Id Gene Expression as a Key Mediator of Tumor Cell Biology

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Abstract

Id genes encode members of the helix-loop-helix (HLH) family of transcription factors that inhibit transcription by forming inactive heterodimers with basic HLH (bHLH) proteins. There are four members of the Id gene family recognized in mammals, and the proteins they encode share homology primarily in their HLH domain. bHLH proteins typically form heterodimers with other bHLH proteins, and their basic domain binds to a DNA sequence element, the E-box, activating transcription. Products of Id genes lack the basic DNA binding domain of the bHLH transcription factors, and when they heterodimerize with bHLH proteins, the complexes are inactive. Generally, high levels of Id mRNA are detected in proliferative undifferentiated, embryonal cells and lower levels are detected in well-differentiated, mature, adult tissues. In vitro, these genes are generally expressed at lower levels in cells after the induction of differentiation. Recently, high levels of expression of Id genes have been identified in cell lines derived from a wide variety of different tumors and in tumor tissues as well. These findings suggest that not only the inappropriate proliferation of tumors but also the anaplastic characteristics that contribute to their malignant behavior may be regulated by Id gene expression.

Introduction

An important challenge for cancer research is to understand how genetic alterations associated with tumorigenesis actually contribute to the complex cellular and biological alterations that can be recognized as malignancy. Genes intimately involved in the regulation of cellular proliferation are known to be altered in virtually all of the commonly occurring tumors, including embryonal tumors. These genes typically belong to the family of cancer-related genes known as tumor suppressor genes. However, the precise role of oncogenes, genes whose normal functions are enhanced in many different tumors, in mediating the key characteristics of malignancy is less clear. Tissue invasion, metastases, neoangiogenesis, immune escape, and therapeutic resistance, all key characteristics of malignancy, seem to occur as a result of cellular transformation, but how this occurs is poorly understood.

Virtually all cancer is characterized by two invariant characteristics, inappropriate growth and a lack of the differentiated features that characterize the tissue in which any given tumor arises. Enhanced proliferation and anaplasia are the cornerstones for diagnostic recognition of malignant tissue. In tumors, this is frequently identified by the appearance of mitotic figures in tumor cells that are oftentimes so undifferentiated that they cannot be recognized as one specific tissue type or another. Interestingly, many of the biological features of malignant cells, such as the ability to invade neighboring tissues, migration, and their lack of recognition by the immune system, are also characteristics of embryonal cells. Understanding the molecular mechanisms by which normal cells mediate the expression of differentiated characteristics and how the expression of these characteristics is inhibited in tumor cells may provide important insights into the pathological processes by which the malignant characteristics of cancer cells are regulated.

Id Genes

Id proteins belong to a class of transcription factors known as HLH proteins (1, 2). The distinguishing feature of this class is an amino acid motif predicted to form a HLH structure mediating the dimerization of these proteins. Many members of the HLH family acting as transcription factors contain a domain of basic amino acids NH2-terminal to the HLH motif. The basic domain of such transcription factors (bHLH factors) mediates their binding to DNA when these proteins dimerize (3). These proteins are structurally and biologically distinct from other, related transcription factors such as bHLH-ZIP factors, which, in addition to the HLH dimerization motif, also contain a leucine zipper (1, 4). bHLH factors have been designated as class I and class II proteins based on their ubiquitous expression in many different cell types (class I) or a highly restricted pattern of expression limited to cells of a single lineage (class II; Refs. 3 and 4). bHLH transcription factors are known to play an important role in the regulation of cell determination and differentiation in many lineages, and almost 70 different genes encoding lineage-specific bHLH transcription factors have been identified.

Id proteins are distinct from bHLH factors in that they lack the basic amino acid domain necessary for DNA binding. Id proteins inhibit bHLH proteins from binding to DNA and inactivate transcription by heterodimerization with bHLH proteins (Refs. 1 and 4; Fig. 1; Table 1). This inhibition of DNA binding, in turn, is closely associated in vitro with the inhibition of induced differentiation in a number of different cell types in which tissue-specific differentiation has been studied, including neuronal cells (11), skeletal muscle (12), mammary cells (13), adipocytes (14), and others (Table 2). Because Id proteins seem to bind class I bHLH proteins more avidly than those expressed in a lineage-specific manner (4), the mechanism by which Id proteins inhibit cellular differentiation may be through the sequestration of class I bHLH proteins (Fig. 1).

