

Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5)

(hemopoiesis/lymphokine/colony-stimulating factor)

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ABSTRACT The human eosinophil differentiation factor (EDF) gene was cloned from a genomic library in λ phage EMBL3A by using a murine EDF cDNA clone as a probe. The DNA sequence of a 3.2-kilobase *Bam*HI fragment spanning the gene was determined. The gene contains three introns. The predicted amino acid sequence of 134 amino acids is identical with that recently reported for human interleukin 5 but shows no significant homology with other known hemopoietic growth regulators. The amino acid sequence shows strong homology ($\approx 70\%$ identity) with that of murine EDF. Recombinant human EDF, expressed from the human EDF gene after transfection into monkey COS cells, stimulated the production of eosinophils and eosinophil colonies from normal human bone marrow but had no effect on the production of neutrophils or mononuclear cells (monocytes and lymphoid cells). The apparent specificity of human EDF for the eosinophil lineage in myeloid hemopoiesis contrasts with the properties of human interleukin 3 and granulocyte/macrophage and granulocyte colony-stimulating factors but is directly analogous to the biological properties of murine EDF. Human EDF therefore represents a distinct hemopoietic growth factor that could play a central role in the regulation of eosinophilia.

Eosinophils, which are bone marrow-derived granulocytes, are capable of killing a wide variety of organisms and cells and appear to be particularly well adapted for killing large parasites. These antibody-dependent cytotoxic effector cells are present in relatively low numbers in healthy individuals, but their numbers increase dramatically in certain diseases, particularly infection by parasitic helminths, and in allergic conditions. The selectivity of their production in specific diseases suggests the possibility that a linear-specific growth factor may control the development of eosinophilia. However, no such factor has been definitively characterized in humans.

Two previously characterized human hemopoietic growth regulators, granulocyte/macrophage colony-stimulating factor (GM-CSF) (1, 2) and interleukin 3 (IL-3) (3, 4), have been shown to stimulate the formation of eosinophils but are multilineage regulators in that they also promote the differentiation of neutrophil, macrophage, and mixed neutrophil/macrophage colonies. The presence of an eosinophil CSF activity in human placental conditioned medium (HPCM) was reported (5), but this activity could be attributable to the GM-CSF also present in HPCM (1).

A murine eosinophil differentiation factor (EDF), has been identified and partially characterized (6). It is a lymphokine produced by T cells and T-lymphoma cells and stimulates only the production of eosinophils in murine bone marrow

cultures. Murine EDF is also active in stimulating human eosinophil differentiation and activation (7). Somewhat surprisingly, this factor, apparently specific for the eosinophil lineage in myeloid differentiation, was found to be active on mouse B cells (8). Molecular cloning of a cDNA for murine EDF (unpublished work) confirmed its identity with the recently cloned B-cell growth factor II/interleukin 5 (BCGF-II/IL-5) (9) and provided a probe to seek the human EDF gene. We report here the cloning and characterization of the human EDF gene[‡] and its expression in mammalian cells. Recombinant human EDF is shown to stimulate eosinophil colony formation but appears to be inactive in other myeloid lineages, suggesting that human EDF may play a significant role in the control of eosinophilia in humans.

MATERIALS AND METHODS

Southern Blotting and Hybridization. A murine EDF/BCGF-II cDNA fragment extending from nucleotide 198 to the *Nco*I site at nucleotide 458 (9) was isolated from a murine EDF cDNA clone (unpublished work). ³²P-labeled probes were synthesized by the random-primer method (10). Human liver DNA was digested with restriction endonucleases (11), electrophoretically fractionated (15 μ g per lane) and transferred to nitrocellulose according to Southern (12), and hybridized to labeled probe (10⁷ cpm/ml; specific activity 7 \times 10⁸ cpm/ μ g) for 20 hr at 60°C in 0.75 M NaCl/0.075 M sodium citrate/50 mM sodium phosphate buffer, pH 6.8/0.5% NaDodSO₄/1 mM sodium pyrophosphate/1 mM ATP/1 mM EDTA/5 \times Denhardt's solution (13)/salmon sperm DNA (100 μ g/ml)/10% dextran sulfate. Washing was at 60°C in 0.75 M NaCl/0.075 M sodium citrate/0.5% NaDodSO₄, then at lower salt concentrations following autoradiography.

