Vitamin A effects on UMR 106 osteosarcoma cells are not mediated by specific cytosolic receptors

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Retinol and retinoic acid at 20 μM altered cell morphology and inhibited cell proliferation of UMR 106 osteosarcoma cells in culture. No specific cytosolic binding proteins for retinol could be detected.

INTRODUCTION

Vitamin A (retinol) plays important roles in the maintenance of normal growth, bone formation, epithelial tissue integrity, vision (function of the retina) and reproductive ability (Barnicott & Datta, 1972). Effects *in vivo* and *in vitro* of retinol on skeletal tissues are well documented but the mechanism of action of the vitamin remains obscure (Wolbach & Howe, 1925; Sherman, 1967; Moore, 1954; Zile *et al.*, 1979; Mellanby, 1947). In part this is due to the complexity of cell-types present in skeletal tissues and the known interactions between the bone-forming (osteoblastic) and bone-resorbing (osteoclastic) cells (Rodan & Martin, 1981; Chambers, 1982). Here we report the effects of vitamin A on UMR 106 osteoblast-like cells. The UMR 106 cell line is a clonal malignant line derived from a ^{32}P -induced, transplantable rat osteogenic sarcoma (Martin *et al.*, 1976; Partridge *et al.*, 1980). It is characterized as an osteoblast-like cell line by hormone responsiveness, osteogenic capacity *in vivo* and alkaline phosphatase content (Partridge *et al.*, 1981).

In the present paper we show that retinol and retinoic acid have direct effects on the growth of UMR 106 cells but that these effects are not mediated by specific cytosolic-binding proteins.

EXPERIMENTAL

Materials

[11,12(N)- ^3H]Vitamin A free alcohol (40–60 Ci/mmol) was from Amersham International. *all-trans*-Retinoic acid, retinyl palmitate, retinol, *p*-nitrophenyl phosphate, alkaline buffer solution (AMP) and phosphatase substrate were from Sigma. Nunc tissue culture flasks were from Gibco; all tissue culture media and newborn calf serum were obtained from Tissue Culture Services (Slough, Berks., U.K.). Foetal calf serum and Dulbecco's minimum essential medium was from Flow Laboratories. HP Ready Solv aqueous counting solution was from Beckman. The rat osteosarcoma cell line, UMR 106, was a generous gift from Drs. N. C. Partridge and T. J. Martin, University of Melbourne, Department of Medicine, Parkville, Victoria, Australia. The mouse NIH 3T3 fibroblasts were obtained from Dr. R. Sutherland, Dunn School of Pathology, Oxford, U.K. Cyanogum 41 and all other reagents were of analytical grade and were obtained from BDH.

Cell culture

UMR 106 and NIH 3T3 cells were grown in culture flasks (25 cm^2), and dishes (9.6 cm^2), in Dulbecco's minimal essential medium containing 10% (v/v) foetal calf serum and 10% (v/v) newborn calf serum respectively. Retinol, retinoic acid and retinyl palmitate in ethanol (final concn. 0.2%, v/v) were added to the media to give various concentrations (10–100 μM) of retinoid. Control cultures contained the ethanol vehicle alone.

Cell harvesting

Adherent cells were washed twice with phosphate-buffered saline (Flow Laboratories) and harvested in 1 ml of distilled water by scraping with a rubber policeman. Harvested cells were lysed by sonication at 20 kHz for 30 s in 10 ml of distilled water in an ultrasonic disintegrator (Ultrasonics, Plainview, NY, U.S.A.).

Measurement of DNA content, protein content and alkaline phosphatase activity of cells

Cell lysates were assayed for DNA content by the fluorimetric technique of Wilfinger & Down (1983), protein content by a commercially available kit using immunoglobulin standards (Bio-Rad) and alkaline phosphatase activity by the method of Partridge *et al.* (1981).

Cell morphometric analysis

UMR 106 cells were grown in culture flasks (25 cm^2) in media containing retinoid or ethanol alone (control) as described above. After 3 and 5 days in culture the flasks were photographed with phase contrast on a Zeiss photomicroscope (Carl Zeiss, Welwyn Garden City, Herts., U.K.) using Kodak Technical Pan film (ASA 100) (Kodak, Hemel Hempstead, Herts., U.K.). Negatives were enlarged by using a photographic enlarger, and cell area and perimeter were measured on a Kontron Quantimet (Kontron Electronics, St. Albans, Herts., U.K.).

