Phorbol Ester Induced Osteoclast-like Differentiation of a Novel Human Leukemic Cell Line (FLG 29.1)

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Abstract. Studies on human osteoclast formation have been hampered by lack of a defined isolated progenitor cell population. We describe here the establishment of a human leukemic cell line (designated FLG 29.1) from bone marrow of a patient with acute monoblastic leukemia. The cultured cells are predominantly undifferentiated leukemic blasts, but addition of 12-*o*-tetradecanoylphorbol 13-acetate (TPA; 0.1 μM) induces irreversible differentiation into adherent, non-dividing, multinucleated cells. TPA-treated cells bear surface antigens typical of fetal osteoclasts, degrade ^{45}Ca-labeled devitalized bone particles, display tartrate-resistant acid phosphatase in both mononuclear and multinuclear cells and receptors for calcitonin. Calcitonin increases intracellular cAMP accumulation in TPA-treated cells. TPA-treated cells show some ultrastructural features of osteoclasts as evidenced by transmission EM. These results indicate that FLG 29.1 cells may represent an osteoclast committed cell population, which upon induction with TPA acquire some morphological, phenotypical, and functional features of differentiated osteoclasts.

THERE is an ample evidence that the osteoclast, the cell that resorbs bone, is derived from some precursor cell carried with the blood vascular system (45). However, the precise nature of the immediate osteoclast precursor is still unknown. Initially it was believed that circulating monocytes were osteoclast precursors for their ability of fusing in multinucleated cells (65). It was later established that tissue macrophages and monocytes isolated from the circulation significantly differ from osteoclasts in morphology, antigenic properties, and bone resorbing activity (17, 29, 30, 63). It was then proposed that the osteoclast originates from an early precursor common to the monocytic and granulocytic lineages (12).

Recovery of a definitive homogeneous osteoclast population from bone tissue has not yet been achieved. Studies in this area generally have used preexisting cells stripped from growing bones (27, 50). Such models, however, do not permit analysis of the sequential steps in differentiation from the precursor to the mature cell. Moreover, cell viability is often poor. In contrast, purification of precursor subpopulations has allowed studies on osteoclast maturation in vitro (12, 32). These progenitor cells fuse spontaneously as well as in response to a variety of agents, including osteotropic hormones (43), transforming growth factors (51, 66), and bone matrix constituents (36, 44, 56). These precursors, however, are not readily identifiable and their features may vary from preparation to preparation. Another approach has used human leukemic cell lines, such as HL60 and U937 cells, induced by TPA and 1,25 dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$)

to differentiate toward monocyte macrophages and which might show some resorbing activity in vitro (7, 28, 59). These in vitro–derived macrophage-like cells considerably differ, however, from osteoclasts both functionally and phenotypically. The development of a clonal population of osteoclast precursors would offer a unique tool for studying mechanisms that regulate replication and differentiation of osteoclasts. The use of pure populations of progenitors and terminally differentiated osteoclasts would further allow the analysis of interactions between homogeneous, but distinct classes of bone cells.

We report here the development of a human leukemic cell line, designated FLG 29.1, that can be induced to differentiate in vitro towards the osteoclastic phenotype.

Materials and Methods

Culture Conditions, Cytochemical, Biochemical, and Ultrastructural Studies

FLG 29.1 cell line was established at the U.O. Hematology of Florence (USL 10/D) from a 38-yr-old female suffering from acute monoblastic leukemia, M5a type according to the FAB classification (9). The clinical course of this patient was characterized, after a 15 mo complete remission, by a fatal pancytopenic episode associated with a peripheral neuropathic syndrome and generalized increase in bone density. Bone marrow mononuclear cells were isolated on Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) and resuspended in RPMI-1640 culture medium (Flow Laboratories Inc., Irvine, UK) supplemented with 10% heath inactivated FCS. Cells (10^5 cells/ml) were grown in Falcon polystyrene tissue culture flasks at 37°C in atmosphere of 5% CO$_2$/95% air. An adherent cell

monolayer developed after 20 d of culture and on top of the adherent cells a homogeneous population of floating cells proliferated. This latter cell population was collected, propagated in suspension culture and cloned by limiting dilution. The cell line was determined to be free of mycoplasma contamination and has been maintained in continuous suspension culture for almost 4 yr. HL60 and U937 human leukemic cell lines were obtained by ATCC and grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. TPA (Sigma Chemical Co., St. Louis, MO) was solubilized in acetone at 0.16 mM. Cell differentiation was obtained by adding TPA to the cell suspension at a final concentration of 0.1 μM for 72 h of culture. Control cultures contained equal amounts of acetone. 1,25 Dihydroxyvitamin D3 (Hoffman-La Roche) was dissolved in absolute ethanol at 1 mM and diluted into culture medium at a final concentration of 10 nM for induction experiments (72 h in the dark). For cooperation experiments 1,25(OH)₂D₃ was added to FLG 29.1 cell cultures after 12-h preincubation with 0.1 μM TPA and then the incubation proceeded for the remaining 60 h in the dark. Control cultures contained equal amounts of acetone and ethanol.

Daily cell counts were performed with a Coulter Counter to assess growth kinetics. Viability was evaluated by trypan blue exclusion. Upon treatment with 0.1 μM TPA for 72 h cell adhesion was calculated on the total number of viable cells. The ability of FLG 29.1 cells to form colonies in soft agar was assessed by plating them in 35-mm Petri dishes in 1 ml of RPMI-1640 medium containing 20% FCS and 0.3% agar (Bactoagar; Difco, Detroit, MI). De novo synthesis of DNA was assessed by a standard [³H]thymidine incorporation microassay (60). Cells were assessed morphologically on cytospin slide preparations stained with May-Grunwald-Giemsa, Sudan Black, PAS, acid phosphatase and tartrate-resistant acid phosphatase (TRAPase), naphtol ASD chloroacetate esterase, and sodium fluoride inhibitable α-naphthyl acetate esterase (19). TRAPase activity in the whole cell population was measured using p-nitrophenylphosphate as substrate as previously described and expressed as mIU/10⁶ cells (41). Intracellular and extracellular tissue plasminogen activator antigen (tPA) was measured by using a highly specific two-site immunoassay (Biopool AB, Umea, Sweden) (55). For the evaluation of the cellular content of TRAPase and tPA the cells were extracted for 10 min with HBSS buffer containing 1% Nonidet. Phagocytic activity was measured with a flow cytometry technique that discriminates between internalized and membrane-bound fluorescein-conjugated heat-killed Candida albicans (24). Generation of superoxide was measured in FLG 29.1 cells by the ability to reduce nitroblue tetrazolium (NBT) in intracellular blue–black formazan deposits (34). FLG 29.1 cells were analyzed by transmission EM after 72 h of culture with or without 0.1 μM TPA. In separate experiments TPA-treated FLG 29.1 cells were also co-cultured with dentine slices. Both suspended and adherent cells were centrifuged at 800 g and the pellet obtained was fixed in 4% cold glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4, at room temperature for 1 h and postfixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4, at 4°C for 1 h. The pellets were then dehydrated and embedded in Epon 812. Semithin sections were used for light microscopy observation. From the same specimens ultrathin sections were also obtained and stained with uranyl acetate and alkaline bismuth subnitrate for EM.

