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Specific binding of 1,25-dihydroxycholecalciferol in human medullary thyroid carcinoma

Hedley C. FREAKE and Iain MACINTYRE
Endocrine Unit, Royal Postgraduate Medical School, Du Cane Road, London W12 0HS, U.K.

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A specific 1,25-dihydroxycholecalciferol-binding protein has been detected in high-salt cytosols prepared from human medullary thyroid carcinomas. The binding protein had the same equilibrium dissociation constant (K_d = 0.17 ± 0.05 nM; n = 4) and sedimentation coefficient on sucrose gradients (3.7 S) as that seen in established vitamin D target tissues. This protein was not detected in normal thyroid cytosols, which may reflect the low proportion of C-cells within the gland.

$1,25(OH)_2D_3$ is thought to function in the classical mode of a steroid hormone. Thus its action is initiated by binding to a specific cytoplasmic receptor-protein, which then translocates to the nucleus to produce its effects on gene expression (Haussler & McCain, 1977; Wecksler & Norman, 1980). The localization and characterization of this binding-protein have recently become a prime area of vitamin D research. It has been detected in tissues that can be divided broadly into three classes, namely: (i) organs involved in transepithelial Ca^{2+} transport, such as intestine, kidney and bone (Wecksler & Norman, 1980) and also breast (Eisman *et al.*, 1980); (ii) endocrine glands producing hormones thought to regulate the renal $25(OH)D_3$ hydroxylases, e.g. parathyroid and pituitary (Pike *et al.*, 1980); and (iii) sites where a function for $1,25(OH)_2D_3$ has yet to be elucidated, e.g., skin (Colston *et al.*, 1980) and brain (Marcocci *et al.*, 1982).

Calcitonin has been shown to stimulate the renal production of $1,25(OH)_2D_3$ (Galante *et al.*, 1972; Kawashima *et al.*, 1981). In mammals, calcitonin is produced principally in the parafollicular or C-cells of the thyroid gland (Foster *et al.*, 1964; Pearse, 1966). However, particularly in human thyroid, C-cells are sparse (Wolfe & Delellis, 1981). Medullary thyroid carcinoma is a tumour derived from C-cells (Williams, 1966) and is associated with high circulating levels of calcitonin (Stevenson & Hillyard, 1980). We chose to examine medullary thyroid carcinomas for the presence of the $1,25(OH)_2D_3$-binding protein to determine whether C-cells might be added to the second endocrine class of $1,25(OH)_2D_3$ target tissues.

Abbreviations used: $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25(OH)D_3$, 25-hydroxycholecalciferol; $[^3H]1,25(OH)_2D_3$, 1,25-dihydroxy[26,27-*Me*-^{3}H]cholecalciferol.

Experimental

Materials

Tumours were obtained immediately upon surgery, quickly dissected to separate malignant and apparently normal thyroid tissue and 'snap-frozen' in liquid N_2. They were stored over liquid N_2 and analysed within 1 month of removal.

$[^3H]1,25(OH)_2D_3$ (sp. radioactivity 148 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). Non-radioactive cholecalciferol metabolites were a gift from Roche Products (Welwyn Garden City, Herts., U.K.). Reagents were of analytical grade and obtained from commerlcial suppliers.

Cytosol preparations

Tumours or normal thyroids were roughly chopped while frozen and freeze-crushed by using a Braun Mikrodismembrator (F.T. Scientific Instruments, Tewkesbury, Glos., U.K.). The resultant powder was weighed and taken up on 6 vol. of buffer (10 mM-Tris/HCl, pH 7.4, containing 0.3 M-KCl, 1.5 mM-EGTA, 1 mM-dithiothreitol, 10 mM-sodium molybdate and 200 kallikrein inhibitory units of Trasylol/ml). The homogenates were spun at 80000 g for 1 h at 4°C in the Ti70 rotor of a Beckman L8 ultracentrifuge (Beckman-RIIV, High Wycombe, Bucks., U.K.) to yield a clear supernatant cytosol. Protein was estimated by the method of Lowry *et al.*, (1951).

