c-myc Oncogene Expression in Estrogen-Dependent and -Independent Breast Cancer

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We demonstrate that c-myc gene expression is essential for growth of breast cancer cells. It also plays an important role in the progression of human breast cancer. c-myc gene amplification may be important for cancer cell invasion, but perhaps not essential for nodal metastasis. We also provide compelling evidence that the c-myc oncogene is an estrogen target gene in hormone-responsive breast cancer. Hormonal progression of breast cancer could be brought about by the enhanced expression of the c-myc gene, with gene amplification and enhanced c-myc mRNA stability being two major mechanisms involved.

Tumor progression encompasses multiple changes including such characteristics as hormone dependence, growth rate, invasiveness, and metastatic potential (1). Our own efforts in the past decade have focused on the molecular basis of some of the above variables as they relate to the progression of human breast cancer. In particular, we have focused our attention on the possible role of the cellular oncogene c-myc in mediating the well-known tumor-promoting effects of the steroid estrogen. These studies have led us to hypothesize that c-myc plays a critical and central role in the progression of breast cancer from hormone dependence to hormone independency; the aberrant expression of c-myc may also play a role in the transition from in situ to invasive carcinoma.

c-myc is the cellular counterpart of the viral transforming gene v-myc. Its encoded protein Myc (two molecular forms, p64 and p67) is a member of the leucine-zipper class of nuclear transacting factors. The activity of Myc is thought to occur through binding to another nuclear protein, Max (c-myc-associated protein x) (2). Although its function is not known, Myc is believed to be an essential factor controlling cell proliferation.

The importance of c-myc in breast tumorigenesis was first demonstrated when transgenic mice overexpressing Myc, under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) enhancer/promoter, developed mammary tumors after a relatively long latent period (3). Overexpression of the c-myc gene as a result of its translocation, mutation, or amplification has been implicated in the genesis and progression of a variety of human tumors, including Burkitt lymphoma (4), small cell carcinoma of the lung (5), leukemia (6), and breast cancer (7). An assessment of primary human breast tumor specimens found amplification of the c-myc gene in 17–33% of the patients (7, 8) a value similar to that for the amplification of c-erbB-2 (9). In human breast cancer, c-myc amplification and increased expression are associated with high-grade tumors (10) and the most aggressive form of breast cancer, inflammatory carcinoma (8). Furthermore, according to one study, c-myc amplification is a better prognostic marker than is c-erbB-2 (11). Thus, alteration in c-myc gene expression may contribute to the rapid progression of human breast tumors.

The above studies of c-myc amplification in breast cancer involved the use of primary tumor samples, which were often heterogeneous with respect to cellular and stromal elements. A given primary tumor frequently contains, in addition to various nontumor elements such as normal epithelial cells, a range of neoplastic elements. These elements are easily identified by pathological examination and include in situ carcinoma, consisting of cells still contained within the mammary ducts or lobules; invasive carcinoma, consisting of cells that have already escaped the original sites and have invaded the surrounding tissue elements; and metastatic carcinoma. Metastasis of breast cancer cells to regional lymph nodes is frequently found during initial diagnosis.

c-myc Amplification in Breast Cancer Cells

To gain insight into the role of c-myc gene amplification in breast tumor progression, we used a quantitative polymerase chain reaction (PCR) to measure the degree of c-myc amplification in cells from the various pathological stages of breast tumors from a given patient: normal epithelium, in situ carcinoma, invasive carcinoma, and lymph node metastasis. We first identified those tumors having c-myc amplification as determined by Southern analysis of total DNA extracted from frozen primary specimens. We observed c-myc gene amplification in ~10% of a relatively small number (100) of primary breast tumors. Next, we examined the paraffin-embedded pathology archival blocks corresponding to the frozen specimens with c-myc amplification. A thin histological section from each block was examined to identify and mark the boundaries of the various cell components. This examination was followed by the preparation of 20-μm-thick sections, which were microdissected to yield subregions containing the different cell components. DNA extracted from these microdissected regions were subjected to quantitative PCR. For each DNA sample, c-mos, a gene on the same arm of chromosome 8 as c-myc but which is not amplified in human breast cancer, was always co-amplified by PCR to serve as a control for DNA input. The c-myc gene copy number observed for a given DNA sample was standardized with

