Prolongation of Fibroblast Life Span Associated with Epithelial Rat Tumor Development

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ABSTRACT

We have examined the proliferative behavior in culture of stromal cells of fibroblast morphology taken from various stages in the development of experimentally induced epithelial tumors in the rat thyroid. In this model long-term stimulation by an elevated level of serum thyroid stimulating hormone leads first to hyperplasia, then to multiple benign, and finally malignant tumors.

Fibroblasts from hyperplastic glands, despite having already undergone more divisions in vivo, consistently showed a longer life span than normal fibroblasts in culture, averaging 16 compared with six divisions before senescing. In the three tumor bearing glands studied, all cultures have undergone more than 40 divisions in one case over 70 and none has yet shown signs of senescence. We conclude that the life span of thyroid fibroblasts observed in culture has been reset to a higher limit by growth stimulation in vivo and furthermore that this limit may be lost altogether in fibroblasts associated with epithelial tumors.

INTRODUCTION

While the multiple genetic events underlying tumor development are now increasingly well understood (1) little is known of the role of the stroma in the development of epithelial cell tumors. It has long been known from histopathological observation that all epithelial tumors contain a stromal component which appears to proliferate along with, and in some cases, e.g., breast, more rapidly than the tumor cells, the so-called "dismoplastic reaction" (2, 3). The importance of this accompanying stroma is shown by the failure of most tumors to exceed a size of a 1 to 2 mm in the absence of stromal blood vessels (4).

Studies on human xenografts in the nude mouse, in which the origins of tumor (human) and stromal (mouse) cells can be readily distinguished, have confirmed that the stroma arises by tumor-induced proliferation of the surrounding normal connective tissue (5). The exact kinetics of stromal growth, however, are less certain. In tumors arising deep within solid organs, it could be envisaged that the stroma is derived by "accretion" at the advancing tumor margin; this, however, cannot be a general mechanism. In those tumors, for example, bladder papilloma/carcinoma, which grow from a mucosal surface to form a polypoid mass protruding into a hollow organ, epithelial cell proliferation is seen far removed from the tumor base, i.e., in regions where there is no surrounding normal tissue.

Clearly in these cases the continued requirement for stroma cannot be met by recruitment of more normal cells but must from very early on in tumor development require the continued expansion of a relatively small number of stromal cell clones present at the site of tumor initiation. This reasoning has led us to consider that tumor growth must often require a number of stromal cell divisions comparable to that of the epithelium, which could be expected to surpass in many large tumors the known limited proliferative potential of diploid fibroblasts (6).

We have therefore examined the hypothesis that heritable changes in control of cell proliferation, either genetic or epigenetic in origin, may be a feature not only of the principal cell type in epithelial cell tumors but also of the supporting stromal connective tissue cells.

We have chosen to study this in a well-characterized experimental epithelial tumor model using the rat thyroid.

Sustained elevation of serum TSH in the rat leads to a characteristic pattern of epithelial and stromal cell growth (7–10). There is an initial dramatic increase in mitotic activity resulting in hyperplasia of both epithelial and stromal cells. The mitotic response, however, is short-lived and returns by 2–3 mo to almost normal levels. The hyperplastic gland remains in this quiescent so-called "plateau" state for 4 to 8 mo until eventually multiple benign epithelial (follicular cell) tumors begin to appear. A few of these eventually progress to malignancy.

We have examined the proliferative behavior in monolayer culture of fibroblasts obtained from primary cultures of thyroids at each stage in this epithelial tumor model.

MATERIALS AND METHODS

Experimental Design. Elevation of serum TSH the stimulus for hyperplasia and tumorigenesis was induced by p.o. administration of the goitrogen ATA, as described previously, (8, 9). Normal thyroid glands for culture were obtained from 32 untreated rats. Hyperplastic glands were obtained from 16 animals treated with ATA for 80 days, by which time thyroid epithelial growth has virtually ceased (8). Three animals which had been on ATA treatment for 11–12 mo were used for tumor studies. Histological examination of 3 random thin slices taken from each of these glands at the time of resection confirmed the presence of multiple benign adenomas in all 3 and in addition a large malignant tumor in one.

Eight separate fibroblast cultures were established from normal glands (4 glands/culture) and 8 from hyperplastic glands (2 glands/culture). Separate cultures were set up from each of the 3 tumor-bearing glands.

Animals. Male Wistar rats aged 10–11 wk at the start of the experiment were fed a standard laboratory diet (iodide content, 4.72 μmol/kg) and were administered the goitrogen ATA at a concentration of 1 mg/ml in drinking water for induction of hyperplasia and tumors.

Primary Cell Cultures. Thyroids were removed from the trachea, washed in Hanks' balanced salt solution (calcium and magnesium free) (Flow), and each lobe was divided into four. For each digest, 4 pooled thyroids were used for normal and 2 for hyperplastic glands. Each tumor-bearing gland was digested separately. The tissue was digested at 37°C with frequent agitation in Hanks' balanced salt solution (calcium and magnesium free) containing collagenase (200 units/ml; Worthington) and dispase (2 mg/ml; Boehringer). Digestion was monitored at 20-min intervals by phase contrast microscopy. As soon as cells began to appear, 20-min digests were harvested and stored on ice and fresh digestion medium was added to the remaining tissue. Using this method we have found (11) that the epithelial population remains largely in the form of follicular aggregates which are recovered in separate (earlier) fractions from the single-celled stromal component.