Binding assays

Tumour and normal thyroid cytosols were assessed for $[^3H]1,25(OH)_2D_3$ binding by saturation

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and sucrose-density-gradient-centrifugation analysis as previously described (Marcocci *et al.*, 1982). However, in this case 5–20% (w/v) sucrose gradients were used and spun at 288000 *g* for 14 h at 4 °C in the SW55 rotor of a Beckman L8 ultracentrifuge. On some occasions, the powdered tumours were taken up in the buffer described, but omitting KCl. Under these conditions, the $1,25(OH)_2D_3$-binding protein associates with the nuclear fraction (Walters *et al.*, 1980). Crude nuclei were sedimented by centrifugation at 800 *g* for 10 min, and washed three times with the hypo-osmotic buffer. They were then incubated with 0·3 M-KCl in the same buffer for 30 min at 4 °C. The nuclei were re-sedimented and the supernatant was used to assess binding of $[^3H]1,25(OH)_2D_3$ by sucrose-gradient analysis as before.

Results and discussion

All four medullary thyroid carcinomas examined contained the specific binding protein for $1,25(OH)_2D_3$. the equilibrium dissociation constants (K_d values) and receptor concentrations were determined by Scatchard analysis of saturation plots (Fig. 1). the K_d values (0.17 ± 0.05 nM; mean ± S.E.M., $n = 4$) are in the range of those reported for the $1,25(OH)_2D_3$ receptor in numerous other tissues (Wecksler & Norman, 1980; Pike *et al.*, 1980; Colston *et al.*, 1980; Marcocci *et al.*, 1982). The receptor concentrations (5.9 ± 1.5 fmol/mg of protein; mean ± S.E.M., $n = 4$) are comparatively low. This may reflect both the inevitable heterogeneity of the tumour tissue, and a degree of degradation of the labile receptor during surgery and storage. It should be noted that concentrations of the $1,25(OH)_2D_3$-binding protein measured in breast tumours are considerably lower than those seen in continuous breast-cell lines (Eisman *et al.*, 1980).

The $1,25(OH)_2D_3$-binding protein in medullary thyroid carcinoma was seen as a single 3.7S peak on sedimentation through sucrose density gradients (Fig. 2*a*). this peak was largely displaced by a 100-fold excess of non-radioactive $1,25(OH)_2D_3$, but a similar amount of $25(OH)D_3$ was without effect. Since the displacement of bound radioactivity in the 3.7S region was not total, two of the tumours were re-analysed by examining high-salt extracts from low-salt homogenates. Under low-salt conditions in this buffer the $1,25(OH)_2D_3$-binding protein associates with the nuclear fraction, and can then be extracted from it with 0.3 M-KCl (Walters *et al.*, 1980). This can be used to effect a partial purification of the binding protein (Simpson & DeLuca, 1982). Sucrose-gradient analysis of $[^3H]1,25(OH)_2D_3$ binding in these preparations revealed a sharp 3.7S peak that was totally displaced by excess $1,25(OH)_2D_3$, but largely unaffected by excess

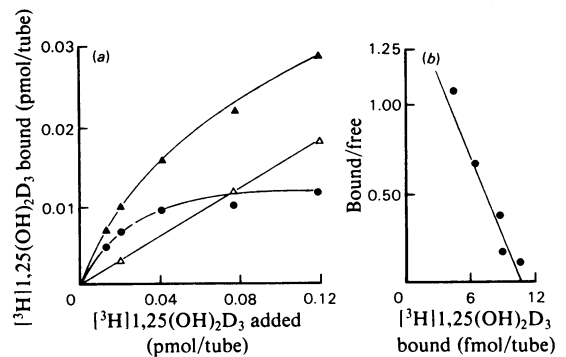

Fig. 1. *Saturation analysis of* $[^3H]1,25(OH)_2D_3$ *binding in medullary thyroid carcinoma cytosol*

Cytosol fractions (0.2 ml) were incubated at 25 °C for 2 h with increasing concentrations of $[^3H]1,25(OH)_2D_3$ in the absence (total binding; ▲) or presence (non-specific binding; △) of a 100-fold excess of non-radioactive metabolite. Free hormone was separated with charcoal. Each point represents the mean of duplicate measurements. The difference between total and non-specific binding, specific binding (●), was used to generate the Scatchard plot shown on the right.

$25(OH)D_3$ (Fig. 2*b*). The peak area in the 3.7S region was smaller when nuclear extracts rather than cytosols were used and so the latter preparations were always employed for binding-protein quantification.

