Insulin-like Growth Factor Binding Protein 2: Gene Expression Microarrays and the Hypothesis-generation Paradigm

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A major goal of modern medicine is to identify key genes and their products that are altered in the diseased state and to elucidate the molecular mechanisms underlying disease development, progression, and resistance to therapy. This is a daunting task given the exceptionally high complexity of the human genome. The paradigm for research has historically been hypothesis-driven despite the fact that the hypotheses under scrutiny often rest on tenuous subjective grounds or are derived from and dependent on chance observation. The imminent deciphering of the complete human genome, coupled with recent advances in high-throughput bioanalytical technology, has made possible a new paradigm in which data-based hypothesis-generation is the initial step in the investigative process, followed by hypothesis-testing. Genomics technologies are the primary source of the new hypothesis-generating capabilities that are now empowering biomedical researchers. The synergistic interaction between contemporary genomics technologies and the hypothesis-generation paradigm is well-illustrated by the discovery and subsequent ongoing study of the role of insulin-like growth factor binding protein 2 (IGFBP2) in human glioma biology. Using gene expression microarray technology, the IGFBP2 gene was recently found to be highly and differentially overexpressed in the most advanced grade of human glioma, glioblastoma. Based on this discovery, subsequent functional studies were initiated that suggest that IGFBP2 overexpression may contribute to the invasive nature of glioblastoma, and that IGFBP2 may exert its function via a newly identified novel binding protein. The IGFBP2 story is but one example of the power and potential of the new molecular methodologies that are transforming modern diagnostic and investigative neuropathology.

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Background

The modern war against cancer has been actively fought for over 30 years. Great strides have unquestionably been made and some victories have been achieved; however, cancer fighters have largely not enjoyed the stunning successes experienced in the fight against other types of formerly lethal diseases such as smallpox and tuberculosis. There have been several obstacles to progress, with 2 of the most significant being that the pathogens underlying cancer often originate from within, and the culprits are highly multiformed and mercurial. To put it in the genetic terms, cancer is a highly variable disease that is inherently characterized by multiple heterogeneous alterations of both a genetic and epigenetic nature (10, 25, 31, 42, 48, 61, 65, 66, 75). These changes alter the balance of cell proliferation and cell death, cell stability, and cell motility. The growth of the list of genes responsible for these myriad alterations shows no sign of abatement.

Diffuse gliomas constitute the most common malignant primary brain tumors. They are derived from neuroepithelial cells and are diverse with respect to anatomic location, morphologic features, differentiation characteristics, and response to therapy. Two major lineages are traditionally recognized: astrocytic and oligodendroglial. Advances are rapidly being made in our understanding of the genetic alterations underpinning glioma genesis and maintenance. For example, loss of heterozygosity (LOH) for large regions at 10p, 10q23, and 10q25, or loss of an entire copy of chromosome 10, constitute the most frequent genetic abnormalities identified in glioblastoma (1, 72). A potential tumor suppressor gene located on chromosome 10, *PTEN/MMAC1*, has been recently cloned and characterized (45, 73).

Similarly, examination of gliomas that exhibit the classical histologic features of oligodendroglial differentiation has revealed an incidence of loss of heterozygosity (LOH) for chromosomes 1p and 19q of 80 to 90% (64). Certain specific genetic alterations may affect treatment strategies. In the case of oligodendroglioma, tumors that exhibit the characteristic 1p and 19q deletions respond better to combination chemotherapy (eg,

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procarbazine, cytoxan and vincristine; PCV) compared to oligodendrogliomas that lack this molecular signature (12, 54).

Other altered genes for which there is evidence for a role in glioma genesis or maintenance include mutation of the p53 and p16 genes and amplification of EGFR and PDGFR (9, 23, 33). Many of the genes that are altered in gliomas have also been found to be abnormal in other types of tumors. Based on this common observation, a currently popular paradigm dictates that when a gene is newly discovered to be altered in one class of tumor, a hypothesis is formulated that the same gene may be altered in another cancer type(s), and experiments are conducted to prove or disprove the hypothesis. Tremendous progress has been made under this traditional hypothesis-driven research paradigm, although the hypothesis generating process is often unimaginative and derivative.

