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At the Cutting Edge

Thyroid hormone regulation of rat pituitary tumor cell growth: a new role for apotransferrin as an autocrine thyromedin

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This cutting edge review has two goals. First, we will describe some of the major advances leading to our present understanding of thyroid hormone-dependent pituitary tumor cell growth. The discussion particularly will emphasize the theme of hormonal/autocrine control of cell proliferation. Second, we will review recent studies in serum-free defined culture which have led to the identification of apotransferrin as an essential regulator of thyroid hormone-dependent pituitary cell growth.

Milestones in thyroid hormone growth regulation

Jacob Furth (b. 1896–d. 1979) made a vital contribution to our understanding of pituitary cell physiology. Along with several colleagues, he established a collection of rodent tumors which retained many of the functional properties of the adenohypophysis (Furth, 1953, 1969; Furth et al., 1956; Yokoro et al., 1961). Most notably, several tumors secreted growth hormone and/or prolactin. One of these, the Wistar-Furth rat derived MtT · W5, was used by Tashjian et al. (1968) to establish the now famous GH_1 and GH_3 cell lines in culture. Later, the GH_4C_1 (Tashjian et al., 1970) and the GC (Bancroft and Tashjian, 1971) subclones were obtained. All have been used in the study of thyroid hormone mechanism of action.

Samuels et al. (1973) were first to report thyroid hormone-dependent growth of the GH_1 cells in culture. This was a milestone in our understanding of thyroid hormone mechanism of action. Later, they showed that growth was mediated by the binding of triiodothyronine (T_3) or L-thyroxine (T_4) to specific nuclear receptors (Samuels and Tsai, 1973; Samuels et al., 1974).

Hayashi and Sato (1976) provided another milestone by achieving GH_3 cell growth in a serum-free defined medium containing a mixture of nutrients and hormones. Between 1976 and 1984, Gordon Sato and colleagues demonstrated that many different cell types grew in serum-free defined medium (Bottenstein et al., 1979; Barnes and Sato, 1980; Sato et al., 1982; Barnes et al., 1984). These studies led to the description of many previously unidentified physiological responses/regulators (Barnes et al., 1987).

At present, there is little doubt that nuclear thyroid hormone receptors mediate the proliferative response of rat pituitary tumor cells in culture (see review Oppenheimer et al., 1987). Nonetheless, the question remained of whether this inter-

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action alone was sufficient to regulate the cascade of events culminating in cell division. The studies of Samuels et al. (1973) were done in serum supplemented medium. Conservatively estimated, this meant that at least 5000 components other than T_3/T_4 were present; sorting through this number for putative new regulatory molecules appeared to be a formidable task.

Fortunately, the reports of Hayashi and Sato (1976) and Hayashi et al. (1978) provided a method to resolve this problem. They grew GH_3 cells in a defined medium supplemented with T_3, diferric transferrin, partially purified insulin-like growth factor I (IGF-I), parathyroid hormone and thyrotropin releasing hormone. Since all five were components of serum, defined culture methods offered a promising means of reducing 5000 possibilities to a much smaller number. However, despite the great success with GH_3 cells, there were two findings of note. Deletion of thyroid hormones from defined media had only modest consequences, compared with removal of some of the other components. Secondly, the magnitude of the T_3 growth response in defined medium was markedly lower than in serum containing culture.

At about this time, another milestone was reached which inspired an entirely different approach to growth regulation. DeLarco and Todaro (1978) found that tumor cells secreted polypeptide mitogens. Later, these were classified as 'autocrine growth factors' (Sporn and Todaro, 1990), and within a few years became the center of the tumor biology universe (see review by Goustin et al., 1986). Very quickly, other investigators applied this concept to endocrine tumors; Dickson et al. (1986), for example, reported the estrogen-induction of autocrine mitogens for human breast cancer cells and we examined pituitary cell conditioned medium and tumor extracts for possible 'autocrine' activities (Danielpour et al., 1984).

Others also were seeking autocrine factors for rat pituitary tumor cells. Fagin et al. (1987) identified IGF-I as a secretory product of GH_3 cells. Because these cells expressed type I (IGF-I) receptors (Rosenfeld et al., 1985), an 'autocrine growth loop' was suggested. However, despite the presence of the basic elements of such a loop, it was not possible to confirm IGF-I regulation of pituitary cell proliferation in serum-free defined medium (Riss et al., 1989; Sirbasku et al., 1991b). In fact, other data suggested that IGF-I instead modulated growth hormone production by negative feedback (Yamashita and Melmed, 1986).

