Loss of dependence on IGF-1 for proliferation of human thyroid adenoma cells

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Summary The proliferative responses to IGF-1 (Somatomedin C) and TSH, as assessed by 3H-thymidine (3H-TdR) incorporation and autoradiographic labelling index (LI), of suspension and monolayer cultures of human thyroid follicular epithelium derived from both normal and adenoma tissue have been compared. In cultures of normal follicles, whilst neither TSH nor IGF-1 alone produced any effect, a combination of TSH (0.1 mU ml⁻¹) together with IGF-1 (10 ng ml⁻¹) induced a highly significant proliferative response as shown by a peak of 3H-TdR incorporation and LI, 4–5 days after growth factor addition. The TSH concentration-effect curve was bell-shaped, a higher concentration of TSH (10 mU ml⁻¹) resulting in a reduced response.

In cultures derived from adenoma tissue, however, TSH alone at 0.1 mU ml⁻¹ was sufficient to permit a highly significant proliferative response (equivalent to, or greater than the normal) in 4 out of 5 adenomas examined; again a higher concentration of TSH (10 mU ml⁻¹) resulted in a diminished response. Addition of IGF-1 (10 ng ml⁻¹) produced no significant change in the response to TSH (0.1 mU ml⁻¹) in 3 of these 4 adenomas, and significantly inhibited the response in the fourth adenoma. It is concluded that escape from the requirement for an exogenous source of IGF-1 may be a key step in the development of human thyroid epithelial (follicular cell) neoplasia.

The development of neoplasia is generally regarded as being a multistep process which, through the aberrant expression of cellular oncogenes, results in the generation of cell clones expressing defects of both differentiation and proliferation (Klein & Klein, 1985).

The mammalian thyroid gland has proved to be a useful in vivo model with which to investigate the development of epithelial neoplasia for several reasons, notably the simple ‘single compartment’ cell kinetics of the follicular epithelial population and the fact that follicular epithelial tumours can be induced in rodents simply by prolonged elevation of the plasma concentration of thyrotropin (TSH), without recourse to carcinogenic chemicals or radiation (Wynford-Thomas et al., 1982).

Investigation of the possible cellular mechanisms which may be implicated in the process of neoplastic transformation, however, requires an in vitro model. The human thyroid is more suitable than that of rodents for this purpose since it yields a spectrum of both benign and malignant epithelial tumours which can be easily distinguished macroscopically and dissected from the surrounding normal parenchyma to provide pure cultures of tumour cells. We have accordingly developed in vitro suspension and monolayer culture models of normal human thyroid follicular epithelium as a basis for investigating the hypothesis that the development of human thyroid epithelial neoplasia is accompanied by specific changes in growth factor requirements (Williams et al., 1987). We have previously demonstrated that in the normal follicular epithelial cell, a proliferative response could be obtained to TSH in vitro, but required the presence of a combination of two permissive factors, namely a supraphysiological concentration of insulin together with a low concentration of foetal calf serum (FCS) and could only be demonstrated in suspension cultures. It was postulated that this combination of insulin and FCS resulted in activation of the cellular IGF-1 (Somatomedin-C) receptor which is known to have an important role in the regulation of proliferation in diverse cell types (Van Wyk & Underwood, 1978). In this communication, we demonstrate that insulin and FCS can indeed be substituted by a physiological concentration of IGF-1, a combination of IGF-1 with TSH being sufficient to induce proliferation (DNA synthesis) in both suspension and monolayer cultures of normal human thyroid follicular epithelium. Furthermore, in 4 out of 5 adenomas (benign follicular cell tumours) so far investigated, there is an apparent lack of requirement for exogenous IGF-1, a proliferative response being obtained to TSH alone. It is postulated that this lack of exogenous IGF-1 requirement by these adenomas reflects constitutive activation of the IGF-growth signal pathway, either by autocrine production of IGF-1 itself or by a change in the activity of the receptor or subsequent intracellular messengers.

Materials and methods

Source of tissue

Normal tissue was carefully dissected from the periphery of fresh lobectomy specimens following surgery for solitary thyroid nodules.

Adenoma tissue was removed from the centre of the solitary nodule. The tissue was judged to be normal or adenomatous retrospectively by histological examination.

All adenomas were solitary encapsulated thyroid tumours composed of follicles lined by moderately- or well-differentiated follicular epithelium. None showed significant pleomorphism or any evidence of capsular or vascular invasion after examination of multiple blocks.