Genomic Library Screening. A human genomic library in λ phage EMBL3A was prepared (14) and amplified. Recombinant phage (8×10^5) were screened under similar conditions to the Southern blots (15). DNA from hybridizing phage was prepared (11) and subcloned into M13mp10 (16) after sonication (17), and this sublibrary was screened by hybridization. Plasmid subcloning and plasmid DNA purification by CsCl/ethidium bromide centrifugation were as described (11).

Abbreviations: EDF, eosinophil differentiation factor; IL-3 and IL-5, interleukins 3 and 5; CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; BCGF-II, B-cell growth factor II; HPCM, human placental conditioned medium; BCCM, bladder carcinoma conditioned medium; CML, chronic myelocytic leukemia.

[‡]The sequence of the human EDF gene is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02971).

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DNA Sequencing. M13 subclones for dideoxy sequencing (18) were generated by sonication (17) or by cloning mixtures of restriction fragments. Sequence was also obtained from plasmid DNA (19) or M13 templates with oligonucleotide primers (20-mers) based on sequence already obtained. These were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer. DNA sequences were assembled and analyzed using computer programs described by Staden (20).

Bone Marrow Cultures. Bone marrow was obtained from normal subjects donating bone marrow for transplantation, following a protocol approved by the Ethics Committee at the Hammersmith Hospital, London. We are grateful to E. C. Gordon-Smith for this material. Low-density cells were recovered after centrifugation over Ficoll-Paque (Pharmacia) and were cultured in liquid medium as described (6). In brief, 10^5 cells were cultured in 100 μ l of RPMI 1640 medium with 15% fetal bovine serum in 96-well microplates. After 16 days, total cells were counted and differential counts were performed after staining with Luxol fast blue/hematoxylin or May-Grünwald/Giemsa stain. The bone marrow proliferation assay was carried out after 3 days of incubation, by adding 0.5 μ Ci (1 Ci = 37 GBq) of [3 H]thymidine 4 hr before harvesting the cells for liquid scintillation counting. Colony assays were in the same medium containing Difco Bacto-agar (0.3%). After incubation for 14 days, the agar was dried on a microscope slide, fixed in methanol, and stained with Luxol fast blue and hematoxylin.

Proliferation of Chronic Myelocytic Leukemia (CML) Cells. Freeze-preserved peripheral blood lymphocytes from an untreated CML patient (kindly provided by J. M. Goldman, Hammersmith Hospital, London) were cultured for 7 days in RPMI 1640 medium containing 15% fetal bovine serum. Blast cells were then recovered and tested with various conditioned media, using 10^4 cells per well in a microplate. [3 H]Thymidine incorporation was measured over the last 4 hr of a 40-hr culture period (21).

Conditioned Media. Human placental conditioned medium (HPCM) (5) and conditioned medium from the human bladder carcinoma U5637 (BCCM) (22) were kindly supplied by A. F. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia). Murine EDF was prepared as described (6).

Transfections. Monkey COS-7 cells were transfected with plasmid DNA by electroporation (23). The culture supernatant was harvested 4–6 days later. Culture supernatant from mock-transfected cells was used as a control.

RESULTS

Isolation of the Human EDF Gene. Southern blotting experiments with human liver DNA were carried out under conditions of reduced hybridization stringency to see whether the human EDF gene was detectable with the mouse EDF cDNA probe. It was found that hybridization at 60°C in the presence of 10% dextran sulfate, followed by washes at 60°C with 0.75 M or 0.15 M NaCl, gave hybridizing bands. Digestion of human DNA with *Bam*HI gave a band at 3 kilobases (kb). Digestion with *Hind*III and *Eco*RI gave bands at 7–8 kb.

A human genomic library in λ phage EMBL3A was screened under similar conditions to the Southern blots, and the phage from two hybridizing plaques were purified for further study. In both phage, hybridization to the murine probe was localized to a 3-kb *Bam*HI fragment, in agreement with the size of the hybridizing *Bam*HI fragment in human genomic DNA.