Hinkle and Kinsella (1986) provided the next piece of the jig-saw puzzle. Using serum containing cultures, they reported thyroid hormone induction of potent autocrine (secreted) factor(s) which completely replaced the T_3 requirement of the GH_4C_1 cells. A subsequent report by Miller et al. (1987) described similar results with GC cells. Not only did these findings provide a timely autocrine mechanism for thyroid hormone induced growth, but they also implied that this new activity might act for T_3/T_4 in the myriad of metabolic processes, including regulation of energy metabolism and control of osmotic pumps, known to be influenced by these hormones (Oppenheimer et al., 1987).

Initial serum-free study of pituitary cell growth

Our initial plan was to grow rat pituitary tumor cells in T_3 supplemented serum-free defined culture and prepare conditioned medium for characterization of this thyroid hormone substituting activity, a strategy beset by many problems (Stewart and Sirbasku, 1987, 1988a, b; Riss et al., 1989).

First, $< 10\%$ of the GH_3 and GH_4C_1 cells survived passage from serum containing culture into defined medium. This problem persisted despite variations in plating density, medium composition and methods of preparation of inoculum cells. Second, the surviving cells required supraphysiological concentrations of T_3 (i.e. 10 nM) and rapidly progressed to autonomy. Indeed, these conditions permitted isolation of hormone-autonomous variants (Riss et al., 1989). Third, the effects of polypeptide growth factors were different from those reported (Hayashi and Sato, 1976; Hayashi et al., 1978; Bottenstein et al., 1979). Deletion of insulin and/or IGF-I had no effect on growth or survival. Members of the epidermal growth factor and the fibroblast growth factor-like families were inhibitory. Transforming growth factor β and platelet derived growth factors were without effect. In summary, none of the five major functional families of non-lymphoid growth fac-

tors supported pituitary cell proliferation in defined medium with or without T_3. By inference, the T_3 substituting activity of Hinkle and Kinsella (1986) probably was not a member of a major growth factor family. In addition, we were unable to identify a T_3 substituting activity from serum-free cultures of GH_4C_1 cells.

When the problem was reconsidered, a different interpretation of the data of Hinkle and Kinsella (1986) appeared possible. Conceivably, one of more component(s) of the serum may have been converted to an active agent during incubation of the cells with T_3. In view of results to be described below, we believe that 'autocrine' apotransferrin generated by recycling of diferric transferrin may be related to the T_3 substituting activity. Finally, thyroid hormone-responsive GH_1 cells failed to grow in any formulation of defined medium including those with T_3 and insulin. In contrast, addition of low concentrations of thyroid hormone depleted serum restored cell survival and thyroid hormone-dependent growth. These data were convincing evidence that serum contained a thyroid hormone mediating activity designated 'thyromedin' (Stewart and Sirbasku, 1987, 1988a, b).

Isolation and characterization of thyromedins

We purified thyromedins from horse serum (Sirbasku et al., 1991a, b) and human plasma (Sato et al., 1991). Bioassays in two related serum-free media were used to monitor the isolations. GH_1 cell growth in PCM-10 containing Ham's F12 and Dulbecco's modified Eagle's medium (1 : 1) supplemented with 2.2 g/l sodium bicarbonate, 15 mM Hepes (pH 7.2), 10 μg/ml diferric transferrin, 50 μM ethanolamine, 10 μg/ml insulin, 10 ng/ml selenous acid, 500 μg/ml serum albumin and 0.1 nM T_3 was compared to proliferation in the same medium without T_3 (PCM-0). Chromatographic fractions supporting growth in PCM-10, but not in PCM-0, were purified to electrophoretic and HPLC homogeneity.

From horse serum, > 600 mg/l of thyromedin was obtained as seven separate forms (Sirbasku et al., 1991a). Human plasma yielded > 500 mg/l represented by three forms (Sato et al., 1991). The quantities obtained, as well as the striking polymorphism, raised the expectation that thyromedin was a previously unrecognized activity of a known serum protein. In summary, all forms/variants or horse and human thyromedins proved to be transferrin by molecular weight estimation, Fe^{3+} binding capacity, Western immunoblotting versus authentic samples, amino acid composition analysis and N^{α}-amino acid sequencing.