Karyotype Analysis

Cells after the 20th and 150th passage were treated with Colcemid for 30 min and then with 0.075 M KCl for 20 min. The preparations were then harvested and fixed in methanol/acetic acid (3:1, vol/vol). Metaphases were stained to obtain GTG and RHA banding (21, 62).

Molecular Rearrangements and Epstein-Barr Virus Detection

Immunoglobulin genes and T cell receptor status in genomic DNA extracted from FLG 29.1 cells were analyzed by probing Southern blots with multiprime-labeled DNA fragments specific for immunoglobulin-JH and -C-μ and T cell receptor-β and -τ constant regions as described (14). Cells were also tested for Epstein-Barr virus DNA (53).

1. *Abbreviations used in this paper*: APAAP, alkaline phosphatase anti-alkaline phosphatase; H-CAM, homing-associated cell adhesion molecule; I-CAM, intercellular adhesion molecule; MHC, major histocompatibility complex; NBT, nitroblue tetrazolium; TRAPase, tartrate-resistant acid phosphatase; TPA, 12-*o*-tetradecanoylphorbol 13-acetate; tPA, tissue plasminogen activator.

Immunological Characterization

The cell surface antigenic pattern was analyzed with a standard immunofluorescence method using a FACStar cytofluorograph (Becton Dickinson and Co., Immunocytometry Systems, Mountain View, CA) as previously described (52). Cytoplasmic antigens were detected with a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) immunoenzymatic procedure (14). Most mAbs used derived from the panels of the IIIrd and IVth editions of the International Conference and Workshop on Leukocyte Antigens (39, 40). Other mAbs were purchased from Ortho (Raritan, NJ), Coulter (Coulter Immunology, Hieleah, FL), Becton Dickinson (Sunnyvale, CA), and Dako (Dakopattz, Glastrup, Denmark).

Western Blotting

The crude membrane fraction of untreated and TPA-treated (0.1 μM for 72 h) were solubilized in SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) overnight at room temperature (38). The solubilized membrane fraction was subjected to SDS-gel electrophoresis on 10% polyacrylamide gels and then transferred electronically to nitrocellulose with transfer buffer (20 mM Tris base, 150 mM glycine, and 20% methanol) overnight at 4°C (13). The protein bands were blocked with HBSS buffer containing 3% BSA and 0.1% Tween-20 overnight at 37°C. The transferred proteins were then reacted with mAb Kn22 (35) in the presence of 1% horse serum and 0.01% Tween-20 30 min at room temperature. The protein bands were detected by Vectastain ABC-AP kits.

Bone Resorption

Resorption of devitalized bone by FLG 29.1 and HL60 cells was quantified using particles of ⁴⁵Ca-labeled bone as described by Teitelbaum et al. (69). Devitalized rat bone particles (⁴⁵Ca labeled, 45–50-μM diam) were added to the FLG 29.1 and HL60 cells cultured in 16-mm wells (10⁶ cells/well) in RPMI-1640 medium containing 10% FCS in the presence or in the absence of 0.1 μM TPA. After 72 h incubation the upper one-half of the medium was aspirated, centrifuged at 15,000 g, and assayed for radiocalcium. The pellet and remaining half of the incubation medium were solubilized in 10% TCA for 24 h and assayed. Net ⁴⁵Ca release was calculated from the isotope released into the medium expressed as percent of total, after correction for the loss of isotope from the particles because of simple physicochemical exchange with ⁴⁵Ca. To assess whether any resorption might be potentially due to release of lytic enzymes from cells, similar experiments were carried out with conditioned media obtained from 3-d-old cultures.

Formation of resorption lacunae on dentine slices was evaluated using sperm whale dentine slices kindly supplied by Dr. A. Boyde (University College, London, UK). FLG 29.1 cells were co-cultured with the dentine slices plated in 16-mm wells (10⁶ cells/well) in 10% FCS RPMI-1640 medium to which 0.1 μM TPA had been previously added. 72-h later the cells were washed twice with RPMI-1640 medium supplemented with 10% FCS and co-cultures were incubated for up to 20 d in growth medium. Dentine slices were processed as previously described (10, 33) for scanning EM.

cAMP Accumulation

Intracellular cAMP content in FLG 29.1, HL60 and U937 cells was measured by radioimmunoassay. Untreated cells were washed with cold HBSS, suspended in assay buffer (TRIS acetate 0.025 M buffer, pH 7.4, containing 0.25 M sucrose, 0.5% BSA, and 3-isobutyl-1-methylxanthine) and transferred to test tubes (10⁵ cells/tube). Differentiated cells were plated in 16-mm wells at a concentration of 10⁵ cells per well and cultured for 72 h in growth medium containing 0.1 μM TPA. Then the medium was carefully aspirated, adherent cells gently washed with cold HBSS, and assay buffer added. Quadruplicate samples of untreated or TPA-treated FLG 29.1 cells were incubated with 1 pM to 0.1 μM salmon CT (Sandoz Pharma, Basel, Switzerland) for 30 min, while TPA-induced HL60 and U937 cells were exposed to 1 nM dose of salmon CT for 30 min. Cells were then washed with cold HBSS, and covered with absolute ethanol overnight at −20°C. The ethanol was removed under vacuum and samples reconstituted with 0.05 M sodium acetate buffer, pH 6.2. cAMP was determined by radioimmunoassay as previously described (11).

Immunocytochemical evaluation of cyclic AMP was carried out as previously described (8). Both untreated and TPA-treated (0.1 μM for 72 h) FLG 29.1 cells were incubated for 30 min at 37°C with or without 1 nM salmon CT in cAMP assay buffer and cytospin slide preparations were then ob-

tained. Immunostaining was performed with the APAAP method as described (8).

Calcitonin Binding Studies

FLG 29.1 cells differentiated with 0.1 μM TPA for 72 h were transferred to test tubes (10^6 cells/tube) and incubated for 120 min with increasing concentrations (0.1-1.0 nM) of ^{125}I-labeled salmon CT (sp act. 2,200 Ci/mmol, Anawa Laboratorien AG, Wangen, Switzerland) with or without unlabeled salmon CT (0.1 mM) at 22°C in binding buffer containing 100 mM Hepes, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 1.3 mM MgSO₄, 5 mM KCl, 120 mM NaCl, and 1% BSA, pH 7.6. After incubation samples were washed with HBSS containing 1% BSA and centrifuged. Cell pellets were then counted in a gamma counter at 70% efficiency. All experiments were carried out in triplicate. Scatchard analysis of binding data from three experiments was performed using the computer program LIGAND (47).

