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Retinoic Acid Induces Cell Proliferation and Modulates Gelatinases Activity in Human Osteoclast-like Cell Lines

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The effect of Retinoic Acid (RA) on human osteoclast-like cell lines, obtained from Giant Cell tumors (GCT) of bone, has been investigated evaluating its action on bone resorption, cell proliferation, microtubular organization and gelatinases expression and activity. Increasing concentrations of RA significantly dose-dependently decreased GCTs bone resorption, while 10^{-7} M RA promoted an increase of cell proliferation. By immunofluorescence we demonstrated that GCTs express A and B gelatinases and, by zymography, that their activity was enhanced in medium collected from GCTs cultured in the presence of 10^{-7} M RA. These data indicate that RA increases cell proliferation and modulates metalloproteinases (MMPs) activity, crucial events during the migration of osteoclast precursors toward bone surfaces.

Vitamin A and its derivatives (retinoids) are required for normal growth. In particular their role in the skeletal development and metabolism have been recognized since long time (1,2). Hypovitaminosis A, in fact, causes abnormal remodelling in growing bones, followed by neural defects due to insufficient wideness of the skull and of the vertebral cavities. The mechanism thorough which these effects are achieved are however not yet been defined. The activities of vitamin A in most biological systems are generally attributed to Retinoic Acid (RA) and its isomers (3). *In vivo* studies demonstrated that retinoids induced an overall increase of bone resorption (4), while *in vitro* on isolated osteoclasts stimulation in an avian model or inhibition in a rat model of resorbing activity have been found (5-7). For this reason we decided to evaluate the effect of RA on several parameters related to bone resorption, utilizing as experimental model human osteoclast-like cell lines, obtained from Giant Cell Tumors of bone (GCT), extensively characterized for their osteoclastic phenotype in our laboratory (8). In this human system RA induced a significant decrease of bone resorption, but increased proliferation and modulated metalloproteinases (MMPs) activity, indicating that this molecule in a human system influences osteoclast activity not during the resorptive phase, but increasing the number of available pre-osteoclasts and probably playing a role in the migration of osteoclasts toward bone surfaces.

MATERIALS AND METHODS

Cell cultures. Osteoclast-like cell lines, obtained from Giant Cell Tumors of Bone (GCTs) and stabilized with passages, have been utilized. The osteoclastic phenotype of these lines has been extensively characterized (8). They consist of cells belonging to the osteoclast lineage that keep dividing and differentiating in culture. Both mononuclear and multinuclear cells are present and capable of calcitonin inhibitable bone resorption and are TRAP positive at various degrees. GCTs are maintained in culture in Iscove medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 IU/ml mycostatin, purchased from GIBCO Limited, Uxbridge, UK, at 37°C in a water saturated atmosphere with 5% CO_2 and fed by medium replacement every 2-3 day. Cells used for the experiments were all within IX and XIV passages.

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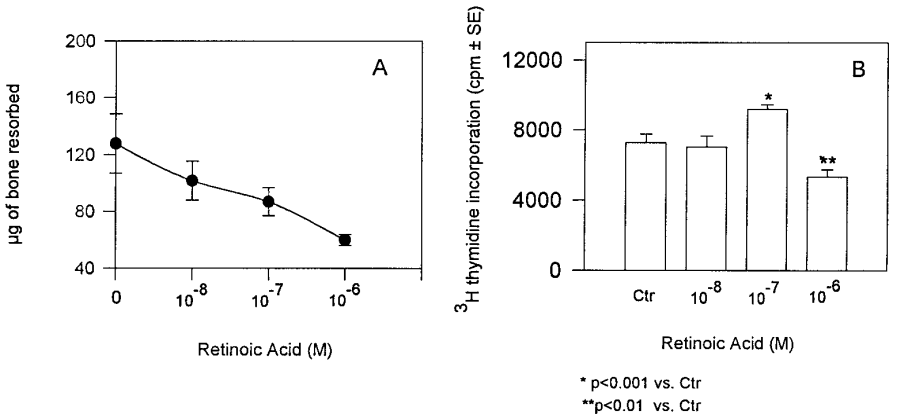

FIG. 1. Bone resorption and cell proliferation. (A) Bone resorption was measured according to Blair et al. ^{3}H-released was detected in the media of GCTs treated for 48 h with increasing concentrations of RA as well as in control conditions. Results are expressed as μg of bone resorbed. (B) 10^{-7} RA stimulates thymidine incorporation by osteoclast-like GCT cells. Cells were pulsed with 1μCi/ml of ^{3}H-thymidine for 24 h, in serum free control medium or in the presence of the indicated concentrations of RA. Values on the ordinate indicate cpm incorporated in trichloroacetic acid precipitates harvested from quadruplicate wells. Bars show the standard error.

