Introduction

I WOULD like to share with you some thoughts regarding the impact molecular biology is having and will continue to have upon the field of surgical pathology. I draw, in part, on my experiences as a member of the College of American Pathologist's subcommittee on molecular pathology. This has given me a unique perspective on the process of incorporating the new technologies of molecular biology into the quality-control-based realm of clinical diagnosis. A major area of medicine that will be affected is that of surgical pathology.

My vision of the future goes something like this: A biopsy of a lesion is taken (the site of the biopsy is essentially immaterial), the morphologic diagnosis is rendered, and - if cancer is diagnosed - the behavior of the tumor is predicted. The pathologist's approach to this question is the subject of my discussion.

How important is this topic? A computer-aided search into the literature on the subject "tumor biology" reveals that, in the past 10 years, 60% of the review articles have been published since the latter half of 1989. This reflects a growing awareness of the potential importance that molecular-based tools will have on, first, predicting the behavior of a cancer (e.g., Will it be locally aggressive? Will it metastasize?) and, second, assessing new therapeutic modalities that will emerge from the study of tumors at the molecular level.

This paper will not deal with the health insurance questions that obviously arise whenever a new technology becomes incorporated into the practice of medicine. However, allow me to be quite straightforward in my assessment of this subject by stating that molecular biology will be the foundation of our future efforts at understanding the disease process in people. No technology, heretofore, has had the capability of delving into the very essence of tumorigenesis as molecular biology has and will continue to have at an increasingly more thorough manner.

Molecular biology is the study of biological systems (in this case the human body and its organs) at the molecular level. It is a reductionist theory of science that evaluates how living systems work based upon fundamental principles of how molecules interact with one another. In a sense, molecular biology is the technique that allows us to decipher the language of cells, i.e., how cells communicate with one another. All one has to do is ponder any of a number of basic activities that cells routinely perform, the constant turnover of new cells within the epidermis of the skin, for example, and one can quickly appreciate the complexity of that inter- and intra-cellular communication vital for the maintenance of life.

My discussion focuses on three points: (1) the Central Dogma of biology; (2) the basics of molecular biology; and (3) the impact molecular studies will have in the practice of surgical pathology.

The Central Dogma of Biology

In order to simplify the approach that molecular biology takes in deciphering these molecular activities, let me re-acquaint you with the Central Dogma of biology which states that deoxyribonucleic acid (DNA) proceeds to ribonucleic acid (RNA) which, in turn, is transformed into protein. To be more precise, DNA, called genes, is transcribed into RNA which is then translated into protein. These proteins can be either structural or enzymes, biological catalysts that help "speed-up" chemical reactions in the cell necessary for a wide variety of duties, including the breakdown of proteins, fats, or carbohydrates for energy. These biochemical processes occur hundreds of thousands of times a second with natural checks and balances built into the design of the cell for a smooth minute-by-minute operation. It is now clear that an alteration in the DNA of a cell produces a change in the RNA which, in turn, affects the ability of a protein to perform its function properly thereby transforming an efficient biological machine into a cancerous growth.}

The techniques in molecular biology utilized to delve into these aberrations are based upon manipulating the
biomolecules in our bodies as chemicals. The first step is to extract the substance of interest from the tissue (or cells) being analyzed. Next, the material is purified so as to remove all potential interfering substances. Once the biomolecule is purified, it can undergo a series of reactions – in principle – not unlike any of those performed in a freshman class of organic chemistry. This defines the substance of interest so that it may be compared with biomolecules from other cells or tissues.

**Keys to Molecular Biology**

The foundation of molecular biology lies in our understanding of the genetic material, deoxyribonucleic acid (DNA), the biomolecule that, along with protein, constitutes the 46 pairs of chromosomes in every somatic cell of our bodies. Molecular biology has evolved from the early days, during which DNA was analyzed for its chemical content and physical properties alone, to the current practice of manipulating large fragments of DNA to address questions of its physiologic or dynamic role in disease pathogenesis. Along the way, several important methods were developed that allowed this evolution to take place, including the discovery and use of restriction enzymes and reverse transcriptase, molecular cloning, DNA sequencing, in situ hybridization, and the polymerase chain reaction. Let me briefly discuss these techniques, keeping in mind that the scope of this paper cannot possibly address all the key concepts and methodologies that define the field of molecular biology as it currently is understood.