Genomics and Genomic Technologies

In order to achieve a fully comprehensive vantage from which to study human biology, an international effort was launched ten years ago to sequence the human genome, which comprises approximately 3.2 billion base pairs (18, 32). By mid-2000 a draft of the human genome was announced (21) and the results have been recently reported in special issues of the journals *Nature* and *Science* (see vol. 409 of *Nature*, 2001, and vol. 291 of *Science*, 2001). The essence of genomics research is the pursuit of biological insight through a comprehensively broad and exhaustively detailed survey of individual genetic and molecular events in a cell or cell population.

Among the roughly 3 billion base pairs comprising the human genome, the number of genes is currently estimated to be in the vicinity of 30 000 (6, 36). In recent years, effective high-throughput screening tools for the evaluation of this gene pool have been developed. Both cDNA microarrays (11, 17, 49, 56) and serial analysis of gene expression (SAGE) (76, 77) can assess simultaneously the expression of very large numbers of genes in a given cell population in a single experiment. By applying similar principles to comparative genomic hybridization (CGH) (38), CGH arrays have also been developed to screen for gene deletions and amplifications at the chromosomal level (58).

Gene expression arrays are available in several different formats; the basic principle is the same in all. Thousands of cDNAs or oligonucleotides are robotically deposited (printed) onto a solid matrix, such as a precoated glass microscope slide or a nylon membrane, in an organized fashion. Oligonucleotides can also be synthesized directly in situ on a silicon chip (47). These attached DNAs, representing thousands of genes, are referred to as probes. To use microarrays to simultaneously profile the expression of genes in a given cell population, total RNA is first isolted, followed by the generation of cDNA by reverse transcription. During reverse transcription, nucleotides labeled with fluorescent dyes (most commonly cy5 and cy3) or radioactive isotopes are incorporated into the cDNAs to make “hot” targets. The targets are then hybridized to the probes on the microarray. After detection of the hybridized signals with either a laser scanner or a phosphorimager an expression profile is produced by quantification and mathematical analysis of the generated image.

One limitation of the cDNA microarray approach is that it constitutes a “closed” system, ie, each microarray can only provide information about the particular set of genes that are included on the array. In contrast, SAGE is an “open” system that can theoretically unmask any gene that is expressed in the cells being analyzed (76, 77). SAGE takes advantage of the fact that a 9-10 nucleotide sequence (a “tag”) often contains enough information to uniquely identify a gene. Generation of gene tag concatemers and their subsequent sequencing reveals the relative frequency at which each tag appears in the library. This frequency represents the relative expression level of that gene in the cell population under study.

Comparative genomic hybridization (CGH) (38) and CGH arrays (58) can be considered complementary genome screening tools. Developed in the early 1990s, CGH provides a physical map of chromosomal region gain or loss throughout the entire genome. To create this map, chromosomal DNA obtained from the sample being tested is labeled with a fluorochrome and DNA obtained from a normal control is labeled with a different fluorochrome. The 2 labeled samples are then cohybridized to normal metaphase chromosome spreads. Differences in signal intensity along the chromosome indicate gene deletion and/or amplification. One limitation of the routine CGH procedure is resolution; the technique is able to detect only relatively coarse regions of chromosomal alteration. In contrast, CGH microarrays possess a significantly higher resolution capability that permits more precise localization of the altered chromosomal region. CGH microarrays are constructed by printing large mapped DNA fragments, such as BAC clones, on a glass slide in an alignment corresponding to their respective chromosomal locations. To search for tumor cell chromosomal regions harboring deletions or

amplifications, the genomic DNAs prepared from tumor cells and from control cells are labeled with different fluorophores and cohybridized to the CGH array. A skewed signal of a particular clone indicates possible deletion or amplification at that locus. The enhanced resolution of CGH arrays enables rapid identification of the specific candidate gene involved in the chromosomal aberration, and is a particularly powerful approach when coupled with complementary techniques such as customized cDNA arrays.