Bone resorption. Bone resorption was measured according to Blair et al., 1986 (9). Semiconfluent GCTs in 24-well multiplates were incubated for 48 hours with or without Retinoic Acid (from 10^{-8} to 10^{-6} M) in serum-free media in the presence of 400 μg of ^{3}H-proline *in vivo* prelabeled rat bone particles. Cell-free wells were used to measure non specific ^{3}H-release. Media were collected after 48 hours, transferred to vials containing 9 ml scintillation fluid and radioactivity measured by scintillation spectrophotometry. Results from three quadruplicate experiments were expressed as mean μg bone resorbed $\pm$ SE.

Incorporation of ^{3}H-thymidine. Cell proliferation was evaluated in semiconfluent monolyers by ^{3}H-thymidine incorporation into trichloroacetic acid (TCA) precipitable material. GCT cells, cultured on 24-well dishes, were exposed to different concentration of Retinoic Acid (10^{-8} to 10^{-6} M) in serum free medium for 24 hours. At the same time control cells were cultured in serum free IMDM containing 0.5% Bovine Serum Albumin (BSA). Cells were pulsed for 24 hours with 1μCi/ml of ^{3}H-thymidine (Amersharm, Life Science), washed with PBS, solubilized in 1 ml of 0.1% SDS containing 1mg/ml BSA and precipitated by addition of 100% TCA. After incubation of 30 min at 4°C, the TCA-precipitable material was pelleted by centrifugation at 955 g, redissolved in 500 μl of 0.1% SDS and counted in a Beckman 6000 scintillation spectrophotometer.

Antibodies. Monoclonal antibodies against α and β tubulin were purchased from Amersham International (Aylesbury, UK); anti-rabbit collagenase 72 (MMP2 #4221) or 92 kd or (MMP9 #110) were kindly donated by Dr. W. Stetler-Stevenson (National Institute of Health - Bethesda, Maryland); anti-mouse or anti rabbit Cy3-coniugated secondary antibodies were from Chemicon International Inc.

Immunofluorescence. Microtubule organization was observed in cells maintained for 2 days in control conditions and then treated with or without 10^{-7} M Retinoic Acid in serum-free medium for 24 hours. After treatment cells were gently washed with (Ca^{2+} Mg^{2+}) PBS, fixed in 3% paraformaldehyde in PBS pH 7.6 for 10 min, permeabilized with methanol and incubated with mouse anti-α and anti-β tubulin antibodies for 45 min at 37°C. GCTs were then rinsed in PBS, further incubated with anti-mouse Cy3-conjugated Antibody, rinsed and mounted in 20% Mowiol 4-88 (Hoechst AG, Frankfurter on Main, Germany).

Gelatinases expression was evaluated in the same conditions with anti-rabbit 72 Kd or 92 Kd collagenase (respectively gelatinases A and B). Immunofluorescence observations were performed on a Zeiss Axiophot microscope. Pictures were recorded on Kodak T-Max 400 films.

Zymography. 72 and 92-kD gelatinases activity was assayed by zymography of media collected from cells cultured, with or without bone fragments in serum-free media with or without increasing concentrations of Retinoic Acid from 10^{-8} to 10^{-6} M. Each sample was dialyzed using spectra/por molecularporous dialysis membranes with 15,000 molecular weight cut off (MWCO) (Spectrum, Houston Texas) and proteins were quantified by Micro BCA protein assay reagent kit (Pierce, Rockford, IL U. and S.A.).