Materials

Collagenase: Cooper Biochemicals Class IV (612 U mg⁻¹); Dispase: Boehringer-Mannheim; Roswell Park Memorial Institute (RPMI) 1640 medium: Flow; Agarose: Sigma type IV; TSH (Thytopar) 0.5 lU ml⁻¹; Armour Pharmaceuticals; IGF-1: Amersham International; Methyl[3H]thymidine (3H-TdR), 41 Ci mmol⁻¹; Amersham International; Foetal calf serum (FCS): Gibco; Gentamycin: Sigma.

Follicle preparation

Both the normal and adenoma tissue was washed and finely minced in cold Hank’s calcium- and magnesium-free balanced salt solution (HBS). Digestion was carried out with a mixture of collagenase (100 U ml⁻¹) and dispase (1 mg ml⁻¹) in 10 ml HBS at 37°C for 1 h with gentle disruption by pipetting every 15 min. At the end of this time,
the supernatant was collected and proteases neutralised by the addition of FCS to 0.5%. Any remaining tissue was digested with fresh enzyme mixture. At the end of the extraction process, the follicles were pooled, filtered through a 200 μm nylon mesh and washed with HBS. The viable cell yield was determined using a haemocytometer and acridine orange/ethidium bromide fluorescence staining.

**Suspension culture** Follicles were resuspended in RPMI 1640 medium containing gentamycin (40 μg ml⁻¹) and aliquoted into flat-bottomed multiwell plate wells precoated with 2% agarose to prevent monolayer formation. The cell density averaged 5 × 10⁴ cells per well.

**Monolayer culture** Follicles were also seeded onto 35 mm Petri dishes in RPMI 1640 medium containing 10% FCS at an average cell density of 2 × 10⁵ cells per dish. After allowing 18–24 h for the follicles to attach, the dishes were washed and refed with the serum-free medium. The cells remained in this medium for 3 days to allow each follicle to completely spread and to eliminate any proliferative effects of FCS. Growth assays were commenced after this 'resting period'.

**Cell growth assays**

Growth was assessed in terms of stimulation of entry into the S-phase of the cell-cycle and DNA synthesis. For suspension cultures, this was measured by ³H-TdR incorporation into the cells as assessed by liquid scintillation counting. For monolayers, the percentage of cells in S-phase was directly assessed by autoradiography (nuclear labelling index).

**Suspension cultures** ³H-TdR incorporation was measured in time courses divided into successive 24 h 'windows' of labeling, commencing at the time of follicle plating. The total length of the time course was 6 days. Each window contained 4 replicates of each combination of growth factors tested. ³H-TdR was added at the beginning of the window to a final concentration of 2 μCi ml⁻¹. At the end of the window, the cells were harvested with water onto glass fibre filters using an automated cell harvester (Titertek). ³H-TdR incorporation was assessed by scintillation counting of the dried filters. Results are expressed as the mean incorporation of ³H-TdR (± s.e.) for each combination of growth factors tested during a given 24 h window.

**Monolayer cultures** Individual dishes containing different combinations of growth factors under test were labelled with ³H-TdR (2 μCi ml⁻¹) over successive 48 h periods, commencing immediately after the serum-free resting period. At the end of each labelling period the monolayers were fixed in methanol:acetic acid (3:1) for 1 h and subsequently air-dried. The dishes were then coated with Ilford K2 emulsion and exposed in the dark (4°C, 4 days) developed and counterstained with Giesma. For each data point, the nuclear labelling index in a random count of 2,000 nuclei was scored. Nuclei having more than 4 grains were counted as positive.

**Results**

**Follicle preparation**

The yield of epithelial cells from both normal and adenoma tissue was ~1.5 × 10⁶ cells g⁻¹ tissue with viability being >95% as determined by acridine orange/ethidium bromide fluorescence. The epithelium was then washed in HBS, trypsinised, and separated into closed follicles or as sheets which reformd closed follicular structures over the first 24 h in suspension culture. Viability of the epithelium remained constant throughout our incubation periods.

No differences were observed by phase-contrast microscopy between normal and adenoma follicles. Electron microscopical studies showed that the released follicles from the extraction process were of normal epithelial polarity with basal nuclei, adluminal microvillous border, supranuclear golgi apparatus and a fragmented basal lamina (Williams et al., 1987).