Quantitative Histomorphometry

FLG 29.1 cells were cultured with 0.1 μM TPA for 72 h. Cells were then washed with RPMI-1640 medium and treated with 1 nM salmon CT for 3 h at 37°C. Phase contrast micrographs were taken before and after the addition of salmon CT. Plan areas of 100-150 FLG 29.1 cells were measured on the enlarged micrographs using a computer program equipped with a NIKON Cosmozone.

Statistical Analysis

Results were evaluated statistically by t test.

Results

Growth Characteristics

Untreated FLG 29.1 cells grew as small floating clumps with a doubling time in the logarithmic phase of ~22 h. The cells produced a population of colonies in semisolid agar directly related to the number of cells plated, with a plating efficiency of ~40%. TPA (0.1 μM) caused an irreversible decrease in [³H]thymidine incorporation with growth arrest in liquid or semisolid medium (data not shown). Trypan blue exclusion, indicated <5% positive cells after TPA treatment.

Karyotype and Molecular Analysis

The FLG 29.1 cell karyotype showed numbers of chromosomes ranging from 45 to 69 with constant expression of the 3p+ marker. Southern blot analysis showed no detectable Epstein-Barr virus in DNA from FLG 29.1 cells and maintenance of a germ line configuration of JH and C-μ immunoglobulin regions and of T cell receptor-β and -τ genes.

Morphology, Cytochemistry, and Ultrastructure

FLG 29.1 cells stained with May-Grunwald-Giemsa appeared like undifferentiated leukemic blasts, with large round nuclei, prominent nucleoli, dispersed nuclear chromatin and basophilic cytoplasm. The diameter of the cells ranged from 15 to 20 μm (Fig. 1 a). Less than 3% of the cells were multinucleated, and commensurately larger in dimensions. Only a minority of the untreated cells adhered to the plastic surface. After 72 h of treatment with 0.1 μM TPA, 36% of the cells were attached to the substrate and >45% multinucleated (3 to 12 nuclei) with size ranging from 50 to 100 μm (Fig. 1, b and c). Multinucleated cells were found attached or in suspension and lacked characteristic peripheral ruffling. Fluorochrome-conjugated Candida particles were not internalized by both untreated and TPA-induced FLG 29.1 cells. No detectable tPA was found in either the conditioned medium or the cytosol of untreated FLG 29.1 cells. After treatment of FLG 29.1 cells with 0.1 μM TPA for 72 h the levels of tPA in the conditioned medium and inside the cells were respectively 0.57 ± 0.08 ng/10^6 cells and 1.29 ± 0.33/10^6 cells. Untreated FLG 29.1 cells did not stain with Sudan Black, naphtol ASD-chloroacetate esterase and PAS reaction, while acid phosphatase and sodium fluoride inhibitable α-naphthyl acetate esterase were present in 100% of the cells with 10 ± 2% of TRAPase-positive cells. TPA (0.1 μM for 72 h) treatment increased by 48 ± 6% the number of cells positive for TRAPase, the TRAPase content of the total population of cells (4.2 ± 0.6 mIU/10^6 cells vs 1.4 ± 0.4 mIU/10^6 cells), the production of acid phosphatase, and sodium fluoride inhibitable α-naphthyl acetate esterase. NBT was reduced by cellular oxidants to formazan in 21 ± 5% of untreated FLG 29.1 cells. The number of cells able to accumulate formazan increased to 85 ± 8% in TPA-induced FLG 29.1 cells. Expression of TRAPase and other enzymes did not correlate with adherence or multinucleation.

