1 Molecular Pathology Laboratory of the Future

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1.1 The Past

The integration of laboratory analysis with human medicine has traditionally been divided into two broad specialties: anatomic/surgical pathology and clinical pathology. Clinical pathology has been primarily the realm of "wet lab" analysis of bodily fluids and secretions, and over time has utilized the full range of laboratory techniques in chemistry, biochemistry, microbiology and molecular biology. The analysis of whole human tissue has been primarily the providence of anatomic pathology, which is much older than laboratory-based medicine, and is a direct descendent of the discipline of gross dissection and examination of human organs of Galen and his contemporaries in ancient Greece. Gross dissection allowed for the basic understanding of the "plumbing" of the human body, and the subdivision of the organs into organ systems of distinct function (circulatory vs. digestive, etc.). During the Renaissance in Italy, autopsies became standard practice in the investigation of human disease. Diseases began to be categorized by the gross changes seen in tissue (e.g., hemorrhage, tumorous growths, caseating necrosis, sclerosis). It is of interest to note that most of these ancient terms have not disappeared, but have been incorporated and integrated into the modern lexicon of clinical medicine, even as the very molecular basis of these diseases is being understood.

With the advent of microscopy in the seventeenth century and the discovery of the cellular basis of life, diseases in which distinctive tissue changes occurred could be categorized on their histologic appearance. Advances in microscopic optics and histologic techniques correlated with increased understanding of disease processes. It was discovered that various substances "fixed" tissues in a way that prevented autolytic breakdown and decay. The development of mechanical microtomes to section tissue as thinly as possible allowed for greater penetration of light through the tissue and provided uniformity of the sections. Embedding of tissue in wax allowed for a rigid matrix that greatly aided in histologic sectioning, but this

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embedding required the extraction of the water from tissue, hence the stepwise processing of tissue through a graded series of alcohol baths into an organic solvent was devised. Since most tissue elements are essentially colorless, many varieties and combinations of stains have been developed to enhance histologic contrast among the various constituents of tissue architecture. By the latter half of the nineteenth century, the basic histologic technology that is still most widely used today had been developed: the hematoxylin and eosin stained formalin-fixed paraffin-embedded tissue section (Fechner 1997).

Histologic analysis of tissue came into its own as a clinical diagnostic test in the nineteenth century, with the German physician-scientist Rudolph Virchow being its greatest early proponent. It was at this time that the "humoral theory" of human disease (disease being the result of imbalances of specific fluids in the human body) began its rapid decline, with the understanding of the cellular basis of human disease, including infectious and oncologic disorders. It is interesting to note that early academic anatomic pathologists were most interested in the scientific discovery of the basis of human disease and were not especially interested in its direct application to clinical medicine. Especially in the United States it was surgeons, many outside academic medical centers, who were the early practitioners of microscopic tissue examination in the clinical management of patients (Rosai 1997). These clinicians needed methods to determine whether surgery was indicated, what type of surgery was indicated, and if the surgical procedure was successful in its intent. This shift in focus from scientific inquiry to clinical practice is reflected in the terminology of "surgical pathology" to describe the subspecialty of anatomic pathology concerned with clinical tissue diagnosis. This very practical need for better ways to inform clinicians on the selection of therapeutic options drove the ascendancy of histologic tissue assessment. This fact is useful to consider when one ponders the future of new technologies in the application of tissue diagnoses.

1.2 The Present

The majority of tissue-based diagnostics in our time is based on the interpretation of the morphologic characteristics of fixed and stained cells and tissues as seen under the microscope. That this technology of over a 100 years of age is still extensively used speaks for its power. New disease entities are still being defined using these methods, and the histologic classification and categorization of diseases are continually revised as our understanding of, and our experience with, these disease processes increases. Although some disease classification requires the assessment of cell ultrastructure by electron microscopy, this requirement occurs in a small minority of clinical tissue examinations. While the strength of histologic analysis is that tissue and cell morphology is often an accurate reflection

of the sum of many molecular alterations, its weakness is that these underlying molecular alterations are not specifically identified.

In general, histologic tissue assessment is concerned with the identification of specific cell types, and the overall organization of the tissue structure. Abnormalities in tissue organization may identify a congenital abnormality, a degenerative process, or an acquired destructive process. Of the latter category, the major etiologies are autoimmune, infectious and neoplastic. Neoplastic processes are usually identified by specific changes that occur on the cell structure level, most commonly an increase in the size of the nucleus, irregularity of the nuclear contours, and/or an increase in DNA content as reflected by uptake of the hematoxylin stain. Architectural changes (abnormal arrangement or placement of cell populations) are also frequently employed in the identification of neoplasms. Once the diagnosis of a neoplasm is established, subclassification is based on differentiation patterns as expressed in cell shape (spindle vs. polygonal, etc.), cell products (mucin, keratin, etc.) and architectural arrangement (formation of glandular structures, single cell infiltration, etc.). The variation from sample to sample due to differences in fixation and staining, as well as the subtlety of many of the pathologic changes has precluded major inroads by computer image analysis, and the interpretation of tissue histology remains the realm of physicians with specialty training in surgical pathology.

There has been some integration of molecular assays into surgical pathology. By far, the most prevalent form of molecular assay is the detection of specific antigens (usually a protein species) by an antibody in the technique of immunohistochemistry. The rapid assimilation of this technique in clinical practice is in part a consequence that the interpretation of this technique is microscopic examination, hence was easily assimilated into the normal practice of surgical pathology. The majority of immunohistochemical analyses employed are for the *classification* of disease processes rather than the determination of *prognosis* of a disease process. For instance, a metastatic poorly differentiated neoplasm may have a panel of antibodies applied to determine if the neoplasm has characteristics of epithelial cells, stromal cells, melanocytes or lymphocytes, hence may be classified, respectively, as a carcinoma, sarcoma, melanoma or lymphoma, with consequences for the selection of subsequent chemotherapy and radiation therapy. While appropriate classification and staging of a clinical disease is important for prognosis, surprisingly there has been little impetus for widespread implementation of molecular tests whose results yield solely prognostic information.