DNA is composed of two strands of nucleic acids held together by relatively weak (hydrogen) bonds between the nitrogenous bases of the nucleic acids. These nitrogenous bases consist of four types: thymidine, adenine, cytidine, and guanidine, linked to each other in a helical fashion by a sugar-phosphate bond backbone. The unique aspect of DNA lies in the fact that the opposite strand of DNA in the double-stranded chromosome is complementary because thymidine hydrogen-bonds (i.e., is "paired") only with adenine on the opposite strand and cytidine pairs only with guanidine. The order of base sequence on a strand of DNA (e.g., TAAGAC, ... etc.) is the foundation of our concept of the genetic code. Base sequences that become translated into amino acids for the production of protein are called genes. Not all sequences are used to make protein. Some DNA sequence is structural in function. There are 6 x $10^6$ base pairs of DNA distributed unevenly in the 23 pairs of human chromosomes.

Restriction enzymes are enzymes that have a "restricted" focus of action, that is they cleave DNA through the double strand of phosphate-bond-linked bases (uridine, thymidine, cytidine, and guanidine) by acting only in areas of a specific sequence of DNA. These enzymes are found in bacteria. For example, the enzyme extracted from the microorganism *Escherichia coli*. Eco RI, recognizes the base sequence GAATTCC. This allows us to combine DNA from human and bacterial origin that has been digested with the same enzyme. This recombinant DNA is then placed in bacteria and the fragment of human DNA is now under the growth control of the microbe, which allows for the production of large quantities of human DNA. This technique, called cloning, represented a quantum leap in the field of molecular biology.

The enzyme reverse transcriptase acts like DNA polymerase in that it takes a template (single strand) of nucleic acid and synthesizes a complementary strand. The difference is that reverse transcriptase uses ribonucleic acid (RNA) as its template rather than DNA. The importance of this is any messenger RNA (mRNA; RNA transcribed from a gene) that could be purified from a cell was the copy of the gene from which it came; thus, reverse transcriptase could make complementary DNA (cDNA), which in turn could be cloned. Therefore, in the "early days" of molecular biology, any cell that made a protein in sufficiently large enough amounts to be purified (hemoglobin in the red blood cell as an example) also had an mRNA that should also be in high enough concentrations to purify. This purified mRNA could then serve as a template for reverse transcriptase, and the resultant cDNA, which represents the base sequence encoding the protein of interest, could be cloned.

Once the DNA product was cloned, it could be re-extracted from its bacterial vehicle and put through a series of chemical reactions in order to determine its base sequence. Sequencing involves dividing a sample of DNA into four groups and then selectively degrading the DNA in each group with chemicals and/or enzymes specific for one of the four bases, G, A, T, or C. By separating these reaction groups on the basis of size using polyacrylamide gel electrophoresis, a "ladder" of fragment-length bands is generated that can be "read" for its DNA sequence. The methodologies of DNA sequencing have evolved such that larger portions of cloned DNA can be sequenced at a much faster rate.