Laser Capture Microdissection and Genomics

A major issue inherent to the study of human clinical samples is purity. In large tissue samples many cell types commonly comingle. To address this problem, one approach is to physically isolate specific cells or cell populations for analysis. A number of methods of differing precision exist for separating target elements from extraneous tissue, including gross manual dissection, scalpel blade scraping of selected tissue from unstained tissue sections on glass slides using an H&E-stained serial section as a guide, selective "core biopsy" of paraffin blocks using a tissue microarrayer, cell sorting by flow cytometry, cell sorting by surface marker selection techniques, and cell culture under selective conditions. Among the most highly selective techniques is laser capture microdissection (LCM) (7, 20), which is particularly suited for molecular genetic applications and was developed specifically for this purpose. With LCM, isolation of individual cells, cell populations, or extracellular components can be accomplished with a high degree of precision under conditions that only minimally perturb the target tissue elements. Although LCM allows the isolation of a highly purified study sample, the material thus isolated is often of exceedingly limited quantity and therefore requires a significantly higher microarray protocol sensitivity. Thus, the use of LCM as a tissue isolation procedure requires the adoption of an amplification procedure. Several protocols that involve a limited PCR amplification and T7 in vitro transcription step have been described. For example, antisense RNA amplification based on T7 transcription may permit analysis of the mRNA population from a few hundred living cells (57). Other techniques combine antisense RNA amplification with a template switching effect (78). Because amplification methods increase the risk of false positive results, confirmatory studies are a necessity.

With regard to the reductionistic LCM approach of isolation and analysis of increasingly smaller and more purely homogeneous samples, from an alternative perspective the targeted analysis of highly purified cancer cells may be overly simplistic and completely miss important cancer component interactions. According to this line of reasoning, cancer cells do not live in a vacuum and an accurate analysis of cancer pathology must address the full mixture and interaction of cancer cells, surrounding stromal cells, infiltrating inflammatory cells and vascular elements. These so-called "normal" cells associated with a neoplasm are clearly distinct from the populations of normal cells residing in non-tumor regions. Support for this contention is provided by the recent finding that tumor endothelial cells exhibit a unique gene expression signature (71). Hence, there is an argument to be made for the genomic analysis of more heterogeneous cancer tissue samples.

From Hypothesis-generation to Hypothesis-testing: the IGFBP2 story

One of the major accomplishments of the genome screening and associated technologies discussed above has been the identification of heretofore unrecognized cancer genes. The discovery of novel genes/markers/targets, however, is only the first step at the beginning of a long chain of scientific investigation. The link between contemporary hypothesis-generating genomic technologies and classical hypothesis-testing pathophysiological investigation is well-illustrated by the recent discovery and subsequent ongoing study of the role of insulin-like growth factor binding protein 2 (IGFBP2) in human glioma biology.

Initial glioma gene expression profiling studies performed in the authors' laboratories revealed that IGFBP2, which is normally expressed in fetal cells and subsequently turned off in adult cells, is highly overexpressed only in the highest grade of human glioma, glioblastoma, compared to lower grade tumors (26). This observation suggested that IGFBP2 expression might be associated with anaplastic progression to glioblastoma. IGFBP2 upregulation in glioblastoma has subsequently been confirmed independently by a second laboratory using a combined cDNA microarray and tissue microarray approach (67). The hypotheses generated by these initial gene expression profiling studies are that IGFBP2 may be involved in glioma progression and may underlie some of the histopathological features of glioblastoma. However, elucidation of the importance and function(s) of IGFBP2 requires research specifically directed at characterization of the role(s) of IGFBP2 in the various critical areas of glioma biology.