Transmission EM showed that most of the untreated cells had cytoplasmic features of undifferentiated blasts, namely abundant free ribosomes, scarce organelles, large and irregularly shaped nucleus with dispersed chromatin and large nucleoli (Fig. 2 a). Rare cells with multiple nuclei were observed. After treatment with 0.1 μM TPA for 72 h FLG 29.1 cells appeared to be in a more advanced stage of differentiation, containing rather numerous rod-shaped mitochondria and a well developed RER (Fig. 2 b), large Golgi areas
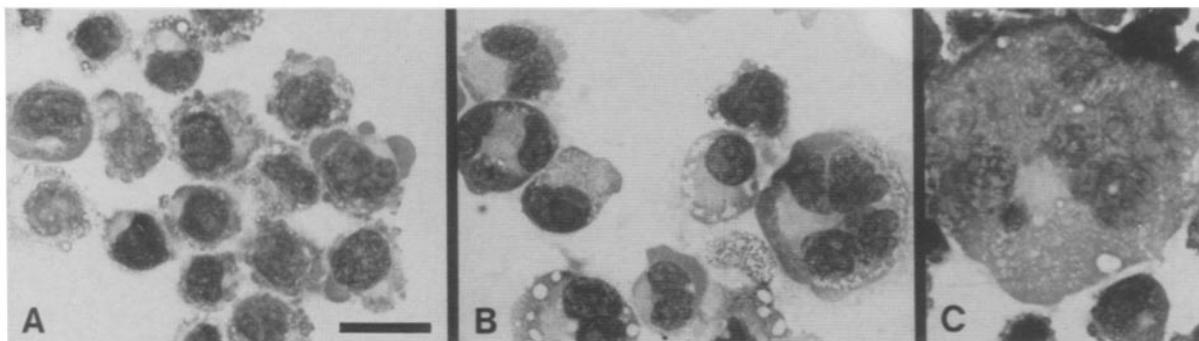
Figure 1. Light microscopy of FLG 29.1 cells. (a) Untreated cells; (b and c) cells treated with 0.1 μM TPA for 72 h (May-Grunwald-Giemsa). Bar, 20 μm.
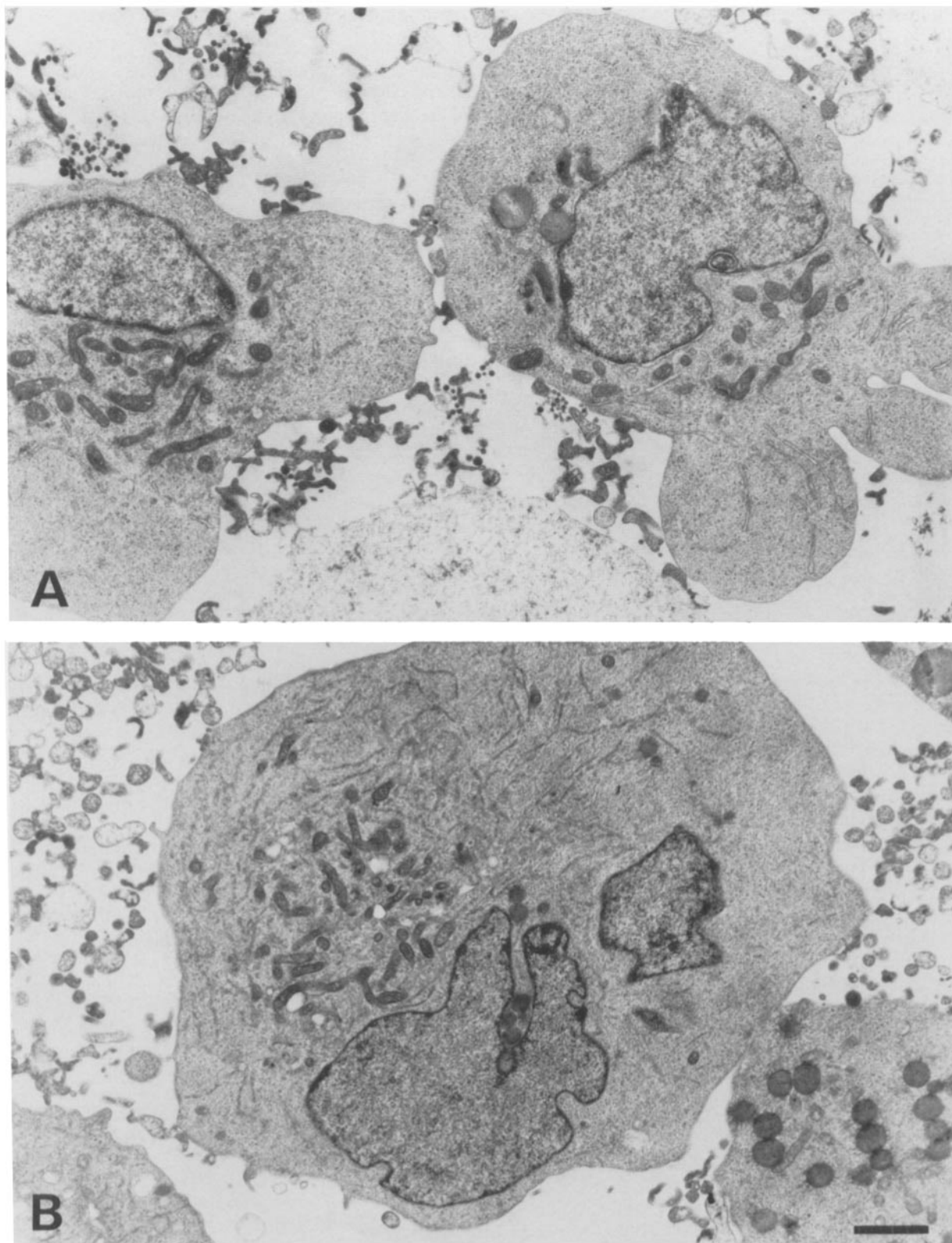
Figure 2. (*a*) Untreated FLG 29.1 cells. The cytoplasm shows scarce organelles apart numerous free ribosomes; the nucleus is large and irregularly-shaped with dispersed chromatin. (*b*) TPA-treated FLG 29.1 cells. The cytoplasm reveals abundant RER and several rod-shaped mitochondria. Bar, 2 μm.
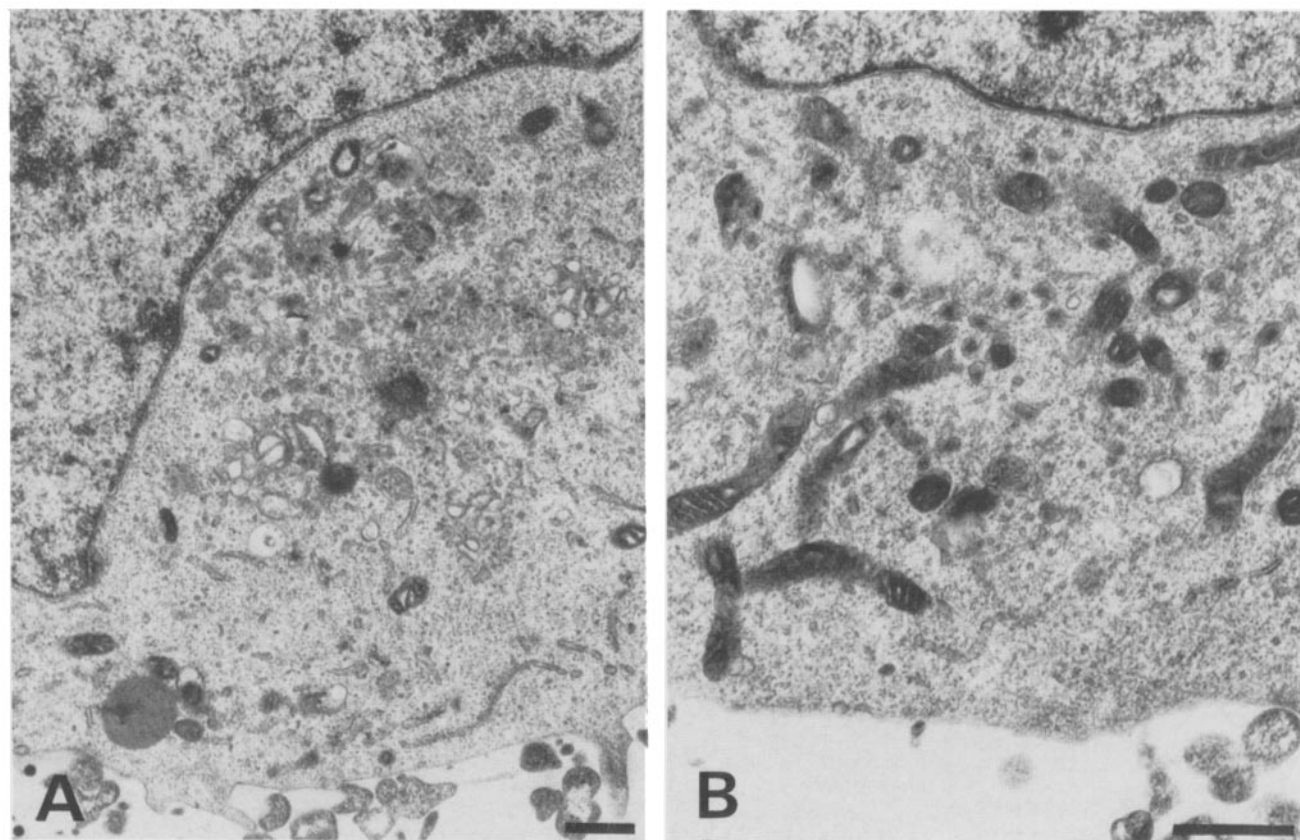
Figure 3. TPA-treated FLG 29.1 cells. (*a*) Multiple Golgi areas and (*b*) several lysosomes can be seen. Bars: 1 μm.

(Fig. 3 *a*), and several dense bodies resembling lysosomes (Fig. 3 *b*).