**In situ** hybridization is a technique applied to cells in which specific base sequences of DNA or RNA are allowed to "pair up" (i.e., hybridize) with their complementary strands of base sequence. The technique involves "tagging" either cloned DNA or RNA with a radioactive or fluorescent marker and then allowing this "probe" to seek out and "hybridize" (i.e., pair up its
base sequences) with its complementary strand of DNA. This allows for the precise localization of DNA sequences in the cells of interest.\(^9\) This technique has been adapted for the analysis of chromosomes in an attempt to map genes to specific chromosome areas.\(^10\)

The latest evolution in the tools of molecular biology has come with the advent of the polymerase chain reaction (PCR)\(^11\) and represents a second quantum leap in the technology of molecular biology. This method employs a unique DNA polymerase that can literally make a billion copies of a short DNA sequence (from 200 to about 2000 bases in length) from a single strand of DNA. The significance of this cannot be underestimated. Prior to the development of this technique, only those genes in which the mRNA expression was sufficient to extract and purify measurable quantities could be cloned (using reverse transcriptase). The polymerase chain reaction now enables the molecular biologist to analyze any fragment of DNA in the human chromosome without the need for cloning. In fact, mass production of any DNA region of choice can be accomplished within days to weeks whereas utilizing the older cloning techniques to amplify DNA may have taken as long as one year. This PCR methodology is the basic principle employed by those scientists involved in the Human Genome Project, the ongoing work to sequence the entire human genome (i.e., all the DNA in our chromosomes).\(^2\) In addition to those techniques described above, medical science has at its disposal a number of other equally sophisticated methods for analyzing the molecular events necessary to transform cells into neoplasms.

**Tumorigenesis**

Once a single cell begins behaving in an abnormal fashion, there are two crucial steps in its transformation into a tumorous growth. The abnormal cell must first perpetuate itself, and then grow in a fashion that allows it to take over the population of normal cells in the immediate micro-environment. As to the first point, the implicit fact of perpetuation is that the alteration in the cell must be inheritable from one generation of cells to the next. The only way that can happen is for the DNA to be altered thereby causing each "offspring" cell to carry the same genetic alteration with it to the next generation of cells.

There are those genes whose protein products are vital to the proper functioning of the cell. If these genes are altered such that their protein products are functioning abnormally, the action of these proteins will morphologically transform the cell into what we in pathology recognize as cancer. We therefore, acknowledge that there are some regions of DNA (genes) on our chromosomes that have an oncogenic potential. In the literature these genes have been designated oncogenes, and over 40 have been identified to date.\(^12\)

Conversely, there are genes whose protein products serve to control or suppress the activity of other genes so that a cell does not "run out of control." Because these genes produce proteins thought to play a role in suppressing tumorigenesis, molecular biologists refer to them as tumor suppressor genes.\(^12\) Picture cells, then, as the morphologic end-result of a series of positive and negative genetic influences. Too much or altered activity in the genes with transforming potential or loss of activity in the suppressor genes will upset the balance, producing abnormal cellular growth activity.

Alteration of these oncogenic DNA regions can take many forms and can be analyzed by different methods. The changes can be at the single base level involving the individual nucleic acids such as base pair deletion in which a base pair is physically lost from the gene sequence or base pair transition in which an A is replaced by a T or vice versa. Only by DNA sequencing\(^\text{13}\) or restriction enzyme analysis\(^\text{14}\) can we identify such changes. The alterations may be of a more "gross" nature such as the physical exchange of long stretches of DNA between two chromosomes or between two segments of the same chromosome. Such alterations may be identified either by DNA restriction digest patterns or karyotype analysis of the tumor cells in which intact chromosomes are analyzed microscopically.\(^\text{15}\)

Once the genetic alteration is in place, stabilization and expansion of the aberrant cells must develop through a process of clonal expansion. It is this step that is perhaps the most complicated to understand since it implies, first, that the aberrant cell makes a product (or products) that offers some advantage for growth over its neighbors' abilities to perpetuate themselves, and second, that the host's response to this abnormal cell is either sub-optimal or impaired for eradication of this malignant clone. The host response to the development of a nearby neoplastic cell would be to activate a defense mechanism (presumably based upon the immune system) that would recruit tumor-killer cells to the area for the purpose of preventing the growth of the cancer. The neoplastic cell's response would be to produce a protein product (or products) that helps circumvent the host's defense screen.\(^\text{16}\) Again we see the role of altered genes and their products in not only the initiation of the tumorigenic event but also in the perpetuation of tumorigenesis.
Molecular Pathology