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respect to c-mos. This subregional analysis revealed a similar level of c-myc amplification in both in situ and invasive carcinoma. The unexpected finding was that c-myc amplification is infrequent (<50%) in lymph node metastases of the same patients. The quality of the DNA samples isolated from all the subregions was similar, as judged by their similar c-mos signals.

This preliminary study strongly suggests that c-myc amplification occurs before the invasion process and that in some tumors c-myc overexpression may be a prerequisite to tumor invasion. However, in these same tumors, infrequent occurrence of c-myc amplification in distal metastasis suggests that c-myc amplification does not confer an advantage to the metastatic phenotype in breast cancer. This observation does not preclude the possibility that the c-myc gene may still be overexpressed by other mechanisms in those metastatic cells lacking c-myc amplification. This latter hypothesis can be tested by immunohistochemical quantification of Myc or by subregional analysis for concentrations of c-myc mRNA by the reverse transcription-PCR protocol with fresh or frozen tumor specimens.

c-myc Expression and the Effects of Estrogen

We next studied the role of c-myc oncogene expression in the estrogen-induced growth of breast cancer and in the progression of breast cancer from a hormone-dependent to a hormone-independent, autonomous state. About 40% of breast cancer patients have estrogen-dependent tumors, tumors that require estrogen for growth. These patients benefit from hormonal therapy involving either the inhibition of estrogen action by antiestrogenic drugs such as tamoxifen or the inhibition of estrogen production. Endocrine therapy rarely achieves long-term effectiveness because most, if not all, estrogen-dependent tumors progress to an estrogen-independent state. As a prerequisite to understanding the molecular basis of hormonal progression, it is necessary to identify those genes that are activated early in response to estrogen.

In estrogen-target cells such as breast cells, estrogen binds to its receptor, a nuclear-transacting factor capable of activating a variety of target genes involved in cell replication. Tamoxifen, a synthetic antiestrogenic substance, competes with estrogen for the receptor site, forming an inactive DNA-binding complex. Our search for early estrogen-responsive genes in estrogen-responsive human breast cancer cell lines MCF-7 and T-47D utilized tamoxifen to arrest cell proliferation. Cell growth resumed when tamoxifen-arrested cells were treated with estrogen. Also, there was a rapid induction of the expression of the c-myc gene in the estrogen-treated cells (12, 13). In vitro nuclear run-on transcription assays revealed a major increase in c-myc gene transcription within 2–3 min of estradiol rescue, reaching a maximum of 11-fold induction of c-myc transcription by 20 min, followed by a gradual decline to ~3-fold above that of arrested cells by 1 h. c-myc mRNA accumulation, 12-fold, was achieved at 1 h after estradiol rescue. The induction of the c-myc protein, revealed by Western immunoblot, compares favorably with that of the c-myc mRNA (14). Also, c-myc induction was specific for estradiol: several other steroid hormones tested had no effect on c-myc gene expression.

In contrast, estradiol did not induce the expression of several other cellular genes (e.g., those for actin and glyceraldehyde-3-phosphate dehydrogenase) and oncogenes including c-H-ras (12). c-fos, an estrogen-responsive gene in the rat uterus (15), was less sensitive to estrogen treatment in these human breast cancer cells, although >10-fold induction of c-fos by insulin was seen in these cells (Watson and Shiu, unpublished; 16). Thus, c-myc is by far the most estrogen-sensitive growth-response gene in human breast cancer cells. Only the pS2 gene exhibits similar estrogen sensitivity in MCF-7 cells; T-47D cells do not express the pS2 gene (17). Unlike pS2, c-myc expression is inducible in all hormone-responsive human breast cancer cell lines so far tested. Thus, c-myc is probably the important gene whose expression mediates the mitogenic effect of estrogen in hormone-responsive breast cancer cells. Our recent transient expression studies with chimeric DNA constructs of 5′-flanking c-myc sequences ligated upstream of a reporter gene showed that the estrogen receptor is a transacting factor of the human c-myc gene (18).