30 μg proteins/samples were loaded, without boiling or reduction, onto 8% SDS/polyacrilamide gel impregnated with gelatin (0.5mg/ml). After electrophoresis, conducted at 35 milliamper and at 4°C, the gel was washed (2 $\times$ 30 min at room temperature) with 2.5 % Triton-X 100 and incubated for 16-20 hours at 37°C in 40 mM Tris-HCl buffer,

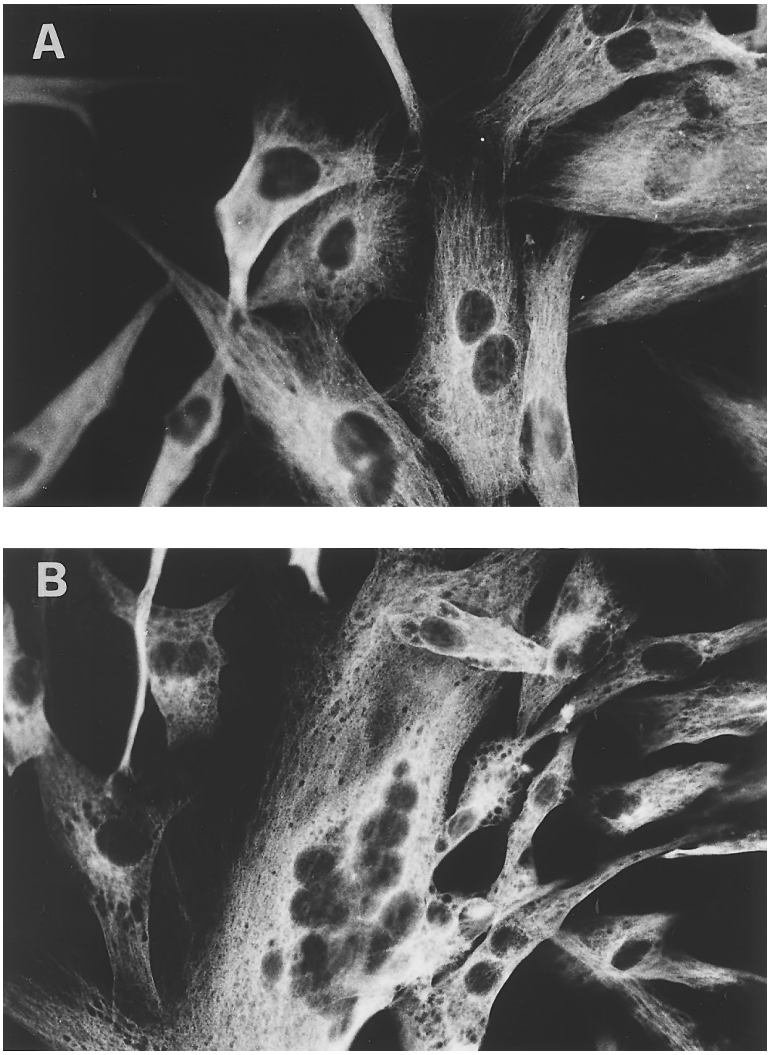

FIG. 2. Immunofluorescence staining for α and β-tubulin. (A) GCTs in control conditions show a network of microtubules radiates from the perinuclear region toward the cell margin. (B) RA-treated GCTs show a dense array of microtubules and a granular distribution of tubulin in the cytoplasm. Magnification 600×.

pH 7.5, containing NaCl 0.2M, $CaCl_2$ 10mM. Clear bands, identifying the position of 72-kDa and 92-kDa gelatinases, were visualized on the blue background after staining with 0.25% Coomassie blue R250 and destaining with 50% methanol and 10% acetic acid.

RESULTS

Bone Resorption

Bone resorption activity was measured after 48 hours in the presence of RA or in control conditions. A significant dose-dependent decrease of resorption activity respect to the control (Fig.1A) was observed.

^{3}H-Thymidine Incorporation

Cell proliferation by ^{3}H-thymidine incorporation into trichloroacetic acid (TCA) precipitable material was evaluated in semiconfluent monolayers. GCT cells, exposed to increasing concen-