M13 subclones were prepared from one of the λ clones, and M13 phage hybridizing to the murine probe were isolated and sequenced. Several showed long regions (\approx 100 bases) of high

nucleotide sequence homology (\approx 80%) with the mouse EDF/BCGF-II cDNA sequence (ref. 9; unpublished work), confirming that a closely related human gene had been cloned. The 3-kb hybridizing *Bam*HI fragment was recloned into the *Sma* I site of the eukaryotic expression vector pcEXV-3 (24, 25) to give pEDFH-1, which has the EDF gene in the same orientation as the simian virus 40 early promoter in the vector, and which expressed human EDF activity following COS cell transfection (see below).

Genomic Copy Number of Human EDF Gene. Southern blots of human genomic DNA were probed with the isolated 3-kb fragment containing the human EDF gene. With *Bam*HI-digested DNA, a single 3-kb hybridizing band was detected that was coincident with the band detected with the murine cDNA probe. A single *Hind*III site is present near the center of the fragment used as a probe (nucleotide 1593, Fig. 1), and, consistent with this, two bands at approximately 1.7 and 7 kb were observed on Southern blots. The intensity of the bands indicated a single copy of this gene per haploid genome (data not shown).

Nucleotide Sequence of the Human EDF Gene. The sequence of the 3-kb hybridizing *Bam*HI fragment, reisolated as an *Eco*RI fragment from pEDFH-1, was determined on both strands. The four exons of the gene (Fig. 1) were readily assigned from the homology with the murine EDF/BCGF-II/IL-5 cDNA (9), and the assignment was confirmed by the recently published sequence of the corresponding human IL-5 cDNA (26). The human IL-5 cDNA sequence (26) is identical with the sequence predicted from the gene structure of human EDF, except for nucleotide 2317 (Fig. 1), which is deoxyguanylate in the human EDF sequence and was reported as deoxyadenylate in the human IL-5 cDNA. The three introns are 208, 950, and 105 base pairs (bp) long and begin with GT and end in AG.

Relative to the murine cDNA sequence, the human gene contains a deletion of 738 bases in the 3' untranslated region, just after the termination codon (Fig. 2). This could have resulted from the insertion of a mobile genetic element in the mouse, and in agreement with this, short inverted repeats flanked by possible duplications of host sequence are present near its boundaries (Fig. 2). Part of the additional mouse sequence shows some low but significant homology with *Alu*-like elements, in particular one located near the site of Epstein-Barr virus integration into Burkitt tumor cell DNA (27).

Transcription probably begins near nucleotide 508 (Fig. 1), since this is the point where the human IL-5 cDNA clones (26) constructed by the Okayama-Berg method (25) commence. A potential "TATA box" (28) is located 29 bp upstream from the start of transcription and is indicated in Fig. 1. A group of potential "CAAT boxes" (28) are located in the vicinity of 80 bp upstream from the start of transcription, at nucleotides 432–440, 426–434, and 416–424 (Fig. 1). Other potential CAAT boxes exist further upstream.

Features of the Protein Sequence. The mouse EDF gene has an exon/intron structure analogous to that of the human EDF gene (unpublished work), and Fig. 3 shows an alignment of the segments of the proteins encoded by the exons of the genes. The precursors of the human and mouse proteins are 134 and 133 amino acids long, respectively. As expected for secreted proteins, long hydrophobic stretches preceded by basic residues are present at the amino termini. The predicted signal-peptide cleavage site (29) occurs after amino acid 19 in the human and 18 in the mouse protein (Fig. 3), so that the predicted size of both processed proteins is 115 amino acids, with M_r values of 13,149 (human) and 13,299 (mouse).

Two potential N-glycosylation sites (Asn-Xaa-Thr/Ser) are present in the human protein (residues 47 and 90) and are conserved in the mouse protein, which also has a further site at residue 75 (Fig. 3). Two cysteine residues are present in