The third and final point I wish to consider is the impact of molecular studies on the practice of surgical pathology. Traditionally, medicine's basic understanding of cancer was founded upon the microscopic assessment of a tumor's morphology. In genetics terminology, what we as pathologists were evaluating was the phenotypic end result of a genotypic aberration. In the days prior to the concept of molecular biology, most of the pathologist's time was spent in the classification of tumors on the basis of histomorphology. While work continues in this area, it has become apparent that we are now in an era of "fine-tuning" the tumor categorization effort. In fact, it will be fascinating to see just how our morphologic classification scheme for cancer is altered with increased understanding of the molecular biology of these tumors.

The role of the surgical pathologist has slowly evolved with the advent of increasingly sophisticated technology. This is based upon two areas of advancement. On one front, diagnostic radiology, particularly mammography and ultrasonography, along with endoscopy and fine-needle aspiration have lead to smaller tissue samples being evaluated sooner in the course of the malignant transformation of a lesion. The second major area is in molecular biology itself. With a better appreciation of tumorigenesis in combination with an earlier morphologic assessment of pre-cancerous or cancerous growths, there is a greater tendency to assess lesions by initially establishing a morphologic foundation followed by detailed molecular studies in the evaluation of whether or not a lesion will become cancerous or is already cancerous. Thus, in earlier days, "tumor biology" (and it was not called this back then) was essentially synonymous with histomorphologic characteristics of the lesion in question. These characteristics included size, border (infiltration or encapsulation of the lesion), cytologic atypia (nuclear size, shape, chromatin pattern, nucleoli), number of mitotic figures, presence of necrosis, etc.

The literature is rich with studies looking at a tumor's clinical behavior and comparing this with histologic features in an attempt to establish a causal link. Why may ask, "Why abandon this method of assessing tumor biology?" The answer is that we have not considered nor should we ever consider discontinuing the traditional morphologic analysis of a surgical specimen. If nothing else, a gross and microscopic assessment of a tumor serves as the perfect example of quality assurance in any surgical procedure. Furthermore, it has been quite readily established that grade of tumor, to take one example of histomorphologic analysis, has a direct bearing on prognosis in many different neoplasms. However, what has happened in the field of oncology is that the questions asked of pathologists have changed. Until recently, if a pathologist diagnosed cancer from a biopsy specimen and he or she informed the clinician that the lesion was a grade 4 example of "cancer X," the clinician could equate this pathologic assessment with prognosis by realizing that, based on data from previous clinical trials, a known proportion of patients with grade 4 lesions of "cancer X" would die from their disease within five years. However, the incorporation of molecular biology into clinical medicine has changed the playing field. Now the questions are: which of the cancer patients will survive the five years; which patients will develop metastases; which group will do better with chemotherapy; which group of patients are responding best after the initial round of treatment; in other words, what can the surgical pathologist do with an individual patient's cancer specimen to help predict the tumor biology of that lesion and in so doing, make a tangible difference in the management of that patient?

My contention is that it is quite logical to begin finding answers to these more sophisticated questions by first establishing a morphologic foundation for a patient's tumor and then employing any and all molecular studies appropriate for that specific tissue in predicting the behavior of that tumor. Perhaps, in the future, a new approach will be superior to the histomorphology and molecular evaluation employed by the surgical and molecular pathologist (e.g., analyzing a patient's immune system in some as-yet-undiscovered manner); but, for now, as a clinician friend of mine is fond of saying, "The issue is tissue."

Now we come to the most complicated aspect of this subject. I liken it to Pandora's Box. Ever since the tools of molecular biology have been unleashed on tackling the question of tumor biology there has been a seemingly never-ending accumulation of data on assessing the molecular aspects of every known human cancer. This is where the concept of biology's Central Dogma helps to keep things in perspective.