Four Id genes, originally termed Id (Id1) (1), Id2 (4), HLH462 (Id3) (20), and Id4 (21), have been cloned from the mouse. Human homologues of each of these have also been characterized (22–25). The four Id genes map to different chromosomes in both man, in whom all four have been mapped, and mouse, in which Id1 and Id2 have been mapped to the proximal region of chromosomes 2 and 12, respectively. In man, the Id1, Id2, Id3, and Id4 genes map to chromosomes 20q11 (26), 2p25 (26), 1p36 (23), and 6p21.3–22 (25), respectively. Despite their unlinked chromosomal locations, these genes share considerable homology to one another and are likely to have been derived from a common ancestral gene (2). The region of greatest conservation is clearly in the HLH domain, which extends for approximately 40 amino acids and is located in the middle region of the Id molecule.

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3 The abbreviations used are: HLH, helix-loop-helix; bHLH, basic HLH; pRb, retinoblastoma gene product.
Id proteins function to inhibit tissue-specific gene expression by binding to bHLH transcription factors. bHLH transcription factors such as E47 are ubiquitously expressed, whereas other tissue-specific bHLH transcription factors (TSF) have a very limited distribution of expression and typically regulate the expression of other tissue-specific genes. Id proteins lack a basic DNA binding domain and, after dimerization, inhibit the binding of active, heterodimeric complexes of bHLH factors to E-boxes (CANNTG) found in the upstream regulatory regions of many different genes. + and − indicate the likelihood of transcriptional activation when HLH-containing transcription factors are present in the molecular conformations indicated.

There is an approximately 85% amino acid identity in this region among the four different Id genes, and several other very short regions of sequence similarity have been recognized among various members of this gene family. Interestingly, alternative open reading frames have been identified for both Id1 (27) and Id3 (28), and a mRNA from an Id2 pseudogene that could encode a peptide corresponding to the first 36 amino acids of Id2 has been identified (29). The functions of the distinctive NH2-terminal and COOH-terminal regions of the different Id proteins are largely enigmatic. The COOH-terminal portion of Id3 is clearly important for the stabilization of this protein (30), although little is known of the mechanisms that regulate Id protein levels in any cell type. Importantly, a role for the NH2-terminal region of Id2 in mediating the apoptotic response of cells to growth factor withdrawal has recently been identified, suggesting that multiple different domains of these proteins will be of functional significance (see below).

**Id Gene Expression**

Investigators using in situ hybridization have sought to examine the expression of Id genes during mouse development (31–35). These studies have been remarkable in demonstrating widespread expression of the Id1, Id2, and Id3 genes throughout the developing organisms from very early times in gestation through birth. Some tissues clearly express multiple different Id genes, and data from cell lines in culture indicate that a single, specific cell type can express multiple, different Id genes simultaneously. In sharp contrast to the other Id genes, the expression of Id4 seems to be limited largely to the peripheral and central nervous systems (34). Also, at the level of resolution afforded by published in situ hybridization studies, it appears that there is considerable overlap in the pattern of expression of Id1 and Id3, which is distinguishable from the pattern of expression of Id2 throughout the organism and from the patterns of Id2 and Id4 expression in the nervous system. Remarkably, the pattern of Id2 and Id4 expression in the nervous system is quite similar. These findings suggest that there may be some functional redundancies of these different Id genes, a finding consistent with the extensive sequence similarity in the HLH regions of the Id proteins.

Although it is clear that mRNA corresponding to several different Id genes is expressed in some adult tissues, the precise cell types in which these genes are expressed and the relative levels of expression of these genes in mature tissues are unclear. It is generally thought that the highest levels of Id gene expression occur in undifferentiated tissues, whereas lower levels of expression occur in their differentiated counterparts. It is not known whether terminally differentiated tissues still express Id proteins, although it seems likely that Id2 is expressed in some postmitotic neurons of the central nervous system (33). One possible interpretation of studies detailing the widespread high-level expression of Id genes during early development is that these genes function to modulate the maturation of cells during

<table>
<thead>
<tr>
<th>Table 1 Effect of Id gene expression on lineage-specific genes</th>
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<tr>
<td>Altered gene expression</td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Osteocalcin</td>
</tr>
<tr>
<td>Insulin</td>
</tr>
<tr>
<td>Cardiac α-actin</td>
</tr>
<tr>
<td>Immunoglobulin genes</td>
</tr>
<tr>
<td>Nerve growth factor receptor (p75NGFR)</td>
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<tr>
<td>α-Glycoprotein hormone</td>
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The expression of each indicated gene is inhibited by Id1 expression, except for the repression of p75NGFR, which is antagonized by Id2.
Id genes in cancer

Id genes contribute to the regulation of cellular differentiation, proliferation, and apoptosis. Id proteins may require interaction with a bHLH protein before they can be transported to the nucleus, where they inhibit the binding of bHLH factors to DNA in upstream regulator regions. Similarly, bHLH proteins and pRb can inhibit Id activity. Because bHLH proteins can function as activators or repressors of gene expression, Id gene expression can function to either enhance or inhibit gene expression. Recent data indicating that the NH2-terminal region (N) but not the HLH region of Id2 is required to enhance apoptosis in myeloid precursors suggest that Id effects may also be mediated through interactions with other classes of proteins as well.

