Functional State of Cells During their Life and on their Journey Toward Inactivity and Death: Search for Morphological Evidence in Thyroid Fine Needle Aspiration Smears

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Abstract

Synthesis and storage of thyroglobulin as well as synthesis of thyroid hormones and their release into the circulation are important functions of thyroid, which were studied in fine needle aspiration (FNA) smears from thyroid lesions. Evidence of thyroglobulin synthesis was demonstrated in neoplastic and nonneoplastic follicular cells, especially in Hurthle cells, in the form of colloid inclusions. Whereas the pinocytic vesicles containing colloid at the luminal end of nonneoplastic and neoplastic follicular cells indicated engulfment of colloid for synthesis of thyroid hormones (T3 and T4), the marginal vacuoles (MVVs) (fire-flare appearance) at the basal aspects of follicular cells suggested their release on way to the interfollicular capillaries. The morphological evidence of secretory activity could also be demonstrated in medullary thyroid carcinoma (MTC) in the form of azurophilic granules, marginal vacuoles, and intracytoplasmic lumina (ICL) with secretions; the secretory material, likely to be amyloid, present in MTC cells, and their release to the extracellular space was confirmed by positive immunocytochemical staining for calcitonin. It was found that nuclear grooves and related intranuclear cytoplasmic inclusions (INCIs) in papillary thyroid carcinoma (PTC) possibly represent an initial step of a degenerative process leading to formation of inactive cerebriform nuclei. Based on observation regarding formation and release of precursor substances for psammoma bodies (PBs), it was also suggested that PBs may not represent a process of dystrophic calcification over infarcted/dead papillae but suggest an active biological process, which leads to inhibition of growth of neoplastic cells and acts as a barrier against spread of PTC.

Keywords: Calcitonin, cerebriform nuclei, colloid inclusion, marginal vacuoles, nuclear grooves

INTRODUCTION

Living cells, neoplastic or nonneoplastic, can synthesize and secrete their products; whereas some of these functions can be observed in cytologic or histologic preparations, they are often not highlighted in diagnostic pathology reports. Review of literature on synthesis, storage, and secretion of thyroid hormones1-3 reveals that the thyroglobulin synthesis and packaging takes place in the long endoplasmic reticulum and large Golgi apparatus, respectively, in the cytoplasm of thyroid follicular cells. Thyroid peroxidize (TPO) oxidizes the trapped iodine from circulation to form reactive I2, which combines directly with amino acid tyrosine in thyroglobulin molecule to form monoiodotyronine (MIT) and diiodotyrosine (DIT), which in turn form triiodothyrosin (T3), and thyroxine (T4). The biologically active T3 and T4 are stored in the central colloid cavity, and under the influence of thyroid stimulating hormone (TSH), pseudopods from the apical surface of follicular cells close around small portions of colloid to form pinocytic vesicles (phagosomes) in follicular cells, which fuse with the lysosomes to form digestive vesicles (phagolysosomes) containing digestive enzymes proteinase, which in turn digest the thyroglobulin molecules in order to release T3 and T4 at the base of the follicular cells on their way to the capillaries (blood circulation). Like thyroid follicular cells, the neoplastic and nonneoplastic calcitonin secreting C-cells also take part in synthesis, storage, and secretory activities. Although secretory products of thyroid stimulating hormone (TSH), pseudopods from the apical surface of follicular cells close around small portions of colloid to form pinocytic vesicles (phagosomes) in follicular cells, which fuse with the lysosomes to form digestive vesicles (phagolysosomes) containing digestive enzymes proteinase, which in turn digest the thyroglobulin molecules in order to release T3 and T4 at the base of the follicular cells on their way to the capillaries (blood circulation). Like thyroid follicular cells, the neoplastic and nonneoplastic calcitonin secreting C-cells also take part in synthesis, storage, and secretory activities. Although secretory products