Properties of thyromedin biological activity

The results presented in this and the next section summarize three recent reports (Sato et al., 1991; Sirbasku et al., 1991a, b). Initially, growth assays were done in medium prepared with standard Gibco F12-DME containing ferric nitrate and ferrous sulfate salts. The total iron content of PCM-10 was 65 ng/ml as measured by atomic absorption spectroscopy. If all of the iron was Fe^{3+}, this cocnentration was enough to saturate 46 μg/ml of apotransferrin assuming two Fe^{3+}/molecule.

The ED_{50} of molecular weight 80,000 horse thyromedin IIIa (53% Fe^{3+} saturated) in 'iron salts containing' PCM-10 was 15–20 μg/ml (285 nM); $\geq$ 25 μg/ml was needed for maximal growth (Fig. 1*A*). In 7 days, cell numbers in PCM-10 exceeded those in PCM-0 by 3–4 cell population doublings (CPD) or 800–1600%. It was notable that even at 100 μg/ml, horse thyromedin IIIa failed to achieve ED_{50} in PCM-0.

The same experiment with molecular weight 80,000 human thyromedin gave comparable results (Fig. 1*B*). Form Ia, which was 9% Fe^{3+} saturated, had an ED_{50} of 12 μg/ml. GH_1 cell numbers in PCM-10 after 7 days exceeded PCM-0 by 5–6 CPD or up to 6400%. Even 100 μg/ml of human Ia did not promote ED_{50} growth in PCM-0.

Although thyromedins from both sources clearly were effective mediators of T_3 induced growth, a number of questions arose. First, the ED_{50} values, as well as the concentrations required to saturate growth, were near μM. This was disconcerting because physiological regulators are expected to be active at lower concentrations. Second, diferric transferrin was present in PCM-10 at 10 μg/ml, yet the medium did not support growth.

Thyromedin activity and apotransferrin

Addressing these problems led to the first identification of a serum-derived mediator of hormone-dependent cell growth since our search began (Sirbasku, 1978). The following is a summary of the evidence supporting the conclusion that thyromedins are apotransferrin:

Iron content of thyromedins and activity. When the seven forms of horse thyromedin were analyzed for iron content versus biological activity, an important pattern emerged. Those showing the lowest ED_{50} concentrations (i.e. highest specific activity) were $\leq 50\%$ iron saturated whereas others approaching 80–90% saturation were inactive, or nearly so, when assayed in standard 'iron salts containing' media. The three human thyromedins showed an identical pattern. Thus, lower iron content clearly correlated with higher biological activity.

Iron saturation and thyromedin activity. All seven horse thyromedins and the three human forms were iron saturated with ferric ammonium citrate as described (Ward and Kaplan, 1987). Assays in standard 'iron salts containing' PCM-10 showed no biological activity with any thyromedin at concentrations of up to 100 μg/ml. Fig. 1*C* shows an example assay of iron saturated human thyromedin Ia.

Iron depletion and thyromedin activity. Next, iron was depleted from the thyromedins and from the tissue culture medium before bioassay. Water was purified to reduce iron content (Sirbasku et al., 1991a). Iron salts free F12-DME was obtained from Gibco. The final concentration of iron in 'iron salts reduced' PCM-10 was < 10 ng/ml. All horse and human thyromedins were dialyzed against 0.1 M sodium citrate, pH 4.0, for 48 h at 4°C to reduce the iron content to $< 4\%$ saturation (Roop and Putnam, 1967). Assays in 'iron salts reduced' medium gave ED_{50} values of 80–180 ng/ml. Fig. 1*D* shows an example assay of citrate-treated human thyromedin Ia in 'iron salts reduced' media. All thyromedins were 100- to 2000-fold activated under iron reduced conditions compared to those with iron present. These low