Any tumor can be assessed at any level of the Central Dogma -- DNA, RNA, or protein. Take breast cancer as an example. The analysis of DNA extracted from breast cancer cells can take on many forms. DNA analysis can be relatively crude. For example, flow cytometry analyzes DNA content in a tumor by injecting a suspension of cells extracted from the tumor and quantifying, by the principle of light scatter, the "ploidy" of the cancer cells. Diploid tumors are those cancers whose DNA content is normal or 2n (i.e., that amount of DNA
calculated to be in cells with the normal complement of 23 chromosome pairs), whereas aneuploid tumors contain DNA amounts in their nuclei that are some factor greater or lower than the diploid content (2.3n for example). The implication is that tumors whose cells contain aneuploid quantities of DNA have an alteration in their genetic material that impacts on the patient's survival. Most studies of breast cancer show an inverse relationship between presence of aneuploid DNA content and disease-free state or survival.19

DNA analysis can also be more sophisticated. Identifying amplification of the neu oncogene in breast cancer has been shown to be correlated with disease progression.20 Other analyses of DNA include point mutations identified by DNA sequencing or by changes in restriction enzyme digest patterns evaluated by gel electrophoresis. Data such as this, derived from the study of breast cancers, is poorly defined as to its significance in helping predict the clinical course of a patient's disease to date.21

RNA, the next level in the Central Dogma scheme, can also be evaluated. For example, it has been shown that a metastasis-related gene designated neu23 has more RNA expressed in breast cancer cells from patients with three or less lymph nodes metastatically involved than in those patients with four or more lymph nodes metastatically involved.22 The implication derived from this data suggests that alterations in the DNA of tumor cells can be indirectly assessed by evaluating the expression of that altered DNA in the form of the tumor's RNA. This approach also can offer insight into the behavior of a cancer.

However, there is a relative scarcity of data in the area of RNA analysis that reflects the lack of technological ease seen in handling RNA as compared to protein and DNA. The advent of monoclonal antibodies (antibodies with singular specificity to antigens that often are expressed on either the cytoplasmic or nuclear membrane of cells) has allowed the molecular pathologist to stain tumor cells with antibodies complexed to a chromogen that imparts color to a positive cell. I.e., just as in the case of RNA analysis, another method of assessing the expression of the altered gene in the tumor cell and advances us to the third and final level of the Central Dogma. By directing antibodies against the neu oncogene's PROTEIN, we can evaluate the presence of that gene's protein product for its clinical significance in breast cancer.23 Thus, it is obvious that the molecular pathologist can approach the biology of a tumor from different levels, either by analyzing the alterations in the tumor DNA itself or by evaluating the expression of that DNA in the form of RNA and protein.

The question then is, "What information is pertinent?" The answer lies in the diagnostic or therapeutic question asked. For example, if the clinical concern is whether a patient has cancer, most of the time a simple histomorphologic assessment of the tumor specimen is sufficient. However, if we proceed up the scale to more complicated questions (e.g., "Will the patient respond to a specific therapy?"), it may then become necessary to employ molecular studies at the DNA, RNA, or protein level. Only by comparing the results of molecular studies on a patient's tumor sample with the clinical course of the cancer can we arrive at the appropriate degree of interfacing between traditional surgical pathology methods and modern-day molecular diagnostics.

Conclusion

As you have already surmised, there are myriad molecular steps that go into the making of a cancer. There will likely soon be a tumor biology algorithm unique to each tumor, by which the pathologist morphologically assesses a tumor's cytologic and two-dimensional architectural features, followed by a series of molecular-based analyses that will predict the behavior of the lesion, its responsiveness to treatment, and even the type of treatment that optimizes responsiveness.

The methods of molecular biology truly are the keys to our understanding of tumor biology. Unless we incorporate this knowledge into the clinical arena we will not progress to a level of understanding cancer beyond today's current standards. The foundation of our understanding tumorigenesis remains the morphologic assessment of the patient's tissue by the pathologist. However, this is now followed by an increasingly sophisticated array of technology-based tests to help the clinician find the best approach to managing his or her patient's disease.

References


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