Immunophenotypical Analysis

Table I summarizes the immunophenotypical characterization of untreated and TPA-treated FLG 29.1 cells. Cells were negative for mature and immature T cell (CD1a, CD2, CD3, CD5, CD7, CD8) and B cell (CD19, CD20, CD21, CD22, CD23, CD24) restricted markers and for anti-CD25 mAb recognizing IL2 membrane receptor, a molecule expressed by activated macrophages, B and T cells. In addition, other mAbs recognizing multilineage hemopoietic precursors (anti-CD33, CD34), myelo-monocytic antigens (CD11a, c, CD14, CD16, CD18, CD35, CD36), certain platelet-associated antigens (CD41, Factor VIII–Related Antigen, PLT1), and class II antigens of the major histocompatibility complex (MHC) (HLA-DR, -DP, and -DQ) showed little (<20% positive cells) or no reactivity with either uninduced or TPA-treated cells. TPA-untreated cells showed a low reactivity for MHC class I determinants (expressed only after TPA induction), displayed CD71/transferrin receptors (down regulated by TPA treatment), and reacted strongly with the panleukocyte mAbs anti-CD45, anti-CD44 (homing-associated cell adhesion molecule, H-CAM), along with other mAbs specifically directed against determinants shared by activated macrophages and osteoclasts. This latter group includes anti-CD11b, anti-CD13, anti-CD15, anti-CD32, and anti-CD54 (intercellular adhesion molecule/ICAM-1) mAbs. The anti-CD68 KiM6 mAb showed a cytoplasmic positivity revealed via the

APAAP method, which was increased after TPA exposure (data not shown). TPA-treated FLG 29.1 cells also expressed CD9 (common to osteoclasts and platelets) and CD42 (common to osteoclasts, macrophages, and platelets) antigens. Finally, antigens recognized by the two mAbs 13C2 and 23C6, putatively specific for human osteoclasts (30), were significantly induced by TPA treatment (Fig. 4). Fluorescence histograms for osteoclast-related antigens before and after TPA treatment were expressed as single peaks, thus excluding the existence of heterogeneous cell subpopulations within the clonal FLG 29.1 cell line. The osteoclast-associated antigens recognized by the 13C2 and 23C6 mAbs were also detected by APAAP staining of TPA-induced FLG 29.1 cells (data not shown). It is of interest to point out that no significant differences in APAAP staining with 13C2 and 23C6 mAbs were noted between mononuclear and multinucleated cells. Conversely, neither antigen was induced by TPA in the other leukemic cell lines HL60 and U937 (Fig. 4).

A crude membrane preparation of both untreated and TPA-treated FLG 29.1 cells was prepared and subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the protein band reactive with the mAb Kn22 was identified. As shown in Fig. 5, a single band of ∼50 kD was detected with mAb Kn22 only in TPA-treated cells, while no bands were detected in untreated FLG 29.1 cells.

Bone Resorption

Untreated FLG 29.1 cells were inactive with ⁴⁵Ca-labeled

Table I. Phenotypic Profile of FLG 29.1 Cells before and after TPA Exposure and Its Relationship with Normal Fetal Bone Osteoclasts

CD	Antibody	Recognized molecule cell distribution*	FLG 29.1		FLG 29.1 + TPA		Osteoclasts‡	Macro poly‡	Induced FLG 29.1
			Percent stained cells	Mean fluorescence	Percent stained cells	Mean fluorescence			
4	OKT4	Th, Macro	5	64	12	85	−	+	−
13	MY7	My, M, Macro	15	91	45	111	+	+	+
13	SJ1D1	My, M, Macro	7	84	23	101	+	+	+
14	MY4	M, Macro	4	62	10	83	−	+	−
14	MO2	M, Macro	16	72	15	91	−	+	−
15	OKM15	X-hapten, M, Macro	10	70	27	105	±	±	±
31	ION31	GpIIa, My, M, plt	5	87	73	127	−	+	+
32	2E1	FcRII, My, M, plt	92	139	99	146	+	+	+
33	MY9	My, M, Macro	25	77	21	99	−	+	±
34	MY10	Precursor cells	36	102	20	87	NT	NT	−
35	E11	CR1, PMN, M, DRC, plt, RBC	15	79	20	89	−	+	−
36	OKM5	M, Macro, RBC	7	64	12	85	−	+	−
68	KiM6	Macro	8	89	25	102	+	+	+
68	KiM7	Macro	24	101	66	119	+	+	+
41a	J15	GpIIbIIIa, plt	3	79	18	87	−	−	−
42b	AN15	GpIb, plt	10	70	30	91	+	−	+
44	F-10-44-2	H-CAM	28	103	99	222	+	+	+
45	H1e1	T200, LCA	31	76	67	121	+	+	+
11a	MHM24	LFA-1α	3	80	20	87	−	+	−
11b	MO1	Mac1α	3	79	36	92	±	+	+
18	MHM23	LFA-1β	5	84	59	118	NT	+	+
54	84H10	ICAM-1	99	145	99	162	+	+	+
51	IOP51	VNR-α	25	102	89	135	+	+	+
61	IOP61	GpIIIa, VNR-β	53	112	75	127	+	+	+
51	23C6	VNR-α	12	93	91	139	+	+	+
−	13C2	−	14	96	80	128	+	−	+
71	OHT9	Trf-R	99	163	65	119	−	+	+
9	FMC8	Act, PMN, B, M, Macro, plt	27	98	99	179	+	+	+
−	W6132	HLA-Class I	2	79	48	112	NT	NT	+
−	HLA-DR	HLA-Class II	4	80	10	86	NT	+	−

Abbreviations used: Macrophage polykaria, *Macropoly*; T helper lymphocytes, *Th*; Macrophages, *Macro*; Myeloid cells, *My*; Monocytes, *M*; platelets, *plt*; dendritic reticulum cells, *DRC*; red blood cells, *RBC*; neutrophils, *PMN*; activated cells, *Act*; B lymphocytes, *B*; not tested, *NT*.
* According to the "IVth International Workshop and Conference on Human Leucocyte Differentiation Antigens" (Wien, 1989) (28).
‡ Data obtained from references 1–4, 22, 23, 32.
mAbs additionally tested and resulting negative, indicated as CD,, include: CD1a, 2, 3, 5, 7, 8, 10, 16, 19, 20, 21, 22, 23, 24, 25, 30, 38, 56, 57, and Factor VIII–related antigen.
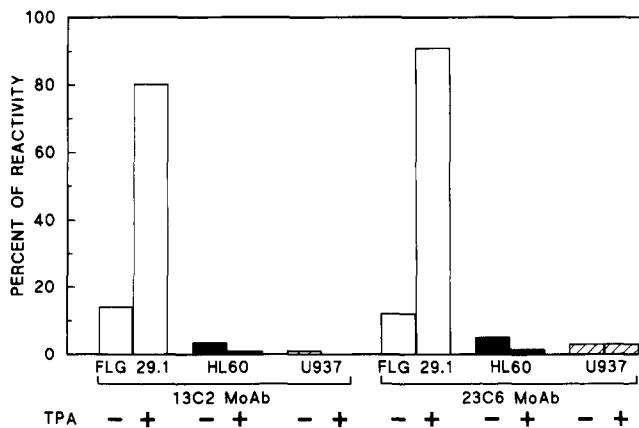