Pooled aliquots of the latter were used for primary culture, each culture consisting of four 35-mm Petri dishes each containing 5 × 104 cells.

2 The abbreviations used are: TSH, thyroid-stimulating hormone (thyrotropin); ATA, aminothiourazole.
Cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and were passaged at confluence at a split ratio of 1:8. Medium was changed every 3 days or at passage. The time to return to confluence after each passage was recorded together with the number of population doublings. Direct hemocytometer cell counts on a small series of cultures confirmed that during exponential growth the number of doublings could be taken as 3 for each one in 8 passage. In the final passage before senescence in which growth stopped short of confluence, direct counts were performed on replicate dishes.

For chromosome counts, metaphase spreads were prepared from trypsinized cultures by suspension in 0.025 M sodium citrate for 30 min, followed by methanol:acetic acid (3:1) fixation, and stained with Giemsa.

RESULTS

All cultures initially were similar in appearance consisting of fibroblast-like cells with scattered islands of epithelium. Over the first 5–7 days, the latter regressed, leaving a relatively uniform monolayer of fibroblasts (Fig. 1a) which initially grew with a doubling time of 1–2 days.

In "normal" cultures, growth rate declined after 3–4 divisions and ceased altogether after an average 6 population doublings (Fig. 2). The senescent cells were thin and flattened with prominent cytoskeletal striations (Fig. 1b) but remained attached to the dishes for 3 to 5 wk.

Cultures of fibroblasts from hyperplastic (plateau phase) glands consistently underwent a significantly greater number of population doublings than did "normal" cultures grown under the same conditions, averaging 16.6 ± 2.1 (SE) and 6.6 ± 1.0, respectively (P < 0.01) (Fig. 2); furthermore, the number of doublings was the same in the progeny of each of the 4 replicate dishes of a given culture. There was no difference between early passage normal and early passage plateau cultures in morphology or growth behavior.

In 2 cultures from normal and one from hyperplastic glands, 1–2 wk after senescence a few foci of mitotically active cells were observed. These cells were morphologically unlike the presenescence normal, hyperplastic, or tumor cultures, grew to a much higher density, and formed multiple layers; they clearly represented spontaneous transformants.

All fibroblast cultures established from tumor-bearing glands displayed a life span greater than any observed in cultures from normal or hyperplastic glands. Up to the present time all have undergone over 40 population doublings (Fig. 2) and one has reached 70 generations without any discernible change in growth rate. The averaging doubling time of these 3 long-lived tumor-derived cultures measured over 7 days of growth was 2.3 days compared with 1.9 days for 6 randomly selected normal fibroblast populations measured during their second wk in culture.

All replicate cultures established from a given tumorous gland have shown closely similar proliferative behavior at all times; furthermore, no intermediate phase of senescence has been observed in any of these cultures (Fig. 1c). It is extremely unlikely therefore that our results are due to "in vitro" selection of spontaneous transformants.

All "tumor" fibroblasts showed physiological growth controls in culture, i.e., density-dependent inhibition and anchorage-dependent growth. All 3 cultures have shown a diploid chromosome content (2n = 42) throughout their recorded life span. We conclude that fibroblasts associated with our epithelial thyroid tumors have a greatly extended life span in culture and may well be truly immortalized but are not transformed. (Interestingly, we have never seen spontaneous transformed foci in these cultures.)

DISCUSSION

Our results with plateau phase fibroblasts demonstrate that a mechanism exists which can apparently reset the proliferative
Fig. 2. Life span of rat thyroid fibroblasts in culture. Eight independent primary cultures were established from normal and hyperplastic (plateau phase) rat thyroids, and 3 from glands bearing epithelial (follicular cell) tumors. Each culture was initiated in 4 replicate 35-mm Petri dishes. The mean number of population doublings recorded before senescence is shown for each culture (○, □, △) together with the overall mean ± SE (bars) for each group (□, △). ○ N, fibroblasts from normal glands; □ P, fibroblasts from hyperplastic (plateau phase) glands; △ T, fibroblasts from glands containing epithelial tumors; †, senescence not observed in any tumor-derived culture.

(prolonged fibroblast life span in epithelial tumors)

potential ["Hayflick limit" (6)] of normal stromal cells in response to a physiological growth stimulus. This can most readily be interpreted as an adaptive response which permits the required expansion of the stroma to keep pace with that of the epithelium. The thyroid stromal cells presumably respond either directly to TSH or more likely to an intercellular signal received from the stimulated thyroid epithelium.

That stromal cells from glands bearing benign epithelial tumors should consistently display a greatly extended life span in culture raises the intriguing possibility that not only can the normal stromal cell's proliferative potential be modulated but that breakdown or inappropriate expression of this control may be a necessary prerequisite for epithelial tumor development, in addition to immortalization of the epithelium (12). Since it is highly unlikely that tumor stroma is monoclonal in origin, we suggest that our findings are explained by induction of extended life span in stromal cells by signal(s) received from neighboring epithelial tumor cells. There is ample evidence of analogous heritable changes in differentiation induced by epithelial-stromal interaction in embryogenesis (13).

These findings have potential implications both for the study of senescence and of epithelial neoplasia: (a) comparison of stromal cells from plateau with those from normal thyroids should provide an excellent model for identifying a senescence-regulating control mechanism; and (b) the epithelial signal which overrides stromal senescence may well represent an additional genetic step in the multistage development of tumors distinct from epithelial cell immortalization.

REFERENCES

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