Background: what is known about IGF and IGFBP2. The IGFs (IGF-I and IGF-II) are polypeptide mitogens that resemble proinsulin in structure and exert metabolic and growth-promoting effects on many types of cells (50, 62, 82). Human gliomas frequently secrete elevated levels of insulin-like growth factors (IGFs) and express increased numbers of IGF receptors compared to normal brain tissue (52). Both IGF-I and IGF-II have been demonstrated to be glial mitogens (44, 46, 50, 69). The biological response of cells to IGFs is regulated by various factors in the microenvironment. Among these factors, the insulin-like growth factor binding proteins (IGFBPs) play an important role in IGF-mediated cellular processes. IGFBPs comprise a family of structurally homologous proteins that bind IGFs with high affinity (37). Six different IGFBPs (IGFBP1 through IGFBP6) with specific binding affinities for IGFs have been identified. Recent studies have indicated that there may be additional members of this family, termed IGFBP-related proteins, which exhibit low-affinity binding to IGFs (37). IGFBPs undergo substantial post-translational modifications, which affects their binding affinity for IGFs. IGFBPs are able to bind to specific cell membrane receptors as well as attach to the extracellular matrix (15, 16, 37, 40).

IGFBPs have multiple and complex functions, which can be either IGF-dependent or IGF-independent. With respect to IGF-dependent function, IGFBPs are variably able to inhibit or to enhance the action of IGFs, resulting in either suppression or stimulation of cell proliferation, respectively (16, 40). When binding to IGFs, IGFBPs serve to transport the IGFs, prolong the IGFs' half-lives by protecting them from degradation, and modulate their biological actions. The IGFBPs normally have a higher binding affinity for the IGFs than does IGF-IR; therefore, the binding of IGFBPs to IGFs blocks the interaction between IGFs and IGF-IR, thereby suppressing IGF action (16, 40). In addition, IGFBPs can also enhance IGF effects by presenting and slowly releasing the IGFs for receptor interactions while protecting the receptor from down-regulation. The association of IGFBPs with the cell membrane or extracellular matrix may also affect the binding affinity of IGFBPs to IGFs. In addition to modifying IGF actions, direct IGF-independent functions have been proposed for the IGFBPs, including modulation of cell adhesion, migration and anti-apoptosis (3, 28). The molecular mechanisms of action of the IGFBPs at the cellular level both in modulating IGF action and in exerting potential direct functions require further investigation.

Unlike the other members of the IGFBP family, IGFBP2 is predominantly expressed in fetal tissues and has demonstrated involvement in the development of the brain (13, 30, 74, 79). After birth, IGFBP2 expression significantly decreases in glial cells (43, 81). Differential induction of IGFBP2 expression in the brain has been associated with a variety of pathological conditions, including hypoxia, regeneration, and trauma. IGFBP2 is the major IGFBP in cerebrospinal fluid due to its production by multiple neuronal populations (27, 41, 68). The IGFBP2 gene has been mapped to 2q33-q34 and encodes a 289 amino acid protein with a molecular weight of 36kDa under nonreducing conditions.

With regard to IGFBP2 functions, both negative and positive effects on IGF-dependent cell proliferation have been observed (14, 22, 29, 55, 63, 70). IGFBP2 transgenic mice show a significant reduction of body weight gain (35). Knockout mice that lack IGFBP2 expression do not show overt phenotypic alterations, which may be due to the concomitant upregulation of other IGFBPs and to functional redundancy within this family of related proteins (59, 60, 80). On the other hand, IGFBP2 augments the IGF-I anti-apoptosis function, and an IGF-independent anti-apoptosis action in the developing mouse limb and brain has also been reported (3, 5).

Clinical studies that have examined IGFBP2 concentrations in a number of different diseases suggest that alterations of IGFBP2 expression may play a crucial role in many of them. A variety of human tumor cells produce IGFBP2, and markedly elevated IGFBP2 levels correlate with the presence of pathological and neoplastic alterations (8). In ovarian cancer, for example, increased IGFBP2 correlates positively with the serum tumor marker CA125 (24). Similarly, increased serum levels of IGFBP2 have been proposed as a prognostic marker for prostate cancer (39). Overexpression of IGFBP2 resulting in increased tumorigenicity in Y-1 adrenocortical tumor cells and epidermoid carcinoma cells has been reported (34, 51). Previous studies have also shown that the introduction of IGFBP2 expression increased tumorigenicity in a nude mice neuroblastoma xenograph model (4), but the molecular mechanism(s) through which IGFBP2 enhances tumor cell growth and increases tumorigenicity remain undefined. Finally, IGFBP-2 levels have been shown to be increased in the cerebrospinal fluid in patients with central nervous system tumors (53).