Figure 4. Reactivity of FLG 29.1 (□), HL60 (■), and U937 (▨) cells to 13C2 and 23C6 mAbs. Analyses were carried out in untreated cells and after treatment with 0.1 µM TPA for 72 h. Results are expressed as percent of stained cells evaluated by quantitative fluorescence analysis in flow cytometry. For details, see Materials and Methods.
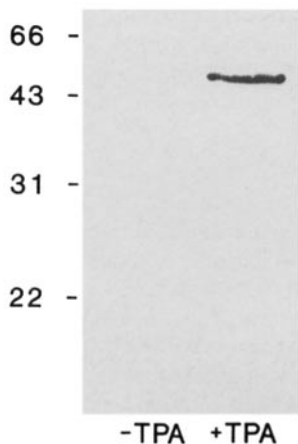
devitalized bone particles; TPA-treated cells resorbed these bone particles effectively ($p < 0.005$; Fig. 6). Neither uninduced nor TPA-induced HL60 cells were active in this assay (Fig. 6). 60% of the effect exerted by TPA-treated FLG 29.1 cells on ^{45}Ca mobilization was due to factor(s) released into the medium (Fig. 6). Under similar experimental conditions, no effect was found with conditioned media from uninduced cells nor from untreated or TPA-treated HL60 cells (Fig. 6). Correcting media pH to 7.4 daily with Hepes or increasing the CO_2 in the air up to 10% did not influence this result (data not shown).

Neither untreated nor TPA-treated FLG 29.1 cells caused resorption of sperm whale dentine slices (data not shown).

Figure 5. Identification of Kn22 antigen by Western blot. The SDS–polyacrylamide gel of the membrane fraction of untreated and TPA-treated FLG 29.1 cells, the protein transfer, and the Western blotting with the mAb Kn22 were carried out as described in Materials and Methods. The arrowhead indicates a single band of ~50 kD detected with Kn22 in TPA-treated FLG 29.1 cells. No reactivity was seen with undifferentiated FLG 29.1 cells.

Figure 7. Cyclic AMP production by FLG 29.1 cells in response to salmon CT. Quadruplicate untreated (O) and TPA-treated (●) FLG 29.1 cells were exposed to 0.5 mM 3-isobutyl-1-methylxanthine and increasing concentrations of salmon CT for 30 min. Cyclic AMP was assayed as described in Materials and Methods.

Calcitonin and Intracellular cAMP

After 30-min incubation salmon CT from a concentration of 1 pM and 0.1 μM caused a slight, but no significant increase in cAMP content of untreated FLG 29.1 cells (Fig. 7). In TPA-induced cells we found a dose-dependent increase in cAMP response to sCT, with a maximal stimulatory effect at 1 nM (p < 0.001) and a lower response at higher concentrations (Fig. 7). Conversely, TPA-induced HL60 and U937 cells did not respond to 1 nM salmon CT after 30-min incubation (Fig. 8).

Immunostaining of both differentiated and undifferentiated FLG 29.1 cells incubated in assay buffer without salmon CT showed very little cAMP signal near the plasma membrane and also very little diffusely in the cytoplasm (data not shown). At 1-nM concentration for 30 min salmon CT caused cAMP accumulation only in TPA-treated FLG 29.1 cells as well in the cytoplasm and along the plasma membrane, with no differences between mononuclear and multinucleated cells (data not shown).
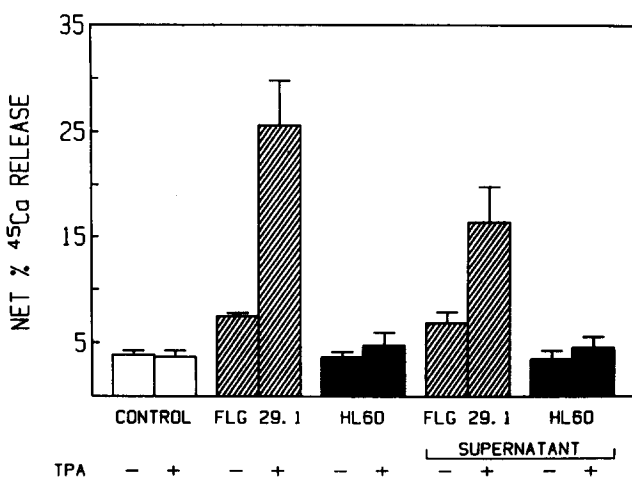
Calcitonin-binding Studies

Scatchard analysis of three binding experiments revealed the presence of two classes of binding sites for CT, one with high affinity (kD 1.1 nM) and low capacity (1.5 × 10³ sites/cell) and one with lower affinity (kD 6.6 μM) and higher capacity (5.6 × 10⁶ sites/cell) (Fig. 9).

Effects of Salmon CT on FLG 29.1 Cell Differentiation Induced by TPA

72-h incubation with 0.1 μM TPA in the presence of 1 nM salmon CT significantly altered cell adhesion (54 ± 8%, p < 0.02) and multinuclearity (59 ± 10%, p < 0.02) of FLG 29.1 cells as compared to control cells exposed to TPA alone (Table II). In similar experimental conditions also the