**Proliferative responses**

**Normal follicles** TSH, when added alone to the cultures (at concentrations up to 10 mU ml⁻¹), produced no increase in ³H-TdR incorporation as measured either by liquid scintillation or autoradiography over any time interval above that observed in the absence of added growth factors (Figures 1a, 3a). Similarly, IGF-1, when present alone in the culture medium (at concentrations up to 100 ng ml⁻¹) did not increase ³H-TdR incorporation above basal values (data not shown).

However, in the presence of IGF-1 at 10 ng ml⁻¹, TSH (at 0.1 mU ml⁻¹) resulted in a highly significant stimulation of ³H-TdR incorporation in all normal suspension cultures tested (total of 4). In a representative experiment (Figure 1b), a peak of 3,722±63 cpm occurred on day 4–5 of the culture period, i.e., ~5-fold greater than the corresponding basal ³H-TdR incorporation of 758±43 cpm (P < 0.001). There was a parallel rise in the monolayer autoradiographic nuclear labelling index from 0.6% (basal) to 18.6% on days 2–4 (Figure 3a). In contrast, at lower or higher concentrations of TSH no significant stimulation of ³H-TdR incorporation occurred in suspension cultures throughout the 6-day culture period, the day 4–5 incorporation being 1,005±114 cpm and 1,132±35 cpm for TSH at 0.01 mU ml⁻¹ and at 10 mU ml⁻¹ respectively (Figure 1b). Similarly, no stimulation of the monolayer autoradiographic nuclear labelling index was seen with these concentrations of TSH – data not shown. (Increasing IGF-1 concentration above 10 ng ml⁻¹ did not lead to any further increase (or decrease) in the response to TSH).

**Adenoma follicles** Three out of 5 adenomas gave the pattern of response illustrated (for one case) in Figure 2a,b. In contrast to the normal epithelium, a single highly significant peak of ³H-TdR incorporation at day 4–5 of the suspension culture period was observed when TSH at 0.1 mU ml⁻¹ was present alone in the culture medium (without IGF-1), the ³H-TdR incorporation rising to 2,900±300 cpm. In contrast to the normal follicles, there was no stimulation of ³H-TdR incorporation of 450±10 cpm (P < 0.001). The corresponding monolayer autoradiographic nuclear labelling index rose from 2% to 12.6% (Figure 3b). However, TSH at lower (0.01 mU ml⁻¹) or higher (10 mU ml⁻¹) concentrations gave a much reduced response, the day 4–5 ³H-TdR incorporation being 580±27 cpm and 900±40 cpm respectively (Figure 2a). The inclusion of IGF-1 (10 ng ml⁻¹) did not significantly affect the response to TSH at 0.1 mU ml⁻¹, the day 4–5 ³H-TdR incorporation in suspension culture being 3,700±900 cpm in the presence of IGF-1, compared with 2,900±200 cpm in its absence (Figure 2a, b). The monolayer nuclear labelling indices were 10.7% and 12.6% respectively (Figure 3b).

The second and third adenomas showed a similar pattern of response, with TSH alone at 0.1 mU ml⁻¹ inducing a single, highly significant peak of ³H-TdR incorporation. In a fourth adenoma, whilst TSH alone (at 0.1 mU ml⁻¹) was again stimulatory, the further addition of IGF-1 (at 10 ng ml⁻¹) resulted in a paradoxical inhibition of ³H-TdR incorporation, the day 4–5 value being reduced from 6,998±600 cpm with TSH alone, to 1,115±70 cpm with TSH plus IGF-1 (P < 0.001).

Finally, a fifth adenoma showed an apparent 'spontaneous' ³H-TdR incorporation, occurring in the absence of any added growth factors. A peak occurred on day 2–3 of the culture period, reaching 3,380±800 cpm. Addition of TSH at 0.1 mU ml⁻¹ with or without IGF-1 at 10 ng ml⁻¹ produced no significant stimulation or inhibition of this
response, the day 2–3 incorporation being 2,750 ± 300 cpm and 2,400 ± 187 cpm respectively.

Discussion

We have previously demonstrated that a proliferative response to TSH could be obtained in cultures of normal human thyroid follicular cells, but required the presence of a combination of two permissive factors, namely a supraphysiological level of insulin coupled with a low concentration of serum. This response was demonstrated in suspension cultures both by measurement of 3H-TdR incorporation by scintillation counting and by direct assessment of the nuclear labelling index by autoradiography, the latter being necessary to exclude artefacts arising from changes in the kinetics of thymidine uptake and synthesis (Williams et al., 1987). However, in this earlier work, no response could be observed in parallel monolayer cultures of normal human follicular epithelium using the same combination of permissive factors.