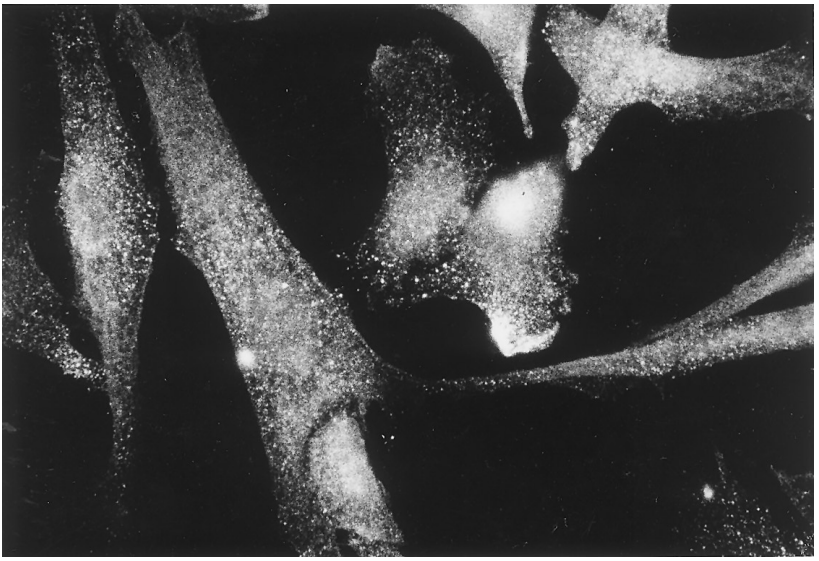

FIG. 3. Immunofluorescence staining for gelatinases-A (72 kDa) and gelatinases-B (92 kDa). MMP-2 and MMP-9 were respectively immunostained with appropriated anti-rabbit antibodies. Both enzymes are organized in intracellular granules crowded at the cell periphery. Magnification 800×.

tration of RA in serum-free medium, showed a significant increase in their proliferation rate peaking at 10^{-7} M RA. This effect was followed by a dramatic decrease at 10^{-6} M RA (Fig. 1B).

Immunofluorescence

Effect of retinoic acid on microtubules. Control GCTs, as already described in chicken and rat osteoclasts (10), presented a well-defined microtubular organization with microtubules

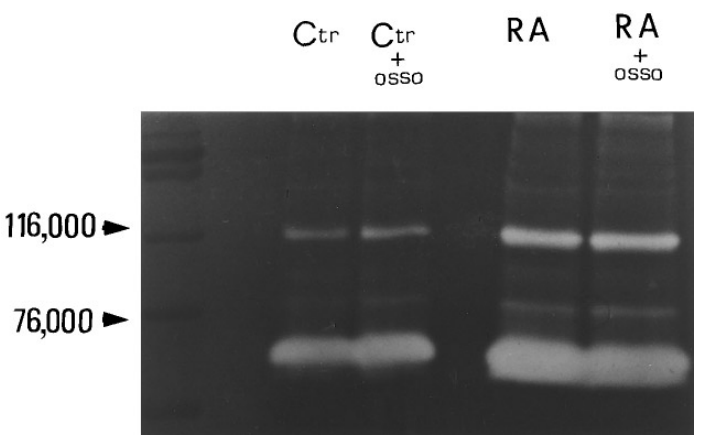

FIG. 4. Detection of gelatinases-A (72 kDa) and gelatinases-B (92 kDa) by zymography. Samples of media collected from GCTs cultured, without or with bone fragments, in serum-free media (line 2-3) or in the presence of 10^{-7} M RA (line 4-5) were subjected to electrophoresis on gelatin-containing SDS polyacrylamide gel. MMP-9 (92 kDa) and MMP-2 (72-kDa) are more active in the treated samples (line 5). Line 4 and 5 show bands at high molecular weigh, poorly evident in controls.

radiating from the perinuclear region toward the cell margin (Fig. 2A). RA treated cells showed a thicker array of tubules together with a granular distribution of unpolimerized tubulins (Fig. 2B).

Effect of retinoic acid on gelatinases expression. CGTs cells immunostained with antibodies against 72 or 92 kd collagenases were strongly positive for both enzymes, that were prevalently organized in granular clusters close to the plasma membrane (Fig. 3).

Zymography. Gelatinase activity in serum free medium in the absence or in the presence of 10^{-7} M RA with or without bovine bone fragment added to the culture was investigated. In the presence of Retinoic Acid the activity of both collagenases was enhanced if compared with untreated culture medium. This effect was more evident in the presence of bone fragments (Fig. 4).