Figure 6. Role of cell–bone contact in FLG 29.1-induced bone resorption. Control (☐) refers to cultures which contained labeled bone alone. FLG 29.1 cells (▨) and HL60 cells (■) were cultured for 72 h with prelabeled bone with and without 0.1 μM TPA. Groups of bone particles were incubated in cell-free wells containing conditioned media recovered from 3-d-old cultures. Six replicate cultures were assessed for each variable, as described in Materials and Methods.
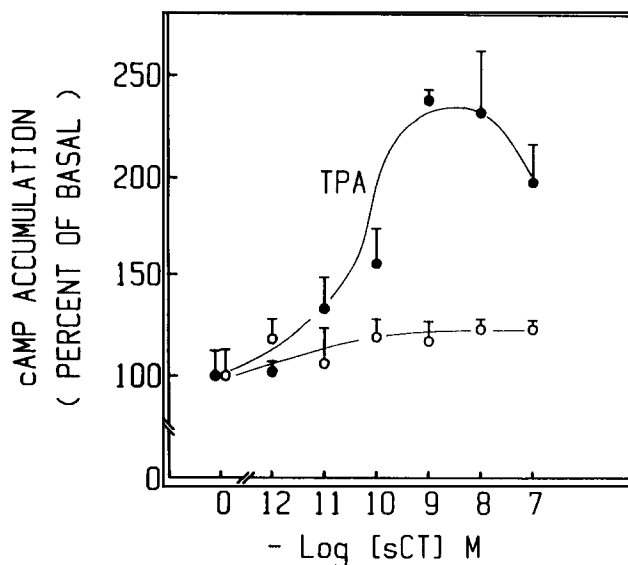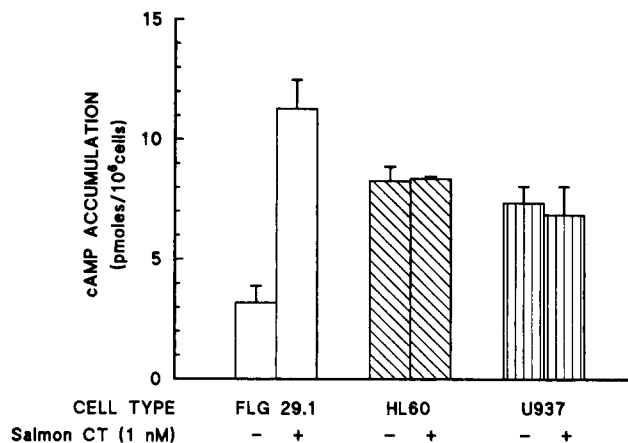

Figure 8. Cyclic AMP responses of TPA-treated FLG 29.1 (☐), HL60 (▨), and U937 (▥) cells to 1 nM salmon CT. Cyclic AMP was assayed in quadruplicate experimental points as described in Materials and Methods.
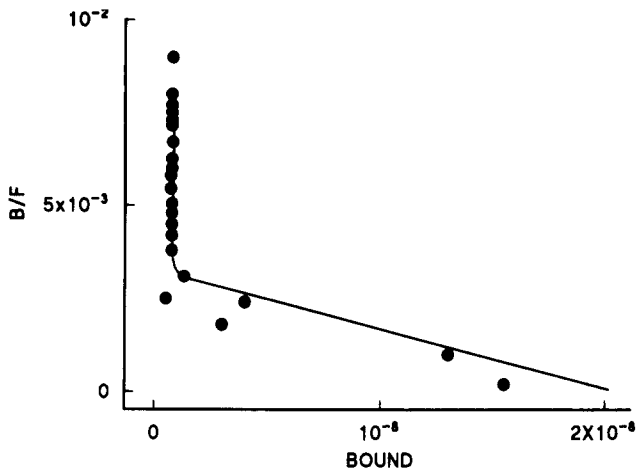
Figure 9. Equilibrium of [¹²⁵I]salmon CT binding to differentiated FLG 29.1 cells. Fitted curve on Scatchard plot was derived from predicted relationship for two-site model for [¹²⁵I]salmon CT, as determined by LIGAND program (47).

TRAPase activity (61 ± 3% of control, $p < 0.01$), the generation of oxygen-derived free radicals (42 ± 5% of control, $p < 0.001$), and the tPA cell content (75 ± 3% of control, $p < 0.01$) of differentiated FLG 29.1 cells were significantly inhibited by 1 nM salmon CT (Table II). Treatment of TPA-induced FLG 29.1 cells with 1 nM salmon CT for 3 h resulted in a marked cellular contraction measured as the change in FLG 29.1 plan area by histomorphometry (65 ± 8% of control; Table II). Similar cellular contraction was observed for mononuclear- and multinucleated-differentiated FLG 29.1 cells.

Effects of 1,25(OH)₂D₃ on FLG 29.1 Cell Differentiation

Treatment of FLG 29.1 cells with 10 nM 1,25(OH)₂D₃ for 72 h increased up to 15 ± 2% the number of adherent cells. Over 20 ± 4% were multinucleated and 15 ± 2% became TRAPase positive. No correlation existed among adherence, multinucleation, and expression of TRAPase. In similar experimental conditions, 1 nM salmon CT significantly increased cAMP accumulation in 1,25(OH)₂D₃-induced FLG 29.1 cells (181 ± 25% of control, $p < 0.02$). Conversely, FACS and APAAP analyses did not evidence induction of the two antigens recognized by the mAbs 13C2 and 23C6 in 1,25(OH)₂D₃-induced FLG 29.1 cells. Moreover no differ-

Table II. Effects of 1 nM Salmon CT on FLG 29.1 Cell Differentiation Induced by 72-hour Treatment with 0.1 μM TPA

	+ salmon CT (Percent of control)§	p
Adhesion	54 ± 8*	<0.02
Multinuclearity	59 ± 10*	<0.02
TRAPase activity	61 ± 3*	<0.01
NBT	42 ± 5*	<0.001
tPA	75 ± 3*	<0.01
Plan area	65 ± 8‡	<0.01

* Salmon CT (1 nM) was incubated with the cells during the entire 72-h TPA treatment.
‡ Salmon CT (1 nM) was added for 3 h at the end of the 72-h TPA treatment.
§ Results are expressed as mean ± SD.

ences were observed in the expression of the above antigens between FLG 29.1 cells treated with 0.1 μM TPA alone or with 0.1 μM TPA and 10 nM 1,25(OH)₂D₃ for 72 h (data not shown).

Discussion

Extensive cytological descriptions of the osteoclast exist, but there is little known about the mechanisms and the kinetics of progression from inactive or immature progenitor cells to actively resorbing elements. In fact, suitable markers for identifying osteoclast progenitor cells to terminally differentiated elements is still missing. Hormones, growth factors, interactions with bone cells of different nature, as well as with organic and inorganic substrata, seem to influence the differentiation and activation processes.

Originally, mononuclear phagocytes were considered to be osteoclast precursors on the basis of their morphological and enzymatic properties as well as their capacity to form multinucleated giant cells (65), but it was concluded later that macrophages per se were not identifiable as the precursor cells of terminally differentiated osteoclasts. In fact, evidence from the fetal bone rudiment co-culture and the osteopetrotic transplant models demonstrated that osteoclasts are not formed by the fusion of mature monocytes and macrophages, indicating that the osteoclast progenitor is a pluripotent hemopoietic stem cell (12, 61). Moreover, macrophages are distinct in phenotype and immunotype (29, 30) and lack calcitonin responsiveness (15, 48). On the other hand, Udagawa and co-workers reported that osteoclastic cells may also be derived from mature monocytes and macrophages in contact with bone marrow–derived stromal cells (71). This suggested that monocytic cells may also give rise to osteoclasts under the influence of local bone factors plus systemic hormones such as 1,25(OH)₂D₃ and dexamethasone (71). Given these conflicting findings, the availability of a clonal cell line of progenitor cells able to differentiate into osteoclast-like elements under appropriate stimulation, might be of great advantage in understanding the pathophysiology of osteoclasts and their relationships with other bone-derived cells. Here we describe the establishment and characterization of a cloned leukemic cell line of putative osteoclast progenitors. Interestingly, the patient from which primary cultures were obtained, suffered in the terminal phase of her illness from an osteopetrosis-like syndrome. This latter finding could be the result of a deficient production of mature osteoclasts because of the neoplastic transformation of their progenitors and leading to an unbalanced bone formation by osteoblasts. This situation closely reminds the lack of the mature neutrophil which accompanies the neoplastic transformation of myelopoietic progenitors in acute myeloid leukemia. Our biological characterization of FLG 29.1 overall indicates that these cells may actually represent osteoclast progenitors.