Determination of [^3H]thymidine incorporation

The method of Ng *et al.* (1983) was used with minor modifications. Cells (1.25×10^5) were subcultured into six-place multiwell plates and left to settle for 24 h. They were washed with culture media (3 ml) and incubated with the appropriate retinoid at 50 nM–50 μM for 3 and 5 days. Media were changed every 2 days and after 70 and

Abbreviations used: CRBP and CRABP, cellular retinol-binding and retinoic acid-binding proteins, respectively.

118 h in culture the cells were incubated for 2 h with [^3H]thymidine (0.5 $\mu\text{Ci/ml}$). The medium was removed and the cells washed with ice-cold phosphate buffered saline and harvested using 0.125% trypsin/0.5 mM- Na_2EDTA (0.9 ml). Calf DNA (0.1 ml, 1 mg/ml) was added to the cell suspension and the suspension precipitated with 10% (v/v) trichloroacetic acid (1 ml). The acid-insoluble macromolecules were recovered by centrifugation at 3000 g for 15 min and washed twice with 5% (v/v) trichloroacetic acid (2 ml) and twice with 95% (v/v) ethanol (2 ml) before dissolving in concentrated formic acid (0.5 ml). Radioactive content of each sample was measured by the addition of scintillation fluid (5 ml of HP Ready Solv) and by liquid scintillation spectrometry.

Determination of CRBP

Preparation of cytosolic fraction. UMR 106 cells (100×10^6) were sonicated at 20 kHz for 30 s in 10 ml of ET buffer (10 mM-Tris/1 mM-EDTA, pH 7.24) and centrifuged at 159000 g (r_{av} 6.12 cm) for 45 min at 4 °C in a Ti 70.1 angle head rotor using an L8-55 ultracentrifuge (Beckman) and the supernatant was taken as the cytosolic fraction. NIH 3T3 cells were treated similarly.

Preparation of tissue fractions. Liver and lung tissue from 12 rat foetuses were collected and stored at -20 °C. These organs were homogenized in 50 mM-Tris/HCl, pH 7.2 (4 ml/g of tissue) and centrifuged at 31000 g (r_{av} 6.12 cm) for 15 min in a Ti 40.1 rotor (Beckman). The supernatant liquids were then centrifuged at 105000 g (r_{av} 6.12 cm) for 60 min in a Ti 70.1 rotor to obtain the cell-free organ extract. Liver extracts were exposed to u.v. light (254 nm) for 24 h to destroy endogenous retinol. Lung extracts were adjusted to pH 5.0 with acetic acid (1 M) to remove low density binding proteins and the precipitated proteins removed by centrifugation at 31000 g (r_{av} 8 cm) for 15 min. The supernatant was adjusted to pH 7.2 with NaOH (1 M), as described by Ong & Chytil (1976).

These extracts and the cell cytosol fractions were assayed for specific CRBP by three methods.

(1) Sucrose density centrifugation. The method of Haussler *et al.* (1983) was used with minor modifications. Samples of cytosol were incubated with 50 nM-[^3H]retinol alone or [^3H]retinol in combination with non-radioactive retinol or retinoic acid (5 μM) and allowed to equilibrate at 4 °C for 24 h. The samples were centrifuged at 234000 g (r_{av} 6.12 cm) on a 5–20% sucrose gradient in polyallomer tubes (7.62 cm $\times$ 1.59 cm) for 20 h. Fractions (0.5 ml) were collected by upward displacement and the radioactive content of each fraction measured by the addition of scintillation fluid (5 ml of HP Ready Solv) and by liquid scintillation spectrometry.

(2) Gel permeation chromatography. Samples of cytosol were incubated as detailed above (method 1) and fractionated on a Sephadex G-25 column (64 cm $\times$ 0.9 cm diam.) (Pharmacia) with ET buffer. Fractions (0.5 ml) were collected on a fraction collector (LKB) and radioactive content measured as detailed above (method 1).

(3) Gel electrophoresis. Samples of cytosol [50 μl of cytosol + 20 μl of 10% (w/v) sucrose containing 0.01% Bromophenol Blue dye] were incubated as detailed above (method 1), and applied to 5% (w/v) and 15% (w/v) polyacrylamide gels, and the discontinuous system of Hjertan *et al.* (1965) was used. Electrophoresis was for 15 min at 1 mA/gel and at 4–6 mA/gel track until the Bromophenol Blue marker had migrated 5 cm. The gels were removed and sliced into 30 fractions (1.6 mm) using a Canaco gel slicer (Canaco, Rockville, MD, U.S.A.). Each fraction was measured for radioactivity content as detailed above (method 1).