Sections of tissue that showed no evidence of tumour invasion were also taken from two thyroids. They were examined in the same way as the tumours but no displaceable binding of $[^3H]1,25(OH)_2D_3$ could be detected, either by saturation or by sucrose-gradient analysis (Fig. 2*c*). This lack of specific $1,25(OH)_2D_3$ binding suggests that the binding seen in medullary thyroid carcinoma cytosols is not due to plasma contamination. Plasma contains vitamin D-binding protein, which is responsible for the transport of all vitamin D metabolites in blood and presumably contaminates all tissue specimens (Franceschi *et al.*, 1981). However, normal thyroid contained more blood than the tumour tissue and yet showed no specific binding of $[^3H]1,25(OH)_2D_3$. In addition the plasma vitamin D-binding protein has a higher affinity for $25(OH)D_3$ than for $1,25(OH)_2D_3$ (Franceschi *et al.*, 1981). Under the conditions used $25(OH)D_3$ was not a successful competitor for $[^3H]1,25(OH)_2D_3$ binding, thus making it extremely unlikely that the plasma protein is the source of binding seen in medullary thyroid carcinoma cytosols.

In adult human thyroid only 0.1% of the glandular epithelium is composed of C-cells (Wolfe

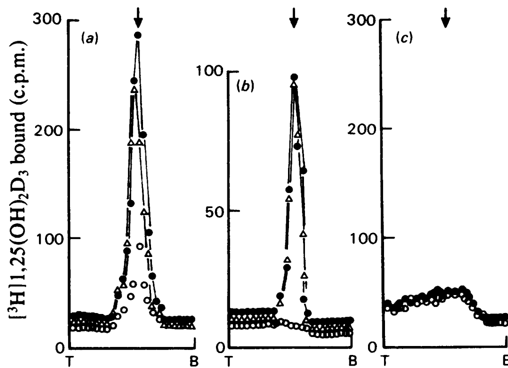

Fig. 2. *Sucrose-gradient analysis of* [^{3}H]*1,25(OH)$_2$D$_3$ binding in medullary thyroid carcinoma and normal thyroid tissues*

Cytosol fractions (*a* and *c*) or high-salt extracts of nuclei prepared in hypo-osmotic buffer (*b*) were incubated with [^{3}H]1,25(OH)$_2$D$_3$ either alone (●) or in the presence of a 100-fold excess of non-radioactive 1,25(OH)$_2$D$_3$ (○) or 25(OH)D$_3$ (△). Free hormone was pelleted with charcoal and 200μl portions of the supernatants were layered on to 5–20% sucrose gradients. The gradients were centrifuged at 288000g for 14h at 4°C and then fractionated from the bottom by downward displacement with mineral oil. (*a*) Medullary thyroid carcinoma cytosol; (*b*) salt-extracted nuclei from an adjacent part of the same tumour; (*c*) normal thyroid cytosol. B = bottom fraction; T = top fraction. The arrow indicates the position (3.7S) of [^{14}C]ovalbumin, run in parallel gradients.

& Delellis, 1981). Thus we would not detect the presence of the 1,25(OH)$_2$D$_3$-binding protein in whole-thyroid cytosols in this assay (limit of detection 0.3fmol/mg of protein), even if it were present in C-cells at levels comparable with those found in receptor-rich homogeneous cell lines such as T47D (Findlay *et al.*, 1980). We have located the binding protein in medullary thyroid carcinomas, which are derived from and represent an enriched population of C-cells (Williams, 1966). This suggests, but does not prove, that the 1,25(OH)$_2$D$_3$-binding protein may be present in normal C-cells, since our findings could be attributed to the transformed nature of the cells.

Feedback loops are a well known feature of endocrine mechanisms. Among the factors that have been shown to influence the renal hydroxylation of 25(OH)D$_3$ are parathyrin (Garabedian *et al.*, 1972), somatotropin and prolactin (Spanos *et al.*, 1981). The 1,25(OH)$_2$D$_3$-binding protein has been located in both parathyroid and pituitary tissue (Pike *et al.*, 1980), raising the possibility that 1,25(OH)$_2$D$_3$ may influence the synthesis or secretion of these hormones. This has been demonstrated in the case of prolactin, by using cloned rat pituitary cells (Murdoch & Rosenfeld, 1981).

Although the effects of calcitonin on 1,25(OH)$_2$D$_3$ production (Galante *et al.*, 1972) have been claimed to be indirect (Lorenc *et al.*, 1977), elegant studies by Kawashima *et al.*, (1981) have shown that calcitonin selectively stimulates the 25(OH)D$_3$ 1α-hydroxylase enzyme in the proximal straight tubule of rat kidney nephron. It is unknown whether 1,25(OH)$_2$D$_3$ has any effects on calcitonin production, but our results suggest that such a link might be usefully investigated.

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