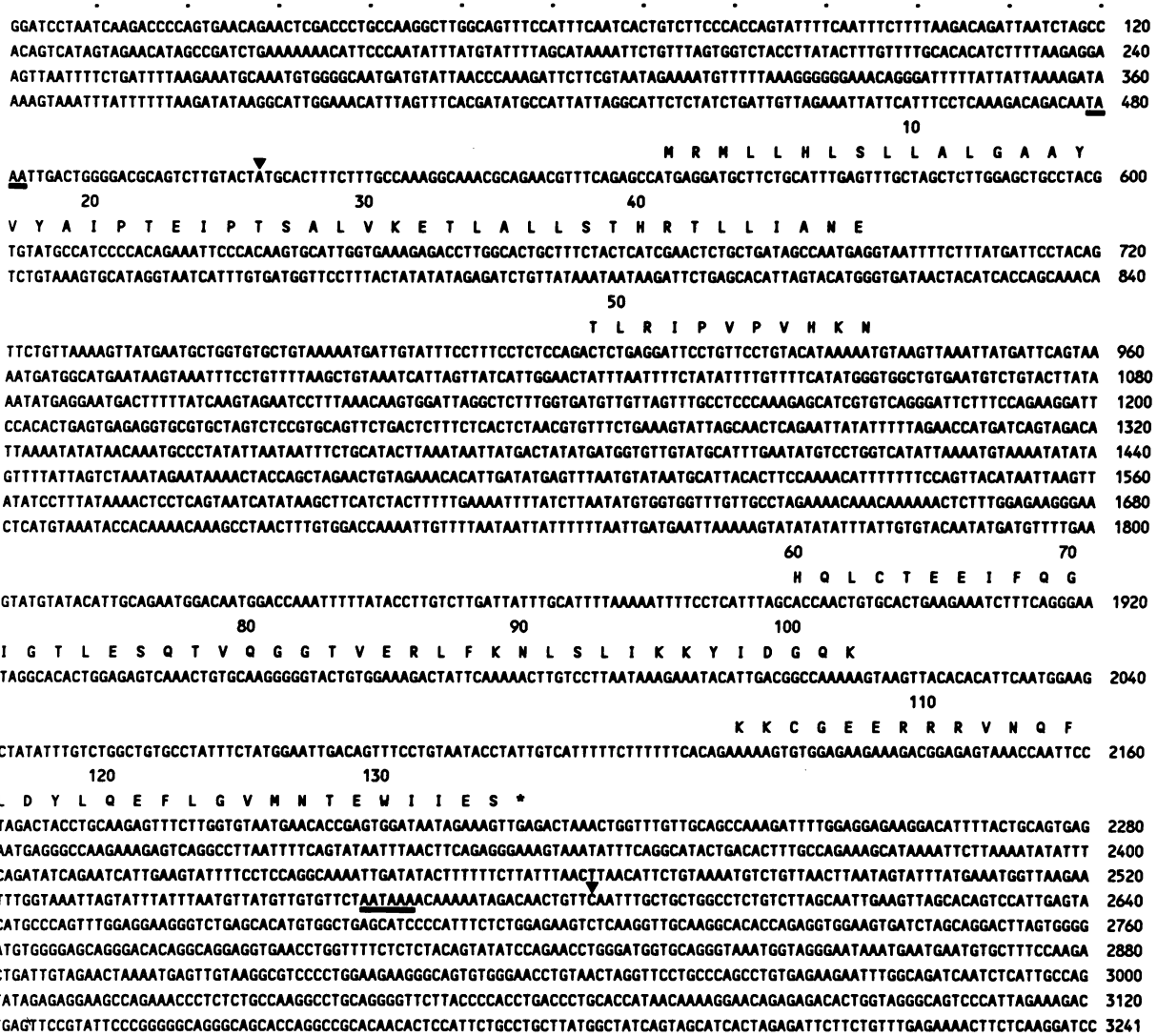


FIG. 1. Nucleotide sequence of the 3.2-kb *Bam*HI fragment containing the human EDF gene. Amino acid sequences encoded by exons of the gene are indicated (standard one-letter amino acid abbreviations). The putative "TATA box" and AATAAA polyadenylation signal are underlined. The transcription initiation site and poly(A) attachment site are indicated by arrowheads.

each mature protein at corresponding positions (residues 63 and 105, human; 62 and 104, mouse) raising the possibility of a single disulfide bond. By analysis using the program DIAGON (30), no significant amino acid sequence homology was detected between human EDF and any other lymphokines and hemopoietic growth factors. The DNA sequences of the protein coding segments were also compared with the GenBank data base[§] according to the method of Wilbur and Lipman (31), and no significant homology with any previously reported sequence was detected.

