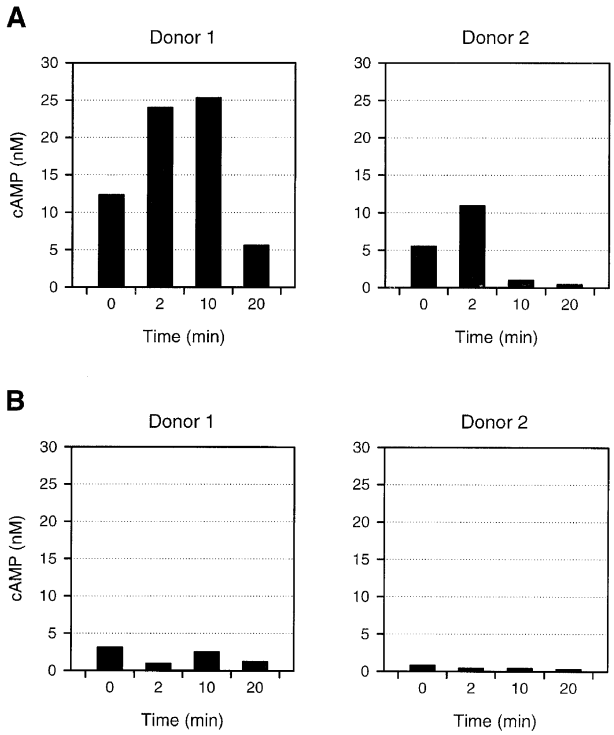

Figure 5. CGRP responsiveness is lost during in vitro granulopoiesis. Human $CD34^+$ cells were cultured for 2 days **(A)** or 14 days **(B)** in the presence of SCF and G-CSF. Cells were stimulated with 100 nM CGRP for the indicated time periods and cellular cAMP levels were determined.

confirmed by inhibition with the selective CGRP receptor antagonist $CGRP_{8–37}$. Furthermore, we show that CGRP responsiveness was downregulated during in vitro granulopoiesis and was completely lost in mature granulocytes.

Table 1. Regulation of RAMP-1 and CRLR during in vitro granulopoiesis

Receptor component	In vitro differentiation	mRNA ratio (normalized against GAPDH)		
		Donor #1	Donor #2	Donor #3
RAMP-1	d 2	0.02	0.11	0.0001
	d 5	0.03	0.04	0.0001
	d 13	0	0	0
	d 20	0	0	0
CRLR	d 2	0.01	0.04	0.0001
	d 5	0.004	0.04	0.0001
	d 13	0	0.004	0
	d 20	0	0	0

Serial 1:3 dilutions of cDNA derived from in vitro differentiated $CD34^+$ cells were used as templates for PCR amplification to detect expression of RAMP-1 and CRLR. Normalized cDNA titers of RAMP-1 and CRLR, defined as the final dilutions yielding detectable amplification products divided by the GAPDH titer derived from the same cDNA template, are given.

These results are consistent with a previous report demonstrating the presence of about 3000 high-affinity CGRP binding sites/cell on mouse bone marrow cells [15]. However, cell populations expressing CGRP receptors as well as the structure of the receptor and its regulation during hematopoiesis have not been defined in this study. Because our results indicate that only a fraction of cells present in bone marrow may express CGRP receptors, the actual number of binding sites on hematopoietic progenitor cells may be considerably higher. Collectively, the regulated expression of CGRP receptors on hematopoietic progenitor cells, along with the presence of CGRP in nerve endings in bone marrow parenchyma [13,14], is consistent with a functional role of CGRP in hematopoiesis.