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of these cells including those of the neuroendocrine system has been demonstrated with the aid of cytochemistry and immunocytochemistry, the morphological manifestations of active secretory activity have hardly been highlighted. During the recent years, through a series of articles on fine needle aspiration (FNA) cytology, an attempt has been made on our part to demonstrate these secretory activities as well as some morphological changes leading to cell death in FNA smears of nonneoplastic and neoplastic thyroid lesions.[3-9]

SYNTHESIS AND STORAGE OF THYROGLOBULIN

The storage of thyroglobulin as part of colloid in the central lumen of thyroid follicles is evident in both nonneoplastic and neoplastic lesions of the thyroid. However, the morphological evidence supporting thyroglobulin synthesis in follicular cells is not adequately described. Begin and Allaire[10] described ultrastructural features of abortive/rudimentary follicular lumina with abundant periluminal dense bodies, and with varying amount of microvilli and colloid intracellularly in insular carcinoma of thyroid, which was positively immune-stained for thyroglobulin as an inclusion/dot-like pattern, often in a paranuclear location. Subsequently, Yang and Khurana[11] reported intracytoplasmic lumen and transgressing vessels as helpful features that distinguishes neoplastic and nonneoplastic Hürthle cell lesions of thyroid. In a report by Das et al.[3] the existence of intracytoplasmic colloid inclusions (CIs) in follicular cells, especially the metaplastic Hürthle cells in Hashimoto thyroiditis (HT) that resembled magenta bodies (intracytoplasmic lumen with secretions in breast cancer cells), was described [Figure 1]. Based on review of the literature and their findings, the authors[3] suggested that the Hürthle cell metaplasia in HT may be a survival response of follicular cells and the presence of CIs in Hürthle cells may represent their limited ability to synthesize colloid and release it.

RELEASE OF THYROID HORMONES

Resorption of colloid because of excessive pinocytosis at the apical end of follicular cells giving rise to scalloped appearance at the edges of colloid in the follicular lumen represents hyperactivity of thyroid, and is typically appreciated in H and E-stained paraffin sections of thyrotoxic state such as Graves’ disease.[12] However, the pinocytic vesicles at the luminal side of follicular cells and the release of thyroid hormones (T₃ and T₄) at the basal end on the way to circulation is not highlighted in histology. In cytologic preparations, the marginal vacuoles (MVs) giving rise to fire-flare appearance has been described as a distinctive feature of thyrotoxic goiter in hyperthyroidism and MVs have also been reported in nontoxic (colloid) goiter, Hashimoto thyroiditis, and neoplastic goiters including follicular neoplasm (FN), metastatic follicular carcinoma, and follicular variant of papillary carcinoma.[13] In earlier studies, MVs were described as markedly dilated cisterns of endoplasmic reticulum playing a central role in the formation of protein in the follicular cells,[14] colloid suds, a manifestation of active pinocytosis of thyroglobulin,[15] or peripheral cytoplasmic vacuoles containing colloid.[16] In a cytomorphological study of thyroid lesions, Das[4] demonstrated the scalloping of colloid, the presence of pinocytic vesicles at the apical end of follicular cells and MVs giving rise to fire-flare appearance at the basal aspect of follicular cells [Figure 2]; the author[4] made the following conclusion: if the scalloped appearance in histology represents hyperactivity because of excessive pinocytosis of colloid at the luminal side of follicular cells, MVs in MGG stained cytologic preparations are likely to represent the events at the opposite end, that is, the process of diffusing out of the thyroid hormones (T₃ and T₄) at the basal aspect of follicular cells on their way to interfollicular capillaries.