The composite pattern of membrane antigen expression of differentiated FLG 29.1 cells is very similar to the surface phenotype of fetal bone osteoclasts: reactivity with the CD9, CD13, CD32, and CD42b mAbs, strong reactivity with two mAbs 23C6 (CD51/VNR-α chain), and 13C2 originally described as osteoclast-specific markers (30), along with the presence of a restricted number of cell adhesion molecules (ICAM-1/CD54, H-CAM/CD44, and CD61/gpIIIa/VNR-β chain) and of the leukocyte common antigen CD45 (3, 5, 6,

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30, 31). The CD11b (Mol) determinant, found on immature precursors but not on mature osteoclasts (46), is expressed in TPA-treated FLG 29.1 cells. The existence of CD31 (gp IIa) in FLG 29.1 cells is the only discrepancy found between osteoclastic and FLG 29.1 cell antigens. Moreover, even though the few published studies find no CD31 expression on mature osteoclast (6, 31), a systematic survey of gpIIa expression in immature osteoclasts or osteoclast precursors has not been performed to date. Therefore, CD31 expression at specific stages of osteoclastic differentiation cannot be excluded. The lack of detectable CD14, CD33, CD4, CD35, CD36, CD11a (LFA-1), CD25, and MHC class II (HLA-DR) molecules characterizes FLG 29.1 as distinct from monocyte–macrophage cell types or macrophage polykaria (6). Similarly, the lack of CD41 (IIb-IIIa) and of factor VIII–related antigen further differentiates the FLG 29.1 cell from megakaryocytes. Note that the only macrophage-specific determinant CD68, intracytoplasmatically expressed by FLG 29.1 cells, also has been found recently on bone osteoclasts (4, 6). Finally, using Kn22, a unique mAb that identifies an osteoclast precursor, we were able to detect the 50 kD antigen, a membrane protein present on pre-osteoclasts and mature osteoclasts (35), in TPA-treated FLG 29.1 cells, while undifferentiated cells were negative. Taken together, these data suggest that the overall immunophenotype of TPA-treated FLG 29.1 cells is fully compatible with that of cells differentiating along the osteoclast-like lineage.

Further evidence of the similarity between differentiated FLG 29.1 and osteoclast-like cells include multinuclearity, TRAPase-positivity, capacity for resorbing mineral from bone fragments, and calcitonin responsiveness. Only the latter feature, however, is considered a reliable and early marker for osteoclastic differentiation in vitro, being absent in cells of the mononuclear phagocyte lineage (25); the other functions are found in macrophages as well (23, 26). Indeed, CT receptors were shown to identify mononuclear cells committed to the osteoclast lineage and are expressed before the acquisition of bone resorptive capacity by osteoclasts (25, 37). Calcitonin treatment of differentiated FLG 29.1 cells was also associated with cellular contraction, as previously shown in mature osteoclasts (49). Accordingly, incubation with CT during the differentiative period induced reduction of cell adherence, of multinuclearity, and of expression of several enzymatic activities. These findings together with the demonstration of specific binding sites for CT in differentiated FLG 29.1 cells clearly demonstrate a specific biological function of the hormone in this cell line. Differently than expression of CT receptors, TRAPase, and multinuclearity are reliable markers for osteoclastic phenotype in bone, but they are unreliable markers in culture (26). And these conclusions are further supported by the lack of correlation of several of the osteoclastic features of differentiated FLG 29.1 cells with multinuclearity. Ultrastructural studies also revealed differentiation of FLG 29.1 cells towards osteoclast-like cells (22, 42). The FLG 29.1 cells indeed acquire in culture some ultrastructural features of osteoclast-like cells (22, 42).

Under the culture conditions used in these experiments, FLG 29.1 cells express only some osteoclastic features. The differentiative agent used in these studies, TPA, is nonspecific and known to prime other leukemic cell lines towards the macrophagic phenotype (59). Interestingly, Su and co-workers have recently shown that activation of protein kinase C

inhibits bone resorption in rat osteoclasts (64). Therefore, it is possible that under other culture conditions, fully developed osteoclasts may form from undifferentiated FLG 29.1 cells. In this regard, Kukita and colleagues (36) suggest that interaction of osteoclasts with bone matrix could influence cell differentiation. It is well known that osteoblasts are critical for hormonally controlled osteoclastic bone resorption (16, 44, 57). Moreover, marrow-derived stromal cells can support osteoclastic cell differentiation in co-culture with spleen cells (70). Finally, one notes that existing in vitro models of osteoclastic resorption represent mixed cell cultures, true osteoclasts accounting for only a fraction of the total cell population.

It is of interest to note that the steroid hormone $1,25(OH)_2D_3$, known to control the differentiation of myelomonocytic precursors toward the macrophage and/or osteoclast lineages (1, 2, 7, 27, 54, 58, 68), acts as weak inducer on FLG 29.1 cells. A number of lines of evidence might justify this lack of hormonal responsiveness. First, in the currently accepted osteoclast differentiation scheme sensitivity to $1,25(OH)_2D_3$ appears at a late pre-osteoclast stage (46, 67). It is, therefore, conceivable that undifferentiated FLG 29.1 cells being arrested at an earlier maturation step are not sensitive to the action of $1,25(OH)_2D_3$. Second, $1,25(OH)_2D_3$ induces an increase in bone resorption in vivo (7), in organ culture (27, 54), and in long-term bone marrow cultures (58, 68), but has no effect on the resorptive activity of isolated osteoclasts (18, 20). Taken together, these results suggest that a clonal cell population, like FLG 29.1 is, may not be suitable for demonstrating the differentiative action of $1,25(OH)_2D_3$ toward the osteoclast lineage, because of the absence of other cell types that may influence the response to the hormone (i.e., osteoblasts and/or stromal cells).

We report here the development of a human leukemic cell line that, upon treatment with TPA acquires some functional and antigenic properties of mature osteoclasts, albeit further stimulation might be required to develop the whole functional repertoire of terminally differentiated osteoclasts, such as cavitating bone resorption. This model should yield detailed identification of many of the steps and mechanisms controlling them, for differentiating toward osteoclasts. Moreover, further experiments manipulating the cell environment may lead to elucidation of the complete pathway for osteoclast development from precursors.

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