A cell surface receptor for CGRP is generated by association of RAMP-1 and CRLR proteins [1]. The cytosolic adapter protein CGRP-RCP binds to CRLR and is required for signal transduction through CGRP receptors [2]. Although mRNAs for RAMP-1 and CRLR were detected in multiple tissues [1,20], expression and regulation of the various CGRP receptor components in hematopoietic cells have not been characterized yet. The present study shows that RAMP-1 and CRLR are coregulated during human granulopoiesis. While both receptor components are detected in $CD34^+$ progenitor cells, expression is lost in mature granulocytes. Expression of RAMP-1 and CRLR was found to be directly linked to

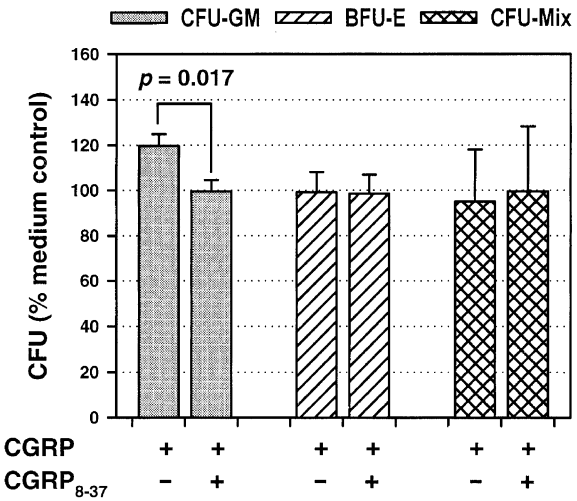

Figure 6. CGRP selectively promotes development of CFU-GM. Purified human $CD34^+$ cells (250/1-mL dish) were incubated with SCF, GM-CSF, IL-3, IL-6, and EPO. Formation of CFU-GM, BFU-E, and CFU-Mix was scored after 14 days. CGRP (1 nM) or $CGRP_{8–37}$ (100 nM) were added to the cultures as indicated. Results are presented as percent colonies observed without addition of CGRP or $CGRP_{8–37}$ (medium control). Absolute numbers of colonies in medium controls were 33.4 ± 5.4 (CFU-GM), 24.8 ± 5.2 (BFU-E), and 4.7 ± 1.7 (CFU-Mix). Results are derived from 8 independent experiments.

CGRP responsiveness, indicating that these proteins are essential components of the CGRP receptor on hematopoietic cells. CGRP-RCP was also detected in $CD34^+$ cells but, in contrast to RAMP-1 and CRLR, expression of CGRP-RCP was maintained on mature granulocytes in 8 out of 15 donors tested. Thus, our results provide evidence that in hematopoietic cells, CGRP-RCP may be regulated independently of RAMP-1 and CRLR. Consistent with this observation, CGRP-RCP was detected in COS-7 cells, which express neither RAMP-1 nor CRLR [2]. It is therefore tempting to speculate that CGRP-RCP may also be involved in signaling events by cell surface receptors that do not associate with the CRLR protein.

In the mouse, in vivo depletion of the neuropeptides, CGRP and substance P, by capsaicin treatment resulted in a substantial decline of neutrophil production [17]. Whereas both neuropeptides significantly stimulated CFU-GM production by unfractionated bone marrow, only CGRP had a direct stimulatory effect on highly enriched progenitor cells. In contrast to CGRP, substance P may therefore stimulate granulopoiesis in an indirect manner, possibly by promoting the release of soluble mediators from bone marrow stromal cells [21] or by regulating adhesive events between progenitor cells and stromal elements. Although demonstrating hematopoietic activity of the neuropeptides CGRP and substance P, the receptors mediating these effects were not identified by these studies.

The present study confirms and extends the findings revealing significant hematopoietic activity of CGRP for human progenitor cells. We show that expression of RAMP-1, CRLR, and CGRP-RCP by $CD34^+$ progenitor cells is directly associated with the presence of functional CGRP receptors. Loss of RAMP-1 and CRLR expression during in vitro granulopoiesis also correlated with the loss of CGRP responsiveness. Moreover, our results indicate that CGRP not only binds to purified $CD34^+$ cells but also directly promotes their CFU-GM production. It should be noted that incubation with CGRP led to elevated CFU-GM numbers even though $CD34^+$ cells were cultured in the presence of optimal concentrations of factors that stimulate granulomonocytic differentiation. Furthermore, we found that primary human bone marrow stromal cells do not respond to CGRP treatment by changes in cellular cAMP levels (data not shown). It therefore appears unlikely that the effects of CGRP may be mediated indirectly through stimulation of stromal cells. Considered together with previous reports [16–18], our results therefore support the concept that neuroendocrine mechanisms exhibit important effects on hematopoiesis and that CGRP is an essential element of the neural input of bone marrow.

Acknowledgments

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