Our present data now demonstrate that the permissive effect of insulin and serum in suspension cultures of normal human thyroid follicles can be substituted for by a physiological concentration of a single permissive factor, namely IGF-1, a combination of IGF-1 (at 10 ng ml⁻¹) and TSH (at 0.1 mU ml⁻¹) producing a highly significant peak of 3H-TdR incorporation occurring on day 4–5 of the culture period.
For the comparison of normal and adenomatous follicular epithelium in this study, we have chosen to employ both suspension and monolayer modes of culture. Suspension cultures were used because there is both ultrastructural and functional evidence to support the view that they provide a more valid in vivo representation of the in vitro state of the follicular epithelium (Chambard et al., 1983; Westermark et al., 1986). Monolayers were used in order to facilitate comparisons between our study and those of other workers, nearly all of whom have employed this mode of culture.

Suspension culture autoradiography is an extremely time-consuming method of analysis. Since our previous work has always shown a close correlation between peaks of $^3$H-TdR incorporation, as measured by scintillation counting, and increases in the autoradiographic nuclear labelling index (Williams et al., 1987; Smith et al., 1986), the analysis of suspension cultures in this study was performed by $^3$H-TdR scintillation counting alone. However, all critical comparisons of growth responses were confirmed by use of autoradiographic nuclear labelling index in the monolayer cultures.

In cultures derived from follicular adenomas, it was found that 4 adenomas showed single peaks of $^3$H-TdR incorporation (occurring on day 4–5) in response to the addition of TSH alone. (Again, the TSH-induced proliferative response showed a bell-shaped concentration-effect curve with maximum stimulation occurring with TSH at 0.1 mU ml$^{-1}$ in both suspension and monolayer culture models). IGF-1 was without significant effect in 3 out of 4 of these adenomas, and was inhibitory in the fourth case. A fifth adenoma exhibited a spontaneous peak of $^3$H-TdR incorporation, the presence of either TSH and/or IGF-1 having no additional effect.

It is known that IGF-1 plays an important role in the mitogenic responses of diverse cell types (Van Wyk & Underwood, 1978). In rodent fibroblasts, for example, entry into S-phase of the cell-cycle is dependent upon the exposure of the cells to a tissue-specific ‘competence’ factor such as PDGF, together with or followed by a non- (or at least less-) tissue specific ‘progression’ factor such as IGF-1 (Stiles et al., 1979; Leof et al., 1982). Our in vitro model of normal thyroid follicular epithelial cell proliferation demonstrates an analogous requirement for both tissue-specific and non-tissue-specific growth factors, TSH and IGF-1 respectively, although no conclusions can be drawn as to their respective temporal roles in regulating entry into S-phase.

Selective loss of the requirement for IGF-1 in 4 out of 5 adenomas strongly suggests that escape from dependence on an exogenous source of IGF-1 confers a selective growth advantage on these tumour cells. The basis for this unexpected observation is not immediately clear since IGF-1 is usually regarded as a permissive factor, produced by liver (D’Ercole et al., 1984) and fibroblasts (Adams et al., 1984), which is present at a fixed, non-limiting concentration in the extracellular fluid and it is assumed that thyroid growth is regulated only by the availability of the specific mitogen, TSH. Our results suggest, on the contrary, that the available concentration of IGF-1 may in fact be ‘growth limiting’ in the presence of normal TSH concentrations and that in adenomas elevation of the IGF-1 growth signal, achieved either by autocrine production of the growth factor itself or by activation of its intracellular messengers, permits a continued growth response to occur to a normal level of TSH thus leading to tumour formation. Evidence for autocrine IGF-1 production by neoplastic cells has, indeed, been documented in both cultures of neoplastic human breast epithelium (Huff et al., 1986) and haemangiosarcoma (Pavelic et al., 1986).

In conclusion, our results demonstrate for the first time a synergism between IGF-1 and TSH in the control of normal human thyroid follicular cell proliferation when cultured in suspension as closed follicles. Furthermore, we have documented major differences in the in vitro growth factor requirements of normal and adenomatous follicles. In particular, 4 of 5 adenomas exhibiting an apparent lack of requirement for exogenous IGF-1. We are currently exploring the possible

![Figure 3](image-url)
mechanisms which have resulted in escape from IGF-1 requirement by these human tumour cells.

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