Figure 1: Hashimoto thyroiditis (HT) with colloid inclusions (CIs): (a) A group of follicular cells, metaplastic Hürthle cells and lymphocytes (MGG × 200). (b) Intracytoplasmic lumen (ICL) in two Hürthle cells (arrows) (MGG × 200). (c) One Hürthle cell with ICL filled with secretion (Pap × 400). (d) One Hürthle cell with multiple ICLs with secretions resembling targetoid bodies (MGG × 1000). (e) One Hürthle cell with ICL and secretions resembling magenta bodies and empty IOLs (MGG × 1000). (f) The Hürthle cells and the secretion in one ICL (arrow) are positive for thyroglobulin (Tg) (×400)
Secretion of Calcitonin

Secretory activity related to hormones is not limited to thyroid follicular cells but has also been described in medullary thyroid carcinoma (MTC), a tumor of C-cell origin. The FNA cytologic features of MTC was initially described by Söderström et al.,[17] which included plasmacytoid or triangular-shaped asymmetrical tumor cells, with one or more eccentric nuclei, spindle-shaped cells, cytoplasmic granules, and amyloid. Five years later the Indian experience on FNA cytologic features of MTC was published, based on six histologically confirmed cases.[18] As per literature review by Das et al.,[5] more than a dozen reports were published on FNA cytodiagnosis of MTC in the following two decades (between 1984 and 2003). Although amyloid was identified in FNA smears in 44–83.8% cases,[19-21] there was limited information on other cytologic features that may represent secretory activity such as intracytoplasmic lumen[22] and red cytoplasmic granules.[17,21,23,24] In the study by Das et al.,[5] the secretory activity in nine samples from eight patients with MTC was highlighted through illustrations, which included fine cytoplasmic vacuoles (in eight cases), azurophilic granules (eight cases), MVs (five cases), and intracytoplasmic lumina (ICL) with secretions (six cases); material, likely to be amyloid based on morphological features, was present both intracellularly and extracellularly in six samples, and in the remaining three it was present in extracellular location only. All the nine samples were positive for calcitonin (the background amyloid in six cases, the coarse cytoplasmic granules in two, and the content of ICL in one) by immunocytochemical (ICC) studies. Staining for cytoplasmic chromogranin yielded positive reaction in all the three cases in which it was attempted. The intracytoplasmic secretory material appeared to be diffusing out of the tumor cells in MGG-stained smears and smears stained for calcitonin [Figure 3].

Papillary Thyroid Cancer and Its Variants

Thyroid cancer is one of the 15 most common cancers in the world,[25] and papillary thyroid carcinoma (PTC) is overwhelmingly the most frequent thyroid cancer.[26,27] As per the “Synopsis of the National Cancer Institute Thyroid Fine Needle Aspiration State of the Science Conference” on “Diagnostic Terminology and Morphologic Criteria for Cytodiagnosis of Thyroid Lesions,”[28] the cytologic criteria for PTC included five major diagnostic criteria (i.e. enlarged, oval “and irregular” nucleus, eccentric and often multiple micronucleoli, fine and pale chromatin, longitudinal nuclear grooves and intranuclear pseudo-inclusions, and eight minor diagnostic criteria (i.e., papillary cytoarchitecture, syncytial monolayers, dense squamoid cytoplasm, “bubble gum” colloid, psammoma bodies (PBs), multinucleated giant cells, histiocytoid cells, and cellular swirls); while enlisting these criteria reference was made to nine publications.[7,29-36] Papillary thyroid cancer has a number of variants besides the conventional or usual variant (UV), which includes follicular variant (FV), tall cell variant (TCV), columnar cell variant (CCV), diffuse sclerosing variant, oncocytic papillary neoplasm (oxyphilic variant), solid papillary carcinoma, papillary carcinoma with nodular fascitis-like stroma, and papillary carcinoma with mucoepidermoid component. Das et al.[30] studied the cytologic features of variants of PTC in FNA smears, which included 17 cases of PTC-UV, 17 PTC-FV, 6 PTC-TCV with ≥30% tall cells, 8 PTC cases with a significant tall cell component (sig. TCC) with 10–29% tall cells, and six miscellaneous variants. According to these authors,[18] a decreasing trend was observed with respect to count (mean) of tall cells, cells with reddish cytoplasm, and intranuclear cytoplasmic inclusions (INCs) from PTC (TCV) to PTC (FV) through PTC (sig. TCC) and PTC (UV); a similar trend was observed for nuclear grooves from PTC (sig. TCC)
to PTC (FV) through PTC (UV). The morphological forms INClIs in FNA smears of PTC and its variants, their mode of formation and association with nuclear grooves have been studied in great detail and the formation of INCI as a cytoplasmic invagination into the nucleus was demonstrated cytomorphologically in rare cells.\textsuperscript{[34,35]}