DISCUSSION

Osteoclast precursors originate in bone marrow from which migrate to reach the sites that have to be resorbed. During this migration they interact with different proteins of the extracellular matrix. The present study was designed to evaluate if RA could exert a direct effect on bone resorption or on other phases of osteoclast activity. Human osteoclast-like cell lines, obtained from Giant Cell Tumors of bone, at present the only available model for human osteoclasts *in vitro*, have been utilized. Bone resorption assays demonstrated, differently from what previously found on an avian system, that RA induced a decrease of bone resorption in a dose-dependent manner. The decrement observed with the highest doses could however be due to a toxic effect. Because *in vivo* observations homogeneously report an increase in bone resorption in hyperviaminosis A, we hypothesized an involvement of RA during other phases of osteoclastic activity, such as proliferation, differentiation and/or (pre)osteoclasts recruitment. Here we demonstrated that 10^{-8} and 10^{-7} M RA significantly increased GCTs proliferation, with a dramatic reduction of 3H-thymidine incorporation in the presence of 10^{-6} M, probably due to a toxic effect. These data let us hypothesize that this molecule could upregulate bone resorption increasing osteoclast number and not the resorptive capabilities of already differentiated osteoclasts. The presence of MMP-9 in normal osteoclasts, associated to MMP-2 in Giant Cell Tumor derived osteoclast-like cells indicate the importance of these two enzymes not only in the removal of mineralized matrix in the subosteoclastic compartment during bone resorption (11-12), but possibly also during migration in the extracellular matrix (13). For this reason we investigated if RA, already known as capable of stimulating lysosomal enzyme synthesis and secretion, could influence the activity of matrix-metalloproteinases. By immunofluorescence we confirmed the presence of both enzymes, prevalently organized in granular clusters close to the plasma membrane.

Zymographies demonstrated a clear increase of MMPs activity in a dose-dependent manner in the presence of RA. Moreover, the observed increased density of the microtubular network could be dependent upon an increase of the secretory activity of the cells induced by RA.

In conclusion this work indicates that RA, increasing GCTs proliferation and gelatinases activity, could positively increase bone resorption and control osteoclast activity.

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REFERENCES

1. Barnicott, N. A., and Datta, S. P. (1972) *in* The Biochemistry and Physiology of Bone (Bourne, G. H., Ed.), 2nd ed., Vol. 2, pp. 197–227, Academic Press, London and New York.
2. Dickson, I., and Walls, J. (1985) *Biochem. J.* **226,** 789–795.
3. Gudas, L. J. (1994) *J. Biol. Chem.* **269,** 15399–15402.

4. Hough, S., Avioli, L. V., Muir, H., Gelderblom, D., Jenkins, G., Kurasi, H., Slatopolsky, E., Bergfeld, M. A., and Teitelbaum, S. L. (1988) *Endocrinology* **122,** 2933–2939.
5. Oreffo, R. O., Teti, A., Triffit, J. T., Francis, M. J. O., Carano, A., and Zambonin Zallone, A. (1988) *J. Bone Min. Res.* **3,** 203–210.
6. Walsh, C. A., Beresford, J. N., Birch, M. A., Boothroyd, B., and Gallagher, J. A. (1991) *J. Bone Min. Res.* **6**(7), 661–671.
7. O'Neill, R. P. J., Jones, S. J., Boyde, A., Taylor, M. L., and Arnett, T. R. (1992) *Bone* **13,** 23–27.
8. Grano, M., Colucci, S., De Bellis, M., Zigrino, P., Argentino, L., Zambonin, G., Serra, M., Scotlandi, K., Teti, A., and Zambonin Zallone, A. (1994) *J. Bone Min. Res.* **9,** 1013–1020.
9. Blair, H. C., Kahn, A. J., Crouch, E. C., Jeffrey, J. J., and Teitelbaum, S. L. (1986) *J. Cell Biol.* **102,** 1164–1172.
10. Lakkakorpi, P. T., and Vaananen, H. K. (1991) *J. Bone Min. Res.* **6,** 817–826.
11. Everts, V., Delaissé, J. M., Korper, W., Niehof, A., Vaes, G., and Beertsen, W. (1992) *J. Cell Physiol.* **150,** 221–231.
12. Okada, Y., Naka, K., Kawamura, K., Matsumoto, T., Nakanishi, I., Fujimoto, N., Sato, H., and Seiki, M. (1995) *Lab. Invest.* **72,** 311–322.
13. Delaissé, J. M., Eeckout, Y., Neff, L., Francois-Gillet, C., Henriet, P., Su, Y., Vaes, G., and Baron, R. (1993) *J. Cell Sci.* **106,** 1071–1082.