A case in point is clinical testing for her-2/neu overexpression in breast cancer. The discovery that amplification of the her-2/neu oncogene was correlated with a more aggressive cancer phenotype and a poorer prognosis was first discovered in 1987 (Slamon et al. 1987), without the significant adoption of this assay in clinical assessment for over a decade. However, widespread clinical testing for her-2 neu overexpression did not occur

until the advent of targeted therapy with trastuzumab (Herceptin) in the late 1990s (Goldenberg 1999; Hanna et al. 1999). The clinical need to assign a therapy has been the driving force in the adoption of a new technique in tissue diagnosis for over a century, and will probably continue to be so for the future.

Other examples of the integration of molecular assays in tissue diagnosis is in situ hybridization, which is slowly being implemented as a standard test to study specific genetic changes that occur in cancer. The indications include subclassification of lymphomas and sarcomas on the basis of specific chromosomal translocations and the analysis of her2/neu gene amplification. Again, note that these determinations usually have implications for the type of anti-neoplastic therapy employed. Polymerase chain reaction (PCR) of genomic DNA or coupled with reverse transcription of messenger RNA (RT-PCR) to identify specific gene rearrangements from tissue samples is also being increasingly used, but remains primarily a technique performed at relatively few specialized laboratories.

1.3 The Future

Leaving the realm of validated clinical tests, there are several technologies currently being used to analyze tissue in the research setting. The most exciting are those that provide a "global" analysis of hundreds to tens of thousands of analytes. This includes DNA microarray analysis, which can be used to assay DNA and RNA content (Duggan et al. 1999; Lipshutz et al. 1999). With a current capacity of tens of thousands of probe sets, the whole of the predicted human gene population can be assayed for the presence and quantity of messenger RNA transcript. Similarly, the probes may query single nucleotide polymorphisms (SNPs) that can determine the presence of specific polymorphic sequences on chromosomes. Such a microarray can be used to determine the gain or loss of specific chromosomal regions in neoplasms. Alternately, microarrays can be used to query all of the nucleotides of a specific gene, allowing detection of all possible mutations in a single assay. For proteins, mass spectroscopy is the most promising global assessment technology. In this technique, protein extracts are ionized, and the constituent proteins are separated in an electric field on the basis of mass and charge. Depending on the specific technique used, either a complex "fingerprint" of the constituent proteins is obtained that may be distinctive for a specific disease process or tumor type, or the actual identity of a subset of the proteins can be determined. All of these technologies impart a catalogue of biomolecules present in a tissue sample, revealing characteristics that may or may not be reflected in the morphologic characteristics of the cells, characteristics that correlate with functional biochemical and biological activity.

The greatest challenge to new technologies is to match the sensitivity of standard histopathology. An experienced surgical pathologist may confi-

dently render an unequivocal diagnosis of a malignant neoplasm with as little as ten cells (or less) from a biopsy that contains thousands or tens of thousands of cells. This sensitivity ranges across most types of the hundreds of cancer subtypes. While some molecular assays are as or more sensitive (PCR for example), none can combine the universality of histologic analysis with such sensitivity. In addition, some tests, especially global analyses of biomolecules, will require a pure population of cells to avoid confounding signals coming from an impure cell population. Given that cancer cells are always present in varying quantities in clinical samples (biopsies, fine-needle aspirates, resection specimens), it is quite likely that a selection or purification step will be required to obtain the cell population of interest. Again, since it will not be known *a priori* what cell type will be present in an initial diagnostic biopsy, histologic analysis will most likely still be employed to make an initial assessment of the disease process.

There have been several studies reported in the literature to show that microarray gene expression profiling can classify cancers as well as traditional histologic examination (Golub et al. 1999; Giordano et al. 2001; Ramaswamy et al. 2001; Su et al. 2001). Are these findings the harbingers of the decline of histology as the major tissue diagnostic modality? Perhaps not. The key fact to remember here is that these genomic analyses were generated from cell populations that were recognized and at least partially purified by histologic examination. Only if molecular markers are identified that robustly and specifically identify discrete cell populations will the need for histologic assessment disappear. The fact that this approach could be used is made clear by the current use of flow cytometry. In this technique, cells in suspension can be identified and separated on the basis of antibody binding, size and cytoplasmic substructure. While flow cytometry on whole cells may be used with ease in noncohesive "liquid" cancers of leukemia and lymphoma, its use in the separation of malignant cells from solid tumors (bound by physical bonds to neighboring cells) is problematic. The manipulations required to dissociate cohesive tissue has major impacts on protein and RNA transcript populations and the selection of separation techniques often requires some *a priori* knowledge of the desired cell population.

The last point brings up the issue of a possible "tissue Heisenberg uncertainty principle" at play. The techniques and procedures that allow assessment of one particular molecular species (e.g., immunohistochemistry, in situ hybridization) often cause changes in other molecular constituents (e.g., RNA and protein degradation, protein dephosphorylation). If it is true that a complex analysis of a large number of specific molecular markers is required to identify a specific cell population, it is difficult to see, therefore, how the need for "simple" histologic examination will be replaced, at least as an initial clinical assessment. Only if two major technical problems can be overcome will be need for histologic assessment disappear: (1) the lack of an adequate *simple* panel of molecular markers that

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