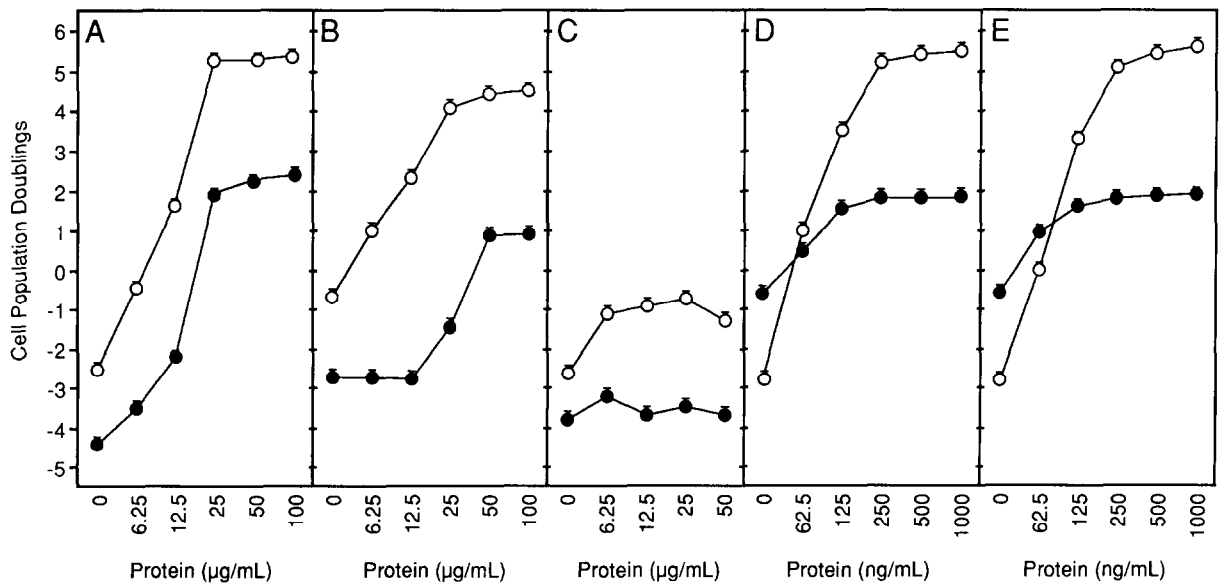

Fig. 1. Dose–response growth of GH_1 cells in PCM-10 (open circles) and PCM-0 (closed circles) with the following preparations/conditions: (*A*) horse thyromedin IIIa (Sirbasku et al., 1991a) in 'iron salts containing' media; (*B*) human thyromedin Ia (Sato et al., 1991) in 'iron salts containing' media; (*C*) Fe^{3+} saturated human Ia in 'iron salts containing' media; (*D*) iron depleted (citrate dialyzed) human Ia in 'iron salts reduced' media; and (*E*) Fe^{3+} saturated human Ia in 'iron salts reduced' media. Assays were over 7 days (± SD, bars).

nM concentrations at which activity is seen are thus consistent with those at which other physiological regulators operate.

Deferoxamine activation of thyromedin activity. Another test was made of the effect of the iron concentration of the medium on thyromedin activity. Deferoxamine mesylate (Sigma) is a low molecular weight chelator which binds one Fe^{3+}/molecule with much higher affinity than apotransferrin (Peter, 1985). When deferoxamine mesylate was added to 'iron salts containing' medium to chelate all available Fe^{3+}, the effect on the biological activity of thyromedins was striking. Horse thyromedin IIIa, which showed an ED_{50} of 20 μg/ml before deferoxamine addition (Fig. 1*A*), increased 100-fold in activity (to an ED_{50} of 200 ng/ml) after preincubation of the medium with the chelator. This observation had two important implications. First, it explained why our initial preparations were less active than expected. With the favorable conditions of tissue culture, the Fe^{3+} of 'iron salts containing' medium associated with apotransferrin to form inactive diferric transferrin. Second, this finding supported the conclusion that apotransferrin was the active mediator of thyroid hormone-dependent cell growth.

Iron salts addition and thyromedin activity. As shown in Fig. 1*D*, citrate-treated human thyromedin Ia gave an ED_{50} of 100 ng/ml in 'iron salts reduced' PCM-10. In another test of the effect of iron on activity, increasing concentrations of ferric ammonium citrate were added to 'iron salts reduced' medium in identical experiments using horse IIIa and human Ia thyromedins. The activities of both citrate treated preparations were inhibited progressively by the iron salt proving that medium composition regulated biological potency.