**Nuclear Irregularity in Papillary Thyroid Cancer and its Significance**

Ultrastructurally, lobulated nuclei in PTC were characterized by multiple indentations that divided the nucleus into several lobules.\textsuperscript{[37]} Combining cytochemical and immunocytochemical parameters to ultrastructure for the study of multilobated ground-glass nuclei in PTC, Escheverria et al.\textsuperscript{[38]} suggested that in PTC cells, changes in distribution of chromatin and ribonucleoprotein, either alone or in conjunction with scarce laminin and perinuclear vimentin and desmin filamentous rings, may be responsible the characteristic ground glass and multilobated nuclei. RET rearrangement (15–33%), BRAF mutation (40–53%), and RAS mutation (0–7%) have been reported in PTC cases.\textsuperscript{[39–42]} PTC is diagnosed mostly on the basis of dispersal of heterochromatin in the nucleus; follicular neoplasm (FN), on the other hand, shows round nuclei and heterochromatin aggregates that are often coarser than normal thyroid epithelial cells. Fischer et al.\textsuperscript{[43]} reported that the irregularity in nuclear shape could derive from either an abnormal postmitotic NE re-assembly or dynamic disturbance of the NE during interphase. In this study by Fischer et al.,\textsuperscript{[43]} RET/PTC1 microinjection induced NE irregularity in 27% of PTC cells at 6 h and 37% of cells in 18–24 h; RAS microinjection on the other hand did not increase the NE irregularity; according to these authors,\textsuperscript{[43]} RAS, which is commonly activated in FNs induces coarsening of chromatin in cells with maintenance of a spherical nuclear shape.

Bell et al.\textsuperscript{[44]} observed that dark cerebriform nuclei, a nucleus with marked convoluted outline and nuclear hyperchromasia was metabolically inactive (negative for PCNA, Ki67, bcl2, caspase 3, S100, CEA, and synaptophysin) but the cell was positive for thyroglobulin; these dark cerebriform nuclei, considered to be derivatives of grooved nuclei with severe infolding of nuclear membrane, were present in 100% usual PTC and 92% of FVPTC. Mallik et al.,\textsuperscript{[6]} studied the frequency of dark cerebriform nuclei in FNA smears of PTC cases along with the pale cerebriform nuclei, which are also derivatives of grooved nuclei but represent a less severe degree of infolding and degenerative changes, and concluded that dark and pale cerebriform nuclei, when used in conjunction with other well-known morphological criteria, could improve the accuracy of diagnosis of PTC, especially PTC-FV. As cerebriform nuclei are indicator of an inactive cell and are related to nuclear grooves, presence of grooved nuclei may indicate the beginning of a degenerative process leading to an inactive state by the time the nucleus assumes a dark cerebriform shape [Figure 4].
Formation of Psammoma Body in Papillary Thyroid Cancer and its Contribution