RESULTS AND DISCUSSION

Distinct changes in cell morphology were observed by phase contrast microscopy after 48 h incubation with retinol and retinoic acid at concentrations of 15–20 μM . Cell morphology changed from the characteristically close packed polygonal cells, as described by Partridge *et al.* (1981), to enlarged and flattened cells with extensive dendritic processes.

Quantification by morphometric analysis showed a

Table 1. Action of retinoids on area and perimeter of osteosarcoma (UMR 106) cells

Values are means $\pm$ S.E.M. ($n = 50$). Significance of difference from control (Student's t test): * $P \leq 0.01$, ** $P \leq 0.001$.

Retinoid	Concn. (μM)	Area (μm^2)	Perimeter (μm)
Control (no addition)		297.9 $\pm$ 11.4	68.8 $\pm$ 1.6
Retinol	0.5	286.5 $\pm$ 11.2	75.4 $\pm$ 5.8
	5.0	278.6 $\pm$ 11.4	79.4 $\pm$ 3.5*
	10.0	376.9 $\pm$ 20.8*	118.9 $\pm$ 4.7**
	20.0	1495.3 $\pm$ 77.4**	171.7 $\pm$ 4.7**
Retinoic acid	0.5	271.5 $\pm$ 12.8	79.5 $\pm$ 9.9
	5.0	402.6 $\pm$ 17.4**	84.6 $\pm$ 3.8*
	10.0	667.8 $\pm$ 36.7**	115.5 $\pm$ 4.6**
	20.0	1046.4 $\pm$ 73.3**	172.8 $\pm$ 7.9**
Retinyl palmitate	0.5	322.1 $\pm$ 17.4	76.7 $\pm$ 2.6
	10.0	318.1 $\pm$ 12.5	75.2 $\pm$ 2.7
	20.0	380.9 $\pm$ 18.3**	93.5 $\pm$ 4.8**

Fig. 1. Effect of retinoids on DNA content (a) and alkaline phosphatase activity (b) of osteosarcoma (UMR 106) cells

DNA content or alkaline phosphatase activity of cultured UMR 106 osteosarcoma cells were measured after 5 days in the presence of retinol (○), retinoic acid (■) and retinyl palmitate (□). For full experimental details see the text. Each point is the mean of paired incubations (DNA) or the mean $\pm$ S.D. of four incubations (alkaline phosphatase).

significant dose-dependent increase over control values in cell area (1.25–4-fold) and perimeter (1.6–2.4-fold) with 10 and 15 μ M-retinol and retinoic acid (Table 1). Retinyl palmitate below 20 μ M had no significant effects but both cell area and perimeter were increased at 20 μ M. Subsequent culture in medium lacking added retinoids led to a reversal of the changes in cell morphology and growth inhibition. Retinol concentrations of 50–100 μ M

were toxic with severe growth inhibition and cell death, and these changes were not reversed by culture in retinoid-free media. Cell viability as determined by Trypan Blue exclusion (85–90%) was unaffected in cultures containing below 50 μ M-retinol and in control cultures containing ethanol alone.

Retinol and retinoic acid concentrations above 20 μ M cause marked inhibition of UMR 106 cell proliferation, as measured by DNA content (Fig. 1a), and this was paralleled by similar effects on alkaline phosphatase activity (Fig. 1b) and measurements of cell number (results not shown).

Thymidine uptake studies at 3 and 5 days (Table 2) showed a significant decrease in thymidine uptake in those cultures incubated with 5 μ M-retinol ($P < 0.01$) and retinoic acid ($P < 0.05$) to about 75% of control values. Retinol and retinoic acid inhibited the observed incorporation of [3 H]thymidine in a dose-dependent manner. Retinoids have been shown to affect differentiation and growth of normal and neoplastic cells using comparable doses of retinoids (Dickson & Walls, 1985; Lacroix *et al.*, 1981; Jetten *et al.*, 1979; Lotan, 1980) and effects *in vitro* on rat chondrosarcoma and human osteosarcoma cell lines have been described (Thein & Lotan, 1982). Our results *in vitro* show similar direct growth inhibitory effects of retinol and retinoic acid on malignant cells, which have some of the characteristics of early precursors of osteoblasts, and pronounced changes in their morphology after retinoid treatment. The action of retinyl palmitate on cell proliferation was less marked, either because this particular retinoid is inactive in culture or because it cannot readily be de-esterified.