Assay of apotransferrins and diferric transferrins from several species. Commercially prepared horse, human, rat, mouse, dog, guinea pig and rabbit transferrins were either Fe^{3+} saturated as described (Ward and Kaplan, 1987) or depleted of iron by exhaustive citrate dialysis (Roop and Putnam, 1967). Assay of the iron depleted proteins in 'iron salts reduced' PCM-10 resulted in ED_{50} values of 80–350 ng/ml and little or no growth in PCM-0. The results paralleled the assay of human thyromedin Ia under the same conditions (Fig. 1*D*) and affirmed that apotransferrins from several species were effective. The iron recharged transferrins were assayed in 'iron salts containing' PCM-10 and PCM-0 exactly as described in Fig. 1*C*; no activity was found at concentrations of up to 200 μg/ml. These results not only proved that diferric transferrins were not thyromedins but also explained why PCM-10 containing 10 μg/ml human or horse diferric transferrin did not support growth.

What form of apotransferrin is present in tissue culture medium? Although these studies demonstrated that ferric ion binding to apotransferrin resulted in the loss of thyromedin activity, they did not address the possibility of association of other metals with the apoprotein. This is an important consideration because apotransferrin binds non-ferrous ions including zinc, copper and trace metals (see review by Harris, 1989). In 'iron salts reduced' medium, 70–80% of apotransferrin is associated with metal ions. Because Zn^{2+} is present at 1.5 μM in PCM-10 and binds to the apoprotein with a K_a of $10^{7.8}$, our present studies are focused on the possible role of Zn^{2+}-apotransferrin in hormone-dependent cell growth.

'Autocrine loop' formation of apotransferrin

Despite the purification of apotransferrin/thyromedin from plasma, we questioned whether this source was important physiologically. Most mammalian cells produce apotransferrin via recycling of the complex of diferric transferrin and the molecular weight 180,000 dimeric cell surface receptor (Ciechanover et al., 1983; May and Cuatracasas, 1985). A schematic summary of this process is shown in Fig. 2. At extracellular neutral pH, diferric transferrin binds with nM affinity to receptors located in clathrin coated pits. The pits are internalized to form coated endosomes followed by acidification of the vesicles to pH 5–6 via action of a H^+-ATPase and dissociation of the Fe^{3+} from transferrin. At acidic pH, apotransferrin remains tightly associated with the receptors. The iron depleted endosomes recycle to the cell surface where fusion exposes the apotransferrin/receptor complex to the extracellular environment. With most cell types studied, apotransferrin rapidly dissociates from the receptor at neutral pH.