Table 2 Representative in vitro lineage-specific differentiation models associated with changes in Id gene expression

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Cell line</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Skeletal muscle</td>
<td>C2C12</td>
<td>12</td>
</tr>
<tr>
<td>Hematopoietic cells</td>
<td>3DE13</td>
<td>15</td>
</tr>
<tr>
<td>Bone</td>
<td>MG3E1</td>
<td>16</td>
</tr>
<tr>
<td>Mammary tissues</td>
<td>Sce2</td>
<td>13</td>
</tr>
<tr>
<td>Trophoblasts</td>
<td>Ro-1</td>
<td>17</td>
</tr>
<tr>
<td>Monocytes</td>
<td>HL60</td>
<td>18</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>3T3-F442A</td>
<td>14</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td>Primary Sertoli cells</td>
<td>19</td>
</tr>
</tbody>
</table>

In mice, the constitutive expression of an Id1 transgene under the control of the immunoglobulin promoter results in inhibited B-cell maturation (42).

A second well-characterized function of Id genes is enhancing cell cycle progression. The hypothesis that Id proteins could have a role in cell proliferation and cell cycle regulation was initially proposed for Id3, which was first described as HLH462 (20). This gene is induced in mouse 3T3 cells as part of the immediate early transcription response to growth factors (20). Subsequently, the induction of other Id genes by serum and isolated growth factors was described, and the ability of Id1, Id2, or Id3 expression to enhance the progression of cells from G1 to S phase was characterized. Antisense oligonucleotides that inhibit Id gene expression delay the reentry of growth-arrested 3T3 cells into the cell cycle elicited by stimulation with serum (36). Interestingly, combined oligonucleotides against Id1, Id2, and Id3 mRNAs were more effective than individual antisense oligonucleotides in these experiments, suggesting that the activities of these Id genes in enhancing cell cycle progression are not completely redundant (36).

The potential of Id genes to enhance cell cycle progression through distinct molecular mechanisms is in accord with the finding that Id2, but not Id1 or Id3, can reverse the inhibition of cellular proliferation mediated by pRb (37, 39). In vivo, a weak physical association between Id2 and pRb has been demonstrated, and in vitro unphosphorylated pRb but not the hyperphosphorylated form binds Id2. The HLH region of Id2 is important for binding to pRb, and wild-type pRb is able to bind to a region of Id2 corresponding to only the HLH domain. Id2 can also bind the pRb-related proteins p107 and p130 and reverse their inhibition of cell cycle progression (39). In agreement with these results, neither Id1 nor Id3 affects the arrest of cell cycle progression mediated by pRb. The growth-suppressive activity of cyclin-dependent kinase inhibitors p16 and p21 is also efficiently antagonized by high levels of Id2 but not by Id1 or Id3 (39). These data provide evidence for Id2-mediated effects on physiological, regulatory events important for cell cycle progression, and they identify distinctive roles for different Id gene products. This interpretation is consistent with recent work indicating that Id1 overexpression in NIH 3T3 cells accelerates cell growth and inhibits p21 expression by antagonizing bHLH transcription-factor-mediated p21 gene expression (43). Interestingly, the function of some Id genes to mediate cell cycle progression may be influenced by cyclin-dependent kinase-mediated phosphorylation (44).

A role for Id genes in mediating apoptosis has also been described.
previously (45). Several different genes important for cell cycle progression, for example c-Myc and E2F1, have been recognized to induce apoptosis when ectopically expressed in cells deprived of mitogenic factors (46, 47). These findings have been interpreted as supporting a model in which activation of the cell death pathway is due to a conflict between growth-promoting and growth-inhibitory signals (48, 49). Transient transfection of Id1, Id2, or Id3 in rat embryo fibroblasts enhances the transition of cells into S phase, and this same population of cells could be identified as containing cells with an apoptotic morphology (45). The conditional expression of Id3 in stable transfectants of the same cell type also induces apoptosis in serum-deprived cells. In both cases, apoptosis was suppressed in the presence of serum. Studying 32D3.5 murine myeloid progenitors, our laboratory has found that Id1 and Id2, but not Id3, can enhance apoptosis that occurs after withdrawal of the survival factor interleukin 3. This activity of Id2 does not require the NH2-terminal region, although the NH2-terminal region is necessary (49).