Direct effects of retinol and retinoic acid on tissues may be mediated by specific intracellular binding proteins (Bashor *et al.*, 1973). CRBP and CRABP have been detected and partially characterized (Ong & Chytil, 1975, 1978a). In agreement with others (Jetten *et al.*, 1979) we have detected CRBP in cultured skin fibroblasts (NIH 3T3) (Fig. 2), and in liver and lung tissue (Fig. 3) by using gel chromatography; specific receptor proteins were shown also by using gel electrophoresis and sucrose density gradient centrifugation (results not shown). In each case [3 H]retinol bound to a macromolecule with an apparent M_r of approx. 15000 as determined by comparison with protein M_r standards, in agreement with the value obtained by Ong & Chytil (1978a,b) for the binding proteins observed in a number of tissues. Furthermore, this binding was reduced to low levels by the addition of excess non-radioactive retinol, but not by an excess of non-radioactive retinoic acid (Fig. 2). Similar results were obtained for liver and lung (Fig. 3). This indicates that the binding macromolecule is specific for retinol and that the binding is saturable. In contrast, CRBP could not be detected in the UMR 106 osteosarcoma cell line by gel filtration procedures (Fig. 2) and further studies using sucrose density gradient centrifugation and gel electrophoresis methods confirmed this (results not shown).

In some cell lines such as 3T3 fibroblasts (Jetten *et al.*, 1979), human breast cancers (Lacroix & Lippman, 1980), embryonic carcinomas (Schindler *et al.*, 1981) and certain human neuroblastomas (Haussler *et al.*, 1983) the retinoid effect is associated with detectable levels of the respective binding protein. Similarly we have observed this effect in the cultured skin fibroblasts (NIH 3T3). In contrast, in a variety of other cell lines the presence of

Table 2. [³H]Thymidine incorporation by UMR 106 cells into acid-insoluble macromolecules after 3 and 5 days

For experimental details see the text. Values are means $\pm$ S.E.M. Significance of difference from control (Student's *t* test): **P* $\leq$ 0.05, ***P* $\leq$ 0.01, †*P* $\leq$ 0.001. N.D., not determined.

Retinoid	Concn. (μ M)	10 ⁻³ $\times$ [³ H]Thymidine incorporation (c.p.m.)	
		Day 3 (<i>n</i> = 6)	Day 5 (<i>n</i> = 4)
Control		28.7 $\pm$ 1.0	28.2 $\pm$ 1.3
Retinol	0.1	28.2 $\pm$ 1.1	N.D.
	5.0	21.8 $\pm$ 0.8	19.6 $\pm$ 0.4**
	10.0	20.1 $\pm$ 0.7†	16.7 $\pm$ 0.9†
	20.0	11.8 $\pm$ 0.4†	9.3 $\pm$ 0.6†
Retinoic acid	0.1	25.1 $\pm$ 1.2	N.D.
	5.0	24.8 $\pm$ 1.2*	24.8 $\pm$ 0.9*
	10.0	21.7 $\pm$ 1.0†	21.6 $\pm$ 1.5**
	20.0	15.2 $\pm$ 1.1†	14.1 $\pm$ 0.7†
Retinyl palmitate	0.1	27.8 $\pm$ 0.7	N.D.
	5.0	26.1 $\pm$ 1.6	28.7 $\pm$ 0.5
	10.0	26.0 $\pm$ 1.4	24.6 $\pm$ 1.4
	20.0	27.2 $\pm$ 0.4	19.5 $\pm$ 0.3**

Fig. 2. Cytosolic retinoid binding by osteosarcoma cells (UMR 106) and fibroblasts (NIH 3T3) determined by gel permeation chromatography

Various concentrations of unlabelled retinoids (10^{-9} – 10^{-5} M) were incubated with 10^{-12} M-[³H]retinol for 24 h at 4 °C to determine specific binding. For full experimental details see the text. Each point represents the mean $\pm$ S.E.M. ○, △, Retinol (○, UMR 106 cells; △, NIH 3T3 cells); ■, □, retinoic acid (■, liver; □, lung). **P* < 0.05 compared with basal level, ***P* < 0.02 compared with basal level (Student's *t* test).

Fig. 3. Cytosolic retinoid binding in rat lung and liver cytosolic fractions determined by gel permeation chromatography

Various concentrations of unlabelled retinoids (10^{-9} – 10^{-5} M) were incubated with 10^{-12} M-[³H]retinol for 24 h at 4 °C to determine specific binding. For full experimental details see the text. Each point represents the mean $\pm$ S.E.M. ○, △, Retinol (○, liver; △, lung); ■, □, retinoic acid (■, liver; □, lung). †*P* < 0.05, **P* < 0.01, ***P* < 0.02 compared with basal level (Student's *t* test).