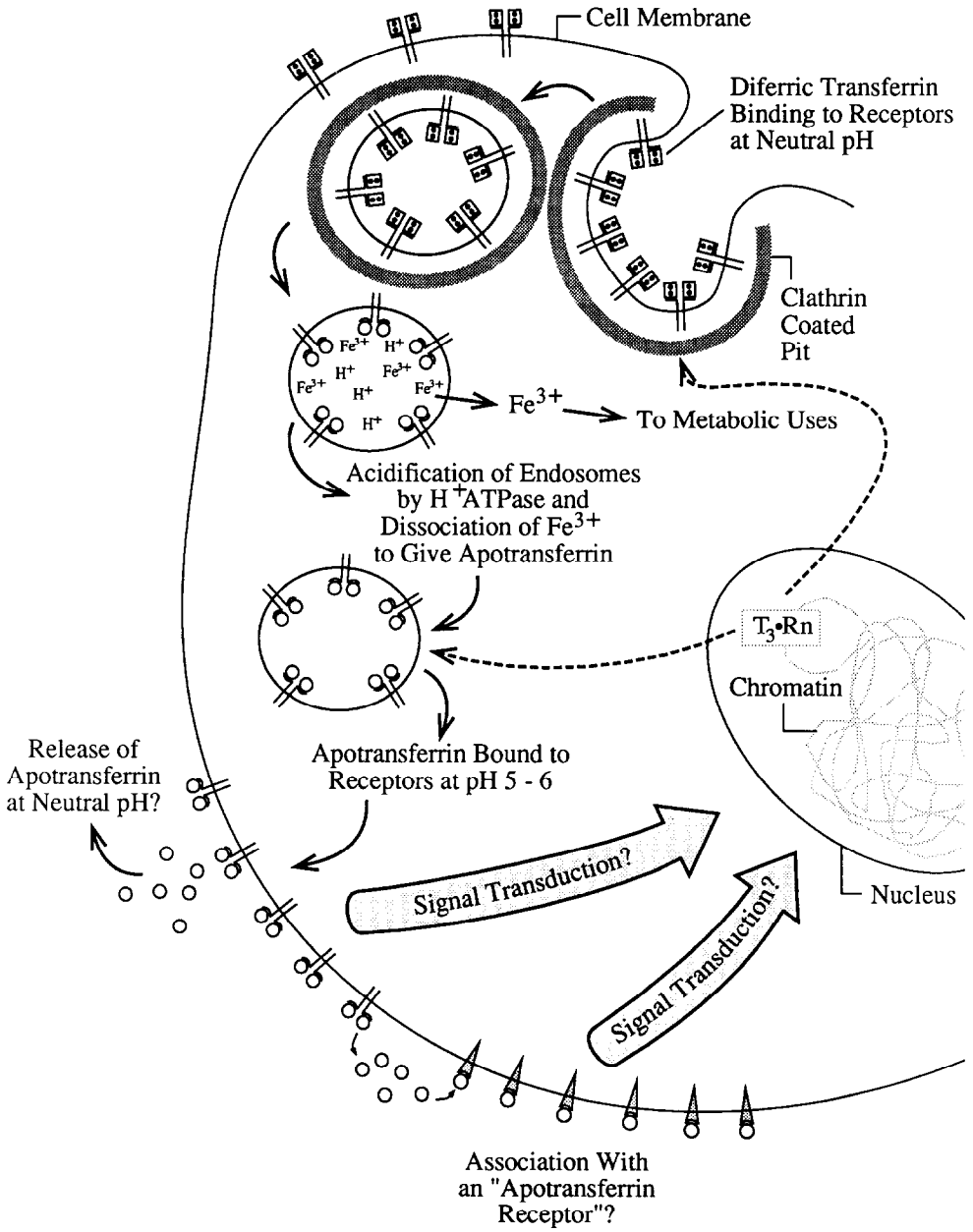

Fig. 2. Schematic summary of the cycling pathway of diferric transferrin internalization to produce apotransferrin.

Although we expected the same cycle in GH_1 cells, experimental confirmation was sought. Iron saturated human thyromedin Ia effectively promoted GH_1 growth in 'iron salts reduced' medium (Fig. 1*E*). It should be noted that this same preparation was inactive in 'iron salts containing' medium (Fig. 1*C*). The results confirmed that apotransferrin generation by recycling of diferric transferrin represented an 'autocrine growth loop' for pituitary tumor cells.

These observations raise important questions. First, do thyroid hormones regulate the rate of diferric transferrin recycling and thereby control 'autocrine' apotransferrin production (Fig. 2)?

Second, does apotransferrin control growth via binding to the molecular weight 180,000 dimeric diferric transferrin receptor or instead function via an apotransferrin specific receptor, as proposed in Fig. 2? In the former case, the usually rapid dissociation of apotransferrin from the diferric transferrin receptor at neutral pH may be altered.

Cell–cell adhesion and apotransferrin

Along with growth regulation, thyromedin/apotransferrin showed another interesting new property. GH_1 cells plated into PCM-0 or PCM-10 alone floated above the dish surface without interactions (Fig. 3*A*). When thyromedin was added to PCM-0, the cells aggregated and attached as clusters to the dish surface (Fig. 3*B*). Neurite-like projects were apparent. Addition of thyromedin to PCM-10 not only stimulated growth, but also the formation of large aggregates attached more firmly to the dish surface (Fig. 3*C*). The morphology in PCM-10 plus thyromedin was identical to that of cultures grown in serum (Fig. 3*D*).

The cell–cell interactions observed in the presence of apotransferrin implied a cell adhesion molecule (CAM) activity. Although most CAMs are in the immunoglobulin superfamily or are lectins, the structure of apotransferrin suggested a new possibility. Transferrin is a bilobular molecule with similar 'N' and 'C' lobes sharing 35–45% amino acid sequence homology (Baker et al., 1987). Each lobe has a '1' and '2' domain positioned so that the same amino acid sequence of the 'N' lobe is facing in the opposite direction as that of the 'C' lobe. This arrangement might recognize equivalent receptors on adjacent cells thereby initiating cell–cell contact.