Properties of Recombinant Human EDF. Conditioned medium from COS cells transfected with pEDFH-1 was compared with HPCM and murine EDF for ability to stimulate production of differentiated cells from human bone marrow cells. Table 1 indicates that whereas HPCM stimulates production of neutrophil and macrophage colonies, with relatively few eosinophil colonies, recombinant human EDF and murine EDF give rise only to eosinophil colonies in relatively high numbers. The high background of mixed neutrophil/macrophage colonies observed appears to be a

result of the particular batch of fetal bovine serum used, which was selected for high eosinophil production in liquid cultures. Although none of the conditioned media increased the number of these colonies over the controls, the colonies were larger in cultures containing HPCM.

Similarly, in liquid cultures (Table 2) recombinant human EDF stimulated eosinophil numbers ≈12-fold after 16 days, with no increase in neutrophils or mononuclear cells. Murine EDF showed a similar effect, whereas HPCM increased

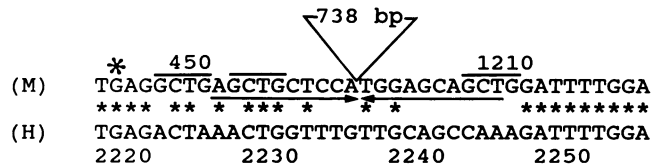


FIG. 2. Features in vicinity of additional 738-bp sequence present in 3' untranslated region of murine EDF. Sequences of the murine cDNA (M) and human gene (H) are shown aligned. The large asterisk indicates the termination codon for both genes. Small asterisks mark identical nucleotides. Homology is extensive 5' and 3' to the area shown. A 10-bp inverted repeat (arrows) and short direct repeats (overlines) are indicated.

[§]EMBL/GenBank Genetic Sequence Database (1987) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 48.

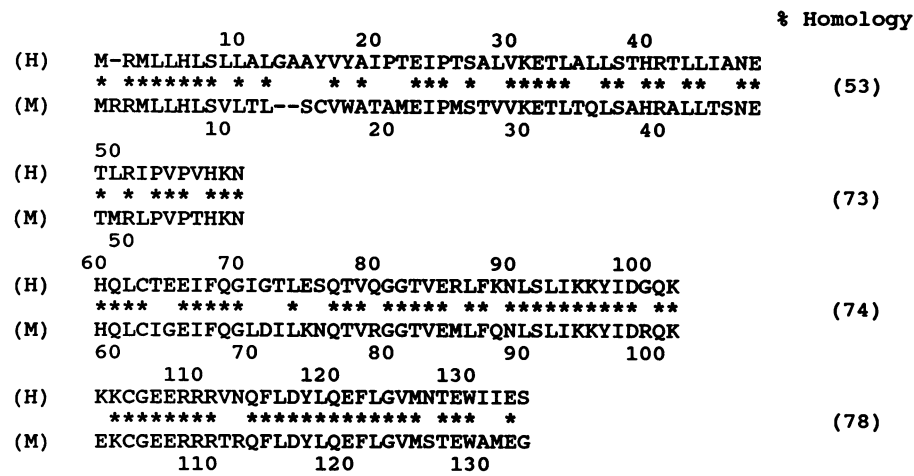


FIG. 3. Alignment of amino acid sequences encoded by the four exons of the human (H) and murine (M) EDF genes. Amino acid residues are represented by standard one-letter symbols. Asterisks indicate identical residues. Dashes indicate gaps introduced to maximize homology. % homology is defined as the number of identical residues minus the number of introduced gaps, divided by the total number of residues (including gaps) and multiplied by 100.

neutrophil and mononuclear cell numbers, with only a small effect on eosinophils. When tested on CML cells or bone marrow cells after incubation for 2 and 3 days, respectively, both HPCM and BCCM stimulated proliferation as measured by [³H]thymidine incorporation, whereas recombinant human EDF had no activity (data not shown).