CRBP or CRABP does not appear to be obligatory for inhibition of cell growth by retinol or retinoic acid (Lotan, 1980; Haussler *et al.*, 1984). For example, human leukaemic cells (HL-60) have no CRABP but their growth is inhibited by retinoic acid and their differentiation into granulocytes promoted (Douer & Koeffler, 1982; Breitman *et al.*, 1980). Our results indicate that the UMR 106 rat osteosarcoma cell line is another example of this latter phenomenon, at least in the case of the action of retinol on these cells. The lack of availability of radioactively labelled retinoic acid precluded our invest-

igation of the presence of CRABP in this osteosarcoma cell line. Further studies are necessary to determine the effects of retinoids on normal bone cells.

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REFERENCES

- Barnicott, N. A. & Datta, S. P. (1972) in *The Biochemistry and Physiology of Bone* (Bourne, G. H., ed.), 2nd edn., vol. 2, pp. 197–227, Academic Press, London and New York
- Bashor, M. M., Toff, D. O. & Chytil, F. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3483–3487

- Breitman, T. R., Selonick, S. E. & Collins, S. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **7**, 2936–2940
- Chambers, T. J. (1982) in *Current Advances in Skeletogenesis* (Silbermann, M. & Slavkin, H. C., eds.), pp. 154–159, Excerpta Medica, Amsterdam, Oxford and Princeton
- Dickson, I. & Walls, J. (1985) *Biochem. J.* **226**, 789–795
- Douer, D. & Koeffler, P. (1982) *J. Clin. Invest.* **69**, 277–283
- Haussler, M., Sidell, N., Kelly, M., Donaldson, C., Altman, A. & Mangelsdorf, D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5525–5529
- Haussler, M., Donaldson, C., Kelly, M., Mangelsdorf, D., Timothy-Bowden, G., Meinke, W., Meyskens, F. & Sidell, N. (1984) *Biochim. Biophys. Acta* **803**, 54–62
- Hjertan, S., Jerstedt, S. & Tiselius, A. (1965) *Anal. Biochem.* **11**, 211–218
- Jetten, A. M., Jetten, M. E. R., Shapiro, S. S. & Poon, J. P. (1979) *Exp. Cell Res.* **119**, 289–299
- Lacroix, A. & Lippman, M. E. (1980) *J. Clin. Invest.* **65**, 586–591
- Lacroix, A., Anderson, G. D. & Lippman, M. E. (1981) *Ann. N.Y. Acad. Sci.* **359**, 405–406
- Lotan, R. (1980) *Biochim. Biophys. Acta* **605**, 33–91
- Martin, T. J., Ingleton, P. M., Underwood, J. E. C., Michelangeli, V. P., Hunt, N. H. & Melick, R. A. (1976) *Nature (London)* **260**, 436–438
- Mellanby, E. (1947) *J. Physiol. (London)* **105**, 382–399
- Moore, T. (1954) in *The Vitamins* (Sebrell, W. H. & Harris, R. S., eds.), 2nd edn., vol. 1, pp. 245–266, 280–294, Academic Press, New York
- Ng, K. W., Partridge, N. C., Niall, M. & Martin, T. J. (1983) *Calcif. Tissue Int.* **35**, 298–303
- Ong, D. E. & Chytil, F. (1975) *Nature (London)* **255**, 74–75
- Ong, D. E. & Chytil, F. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3976–3978
- Ong, D. E. & Chytil, F. (1978a) *J. Biol. Chem.* **253**, 828–832
- Ong, D. E. & Chytil, F. (1978b) *J. Biol. Chem.* **253**, 4551–4554
- Partridge, N. C., Frampton, R. J., Eisman, J. A., Michelangeli, V. P., Elms, E., Bradley, J. R. & Martin, T. J. (1980) *FEBS Lett.* **115**, 139–142
- Partridge, N. C., Alcorn, D., Michelangeli, V. P., Kemp, B. E., Ryan, G. B. & Martin, T. J. (1981) *Endocrinology (Baltimore)* **108**, 213–219
- Rodan, G. A. & Martin, T. J. (1981) *Calcif. Tissue Int.* **33**, 349–351
- Schindler, J., Matthaei, K. I. & Sherman, J. I. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1077–1080
- Sherman, B. S. (1967) *Calcif. Tissue Res.* **1**, 192–196
- Thein, T. R. & Lotan, R. (1982) *Cancer Res.* **42**, 4771–4775
- Wilfinger, W. W. & Down, T. R. (1983) *Anal. Biochem.* **131**, 538–547
- Wolbach, S. B. & Howe, P. R. (1925) *J. Exp. Med.* **42**, 753–777
- Zile, M. H., Bunge, E. G. & Deluca, H. E. (1979) *J. Nutr.* **109**, 1787–1796

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