In addition to the possibility of cell–cell bridges formed by apotransferrin, another adhesion mechanism was conceivable. Ramsdell (1990) identified

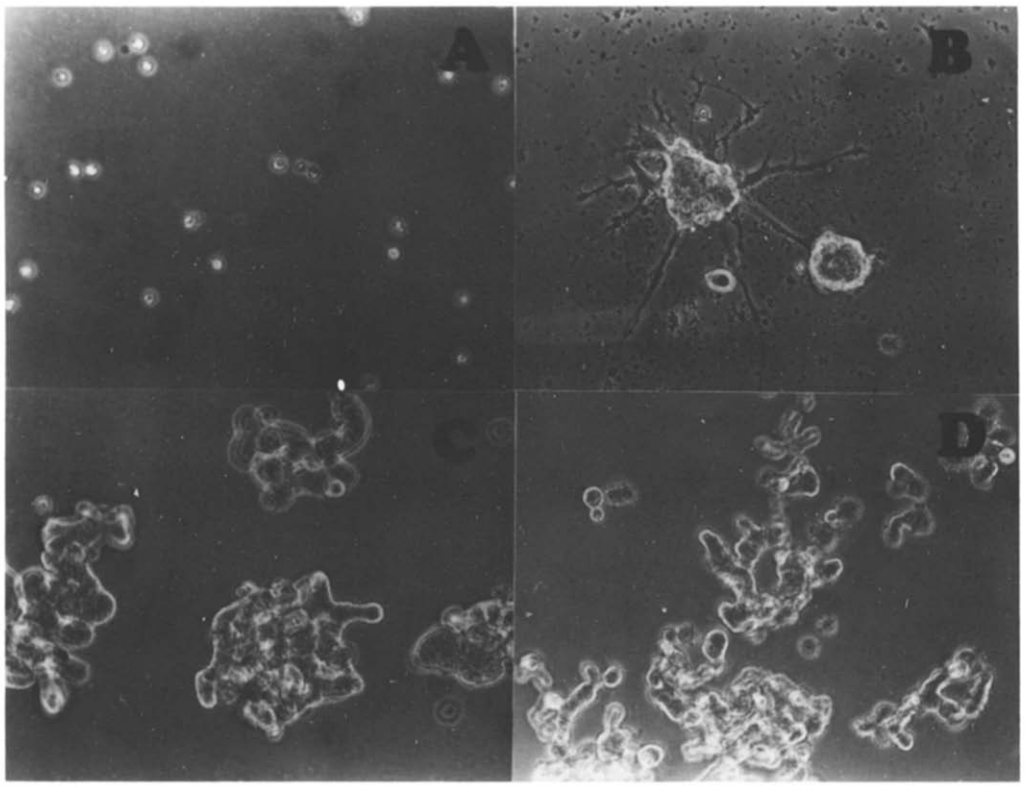

Fig. 3. Photomicrographs of GH_1 cells growing/inoculated into serum-free defined PCM-10 (or PCM-0) only (*A*), PCM-0 plus horse thyromedin (*B*), PCM-10 plus thyromedin (*C*), and PCM-10 plus 5% horse serum (*D*). ×100.

neural cell adhesion molecules (N-CAMs) on GH_4C_1 cell surfaces and studied their role in cell–cell contact. Apotransferrin might induce N-CAM expression and thereby promote cell–cell interactions. Whichever mechanism(s) proves important, the adhesion observations are especially exciting because they raise the possibility that cell–cell interactions might be important in thyroid hormone induced cell proliferation.

Summary

In the 40 years of transferrin research, no previous role for apotransferrin has been recognized other than to serve as a plasma carrier for dietary and storage iron. Our studies have revealed a new 'autocrine' growth role for this molecule as well as a possible new cell–cell bridge/CAM function. Certainly, these observations have opened many new areas of investigation both with regard to thyroid hormone action and the function of apotransferrin. In addition, there is now accessible the broader question of tissues other than pituitary which might utilize apotransferrin to regulate responsiveness to thyroid hormones.

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