DISCUSSION

In this study, we used cross-species hybridization with a murine cDNA probe to detect and clone the gene for human EDF. The DNA sequence shows that the human EDF gene is quite small, comprising 2108 bp from the TATA box to the poly(A) addition site. The coding region is interrupted by three introns (208, 950, and 105 bp) and encodes a precursor protein of 134 amino acids. As expected for a secreted protein, there is a typical signal sequence at the amino terminus. The human and murine proteins are highly homologous (70% identity), and the predicted size of both mature proteins is 115 amino acids. Relative to the human sequence, the 3' untranslated portion of the murine mRNA contains an insertion of 738 bp with properties reminiscent of a mobile genetic element. We have obtained recombinant human EDF by transfection of the human EDF gene, recloned in an expression vector, into monkey COS cells. Recombinant

human EDF is lineage-specific for eosinophils in both liquid bone marrow cultures and the bone marrow colony assay. In contrast, HPCM, which contains GM-CSF and granulocyte CSF, stimulates the production of neutrophils and monocytes with relatively few eosinophils. Similarly, whereas HPCM and BCCM give readily detectable stimulation of proliferation of bone marrow cells and CML blast cells, in short-term cultures, recombinant human EDF has no detectable effects in these assays. The lack of response by CML cells, which respond to GM-CSF and human IL-3 (3), clearly differentiates recombinant human EDF from these other growth factors.

The properties of recombinant human EDF are therefore completely analogous to those of murine EDF, which also appears to be active only in the eosinophil lineage of myeloid hemopoiesis and shows similar activity in the corresponding human assays. Murine EDF is produced by T lymphocytes and T-lymphoma cells and has been extensively characterized in the mouse system (6, 8, 32). Murine EDF also possesses BCGF-II activity (8, 9, 32); whether human EDF also possesses this property remains to be determined. The lineage specificity of human EDF suggests a key role in the regulation of eosinophilia, which is observed during parasitic infections and certain allergic responses. In this connection the isolation and characterization of the human EDF gene

Table 1. Production of bone marrow colonies in semisolid medium by recombinant human EDF

Addition	Dilution*	Numbers of each colony type [†]			
		Eosinophil	Neutrophil	Monocyte	Neutrophil/monocyte
rhEDF [‡]	10	70 ± 12	0	0	38 ± 7
	30	65 ± 1	0	0	36 ± 4
	100	82 ± 12	0	0	42 ± 7
	300	50 ± 2	0	0	42 ± 6
Sham [§]	10	0	0	0	4 ± 1
	30	0	0	0	33 ± 5
	100	0	0	0	40 ± 9
	300	0	0	0	45 ± 8
Murine EDF	100	64 ± 9	0	0	43 ± 5
HPCM	20	5 ± 2	10 ± 2	5 ± 3	32 ± 5
None (control)	—	0	0	0	42 ± 1

*Reciprocal of dilution of conditioned medium in assay.

[†]No. per 8 × 10⁴ human bone marrow cells cultured; values are means ± SD.

[‡]Recombinant human EDF (culture supernatant from COS cells transfected with pEDFH-1).

[§]Culture supernatant from mock-transfected COS cells.

Table 2. Cell types produced in liquid bone marrow cultures by recombinant human EDF

Addition*	Dilution†	Cell type, no. × 10 ⁻⁴ per well‡		
		Eosinophil	Neutrophil	Monocyte
rhEDF	10	5.9 ± 1.2 [§]	2.1 ± 0.8	2.6 ± 1.2
	30	5.0 ± 0.4 [§]	2.1 ± 0.2	3.5 ± 0.3
	100	3.2 ± 2.3 [§]	3.5 ± 0.11	2.2 ± 1.8
	300	1.7 ± 1.5	2.8 ± 1.1	3.6 ± 0.6
Sham	10	0.5 ± 0.5	2.1 ± 0.9	3.3 ± 1.0
	30	0	3.6 ± 1.0	4.0 ± 1.3
Murine EDF	10	3.6 ± 1.2 [§]	1.4 ± 0.7	5.4 ± 3.4
HPCM	10	1.4 ± 1.9	4.0 ± 0.7	8.0 ± 1.0 [§]
Control	—	0.5 ± 0.05	2.8 ± 2.4	4.2 ± 0.4

*Additions as in Table 1.

†Reciprocal of dilution of conditioned medium in assay.

‡Cells obtained from 10⁵ bone marrow cells after 16 days in culture; values are means ± SD.

§Values significantly different from control at 1% level.

provide the basis for future studies on the regulation of human EDF expression. The chromosomal location of this gene may be relevant to the atypical eosinophil morphology and bone marrow eosinophilia observed in leukemia associated with abnormalities of human chromosome 16 (33).

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