PBs are one of the most important diagnostic criteria of PTC in both histology and FNA smears. As regards their mode of formation, following hypotheses have been proposed: (1) progressive infarction of the papillae and ensuing calcification leads to lamellation characteristic of PBs in PTC. (2) PB results from hyalinization and calcification of meningocytic whorls and fibrous septae; the ultrastructural study by Tsuchida et al. revealed round bodies with concentric laminations like a transverse cut onion made of collagen in the extracellular space of meningothelial whorls and the source of collagen was found to be meningothelial cells through cell processes, (3) PBs are formed intracellularly in serous adenocarcinoma of ovary, resulting in cell death and the liberation of small PBs. Ultrastructural study of PTC has also shown that thickening of the base lamina in vascular stalk of neoplastic papillae followed by thrombosis, calcification, and tumor cell necrosis leads to formation of PBs. Studies on serous cystadenocarcinoma of ovary and meningioma, however, revealed that collagen production by neoplastic cells and subsequent calcification was responsible for formation of PBs; mineralization of membrane bound vesicles, liberated from tumor cells, was found to play a key role in the process. During 1990s, the existence of some precursor forms of PBs was reported in meningioma and more recently in PTC. It was suggested that large hyaline globules (LHGs), small hyaline globules (SHGs), and branching hyaline cylinders (BHCs) were possible precursors of PB and irregular hyaline deposits (IHDs) were precursor of irregular calcification.

PBs are believed to represent a process of dystrophic calcification over nonviable and dying tissues. Contrary to general belief, Das et al. for the first time, demonstrated through cytomorphology the intracytoplasmic formation of targetoid bodies as precursor substance for calcification and their release from well-preserved cells in PTC. These intracytoplasmic magenta-colored targetoid bodies (in MGG stain), on their release from the neoplastic cells formed pools of matrix material, some of which showed evidence of calcification. The cytologic findings were confirmed by histopathology of the tumor in the thyroidectomy specimen. Das also demonstrated formation of PBs over its precursor form, and it was also shown that there was cellular degeneration and necrosis, leading to disappearance of neoplastic cells around PBs. It was suggested that rather than being the outcome of dystrophic calcification of dead or dying tissue, PBs may indeed represent an active biologic process involving production of collagen and membrane bound vesicles in alternate layers by the neoplastic cells and subsequent calcification of the layers containing these vesicles, leading to formation of a barrier against spread of neoplasm and/or inhibition of its growth due to death of neoplastic cells.

PTC is a malignancy with excellent prognosis with 20-year-survival ranging from 82.0% to 88.7%. It is diagnosed mostly in the third and fifth decades and is often indolent and slow growing, but behaves aggressively in the older individuals. Tall cell variant of PTC (PTC-TCV), which is the most common among the aggressive variants and the most aggressive among all variants, tends to occur among elderly patients. Das observed that the combined age of PTC (TCV) and PTC (sig. TCC) cases was significantly higher than PTC (UV) cases and PTC (FV) cases and the age of patients with PTC in general showed a significant correlation with tall cell count. The author reached a conclusion that patients’ age in PTC seems to play a role in the morphological manifestations of the neoplasm, which is related to the prognostic outcome.
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Whereas evidence of thyroid hormone release in the form of fire-flare appearance is most pronounced in FVPTC, grooved nuclei and INCI count are minimal indicating a vibrant state of this variant. Grooved nuclei and INCI are most frequent in PTC-UV and the PBs are most frequent in PTC-sig TCC. This observation supports the concept that all PTC cases possibly start as a neoplasm with follicular pattern such as PTC-FV, and PTC-TCV found in advanced age and associated with grave prognosis, represents ultimate emergence of a new clone that can evade most of the protective mechanisms against survival and spread of neoplastic cells.

As PBs and their precursors are found in a limited number of PTC cases, the degeneration and death of tumor cells may be ascribed to other mechanisms including that leading to formation of pale and dark cerebriform nuclei and subsequent apoptosis. It is observed that formation of precursor of PBs such as hyaline globules in the extracellular space and at the center of a group of neoplastic cells, giving rise to an adenoid
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cystic pattern in extreme case, can cause cell death only when calcification occurs. On the other hand intracellular formation of precursor substance of calcification, which may takes the shape of small round targetoid bodies, on their release may flow like lava and when they surround a blood vessel, there may be degeneration and death of neoplastic cells even before calcification occurs.

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