IGFBP2 in Human Gliomas

As stated previously, using cDNA microarray gene expression profiling, we found that up-regulation of IGFBP2 is the most consistent and distinct gene expression change in a comparison of different classes and grades of human diffuse gliomas. IGFBP2 is expressed at high levels only in glioblastoma and not in mid-grade or low-grade gliomas (26). Furthermore, strong expression of IGFBP2 is associated with poor patient survival in diffuse gliomas (19, 67). These data suggest that IGFBP2 expression reflects biologically aggressive behavior and may be associated with the formation of or progression to glioblastoma. However, the role(s) of IGFBP2 in specific aspects of tumor biology, such as anti-apoptosis, angiogenesis and tumor invasion, and the mechanism of action of IGFBP2 at the cellular level, both in modulating IGF action and in exerting potential direct actions, remain to be defined.

Because IGFBP2 is preferentially associated with glioblastoma compared to lower grade gliomas, it may contribute to the proliferative potential of cells. Supporting this contention is the findings that IGFBP2, when transfected, increased the proliferation of adrenocortical tumor cells and epidermoid carcinoma cells (34, 51). To further investigate this issue in gliomas, we transfected IGFBP2-expressing vectors into 2 different glioblastoma cell lines, U87MG and LN229. After transfection, stable clones expressing different levels of IGFBP2 were selected. Monolayer growth curves, spheroid growth curves, and cell cycle profiles were analyzed. Results show that there is no major difference between the growth capability of cells expressing different levels of IGFBP2, which suggests that IGFBP2 does not confer enhanced growth potential in glioma cells.

Migration and invasion are prerequisites for the infiltrative growth pattern seen in malignant gliomas and are crucial aspects of the biology of this class of tumors. Infiltrative growth prevents complete tumor resection and causes significant neurological morbidity and mortality. To evaluate whether IGFBP2 may contribute to invasion in gliomas, we carried out an in vitro chemoinvasion assay using the procedure of Albini et al (2), with minor modifications, using stable clones with different levels of IGFBP2. Preliminary results show that clones expressing high levels of IGFBP2 have a higher cell invasion index compared to vector-alone transfectants (unpublished results), indicating that IGFBP2 may play a role in cell invasion. Tumor cell invasion is a complicated process involving a sequential series of critical steps, including tumor cell adhesion, proteolysis and migration. Current ongoing experiments are directed towards identification of which invasion aspects are affected by increased expression of IGFBP2.

Proteins in general do not function alone—they interact with one or more partners to accomplish a biological mission. Biological signals are transducted via the interaction of the protein with its downstream partner(s). The nature of these partner interactions can be either protein-to-DNA, protein-to-RNA, or protein-to-protein. The study of protein-to-protein interactions is complex but provides a global perspective on cellular processes and networks; these analyses fall under the emerging rubric of proteomics. One tool for identifying and studying the potential interacting partners of IGFBP2 is the yeast 2-hybrid system. To identify potential proteins that interact with IGFBP2, we screened a human fetal brain cDNA library using the yeast 2-hybrid system. After 3 levels of selection, 2 positive clones have been isolated that interact with IGFBP2. Analysis of these associated proteins and their interactions is currently in progress. The discovery, identification, and characterization of new proteins such as these should further our mechanistic understanding of the role of IGFBP2 in cancer progression.

Conclusions

To achieve an understanding of any biological process, 2 steps are involved: *i)* selection of a target for study, and *ii)* functional characterization of the selected target. Contemporary genomic technologies provide a powerful tool for intelligent target selection and hypothesis generation. The second step, commonly referred to as the hypothesis-driven phase, involves functional studies designed to gain deeper insight into molecular relationships and interplay. As illustrated by this review of the identification and study of IGFBP2 in gliomas, both steps play an important role in the discovery process. It is anticipated that genomic strategies, based on rapidly advancing technologies, will play an even larger part in re-shaping research paradigms over the coming decade.

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