Id Gene Expression and Activity in Human Cancer

Expression of mRNA encoded by the Id1, Id2, Id3, and Id4 genes has been examined in a variety of human tumor cell lines, including those from the lung, colon, and pancreas, and in both neuronal and astrocytic tumors of the nervous system (23, 24, 33, 50, 51). In each of these cell lines, one or more Id genes were found to be expressed. Interestingly, in nervous system cell lines in which Id4 is highly expressed, Id2 is also frequently expressed. Only a limited number of embryonal tumors have been examined to date, and these include only nervous system tumors such as neuroblastoma and medulloblastoma. Neuroblastoma-derived cell lines tend to express generally high levels of multiple Id genes, whereas very low levels of Id expression were observed in the cells derived from medulloblastomas. Importantly, all of these experiments have examined RNA from cells grown in the presence of serum, a known inducer of Id gene expression. In any case, it seems likely that in cell lines derived from most tumor types, one or more Id genes will be highly expressed, although there may be occasional exceptions to that rule (33).

Experiments examining the morphological characteristics of NIH3T3 cells stably transfected with Id3 indicated that Id3 expression can induce a transformed morphology in these cells, although the cells did not acquire other characteristics of malignant transformation and did not undergo apoptosis (52). This finding is consistent with the observation that primary rat embryo cells can be immortalized by transfection of Id3 and either Bcl2 or BclX, but not by transfection of Id3 alone; it has been possible to induce stable foci formation of primary rat embryo cells with Id3 (45), and in unpublished studies, Id1 has been shown to immortalize primary human keratinocytes. Id genes are strong candidates to contribute to the malignant transformation of some cell types and to regulate the expression of the malignant characteristics of human cancer.

Conclusion

Generally, high levels of Id mRNA are detected in proliferative, undifferentiated embryonal cells, and these genes are expressed at lower levels in cells induced to differentiate or in cells found in the mature tissues of adults. Recently, we have found high levels of Id gene expression in tumor cell lines derived from many different tissues (23, 24, 33, 50, 51). Overexpression of Id3 induces a morphologically transformed phenotype in NIH3T3 cells, and Id1 and Id3 can contribute to the immortalization of primary cells (52). Also, the constitutive expression of Id1 in developing B cells contributes to the development of lymphoma in vivo (42). Given these observations and the activities of Id genes to induce anaplasia and increase cellular proliferation, these genes are strong candidates for mediating oncogenesis and the accompanying embryonal features of malignancy.

Acknowledgments

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Discussion

**Speaker:** If Id2 is so important for tumorigenesis, wouldn’t you expect to see it occasionally genetically altered in translocation and overexpressed by amplification? That would be the real convincing evidence, wouldn’t it?

**Dr. Israel:** Well, that would be convincing evidence that Id2 was an oncogene, but it’s not clear to me that it is an oncogene. The other 10 slides in my carousel described how overexpression of Id2 can also cause apoptosis, as do many genes that enhance proliferation. Our feeling is that there is a sort of a finely tuned mechanism through which different Id genes in different environments can contribute to malignant transformation. However, I would say that whereas it hasn’t yet turned up and hasn’t been found in translocations, I would be reluctant at this point to say that there are no gain of function mutations that exist. Data from other laboratories do suggest that Id genes can contribute to transformation.

**Dr. Phillip Sharp:** If the Id genes are working primarily through

Rb, do you see a difference in the level of expression of Id genes in Rb tumors versus Rb+ tumors?

**Dr. Israel:** That’s a fair question. We haven’t done that, so I don’t know the answer, but I would say that I personally think the primary function of the Id genes is to contribute to anaplasia, which is invariably a characteristic of human cancer. If you ask a pathologist how he recognizes that a tissue is malignant, he doesn’t say that it’s growing inappropriately. He will say that there is evidence of proliferation, an increased number of mitotic figures, and so forth and that it is anaplastic. So, my own feeling is that this could be the mechanism by which Id2 contributes to the anaplastic features of cancers and, as part of that, contributes to the malignant characteristics of tumors.

**Dr. Sharon Murphy:** Thank you. It’s wonderful to hear how the study of pediatric cancer provides models for understanding all forms of cancer.
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