Having established that the c-myc gene is a hormone-responsive gene in breast cancer, we next wanted to compare its expression in hormone-independent breast cancer cells. Such study could provide insight into the role that c-myc plays in the progression of breast cancer from hormone-dependency to hormone-independency. We compared and contrasted c-myc expression in the hormone-responsive MCF-7 human breast cancer cell lines and the hormone-insensitive, autonomous MDA-MB-231 line. The growth of the latter cells was totally insensitive to estrogen or antiestrogen; they grew with the same rate under these different conditions (12, 13). The pattern of c-myc expression in these two cell lines under various culture conditions mirrored that of cell growth (12). Thus, in the hormone-responsive MCF-7 cells, estradiol stimulated and tamoxifen inhibited accumulation of c-myc mRNA. In contrast, c-myc expression in the hormone-independent MDA-MB-231 cells was constitutively high; the amount of c-myc mRNA produced in these cells in the absence of estrogen was similar to that found in estrogen-stimulated MCF-7 cells. Neither estrogen or tamoxifen affected c-myc expression in the MDA-MB-231 cells. This observation supports the notion that the high c-myc expression confers the hormone-independent, autonomous growth behavior of the MDA-MB-231 cells.

What then is the mechanism responsible for the increased c-myc gene expression in the hormone-independent MDA-MB-231 cells, given that the c-myc gene in these cells is not amplified? An enhanced transcriptional rate and (or) an increased stability of c-myc mRNA could contribute to the increase in c-myc mRNA in the MDA-MB-231 cells. However, the c-myc gene
transcription rates for the two cell lines, as determined by nuclear run-on assays, were similarly low in the absence of estrogen (13). Thus, transcription cannot account for the enhanced amount of c-myc mRNA in the MDA-MB-231 cells.

The stability of the c-myc mRNA in the two cell lines was then compared (13). We used actinomycin D to arrest RNA synthesis and determined by Northern analysis the amounts of c-myc mRNA at various times after actinomycin D addition. In the estrogen-treated MCF-7 cells, the c-myc mRNA decayed with a half-life of ~20 min, a value similar to that reported for a variety of cell types and generally accepted as the "normal" half-life for c-myc mRNA. In contrast, the half-life of the c-myc mRNA in the hormone-independent MDA-MB-231 cells was close to 1 h, indicating that the c-myc mRNA in the hormone-independent cells was about three times as stable as that in the hormone-responsive cells and most other cells and tissues. This threefold increase in half-life is probably sufficient to account for the observed enhanced amounts of c-myc mRNA in the MDA-MB-231. Thus, we have described another novel mechanism by which c-myc gene expression can be enhanced in human breast cancer.

Mechanisms for Expression

There are at least three different mechanisms by which human breast cancer can achieve increased c-myc gene expression: transcription activation by estrogen, gene amplification, and increased mRNA stability. Hormone-responsive breast cancer cells, irrespective of their c-myc gene copy numbers, require estrogen for c-myc activation and growth. Hormone-independent breast cancer cells can achieve high c-myc gene expression through gene amplification, increased mRNA stability, or both. We have therefore proposed (14) that the hormonal progression of human breast cancer involves an alteration of c-myc gene expression and that enhanced c-myc expression may be sufficient to activate downstream cellular events necessary for cell growth.

If this hypothesis is correct, one would expect that cell growth would be arrested if c-myc gene expression alone is inhibited. We therefore selectively inhibited c-myc expression by the use of c-myc mRNA sequence-specific antisense phosphorothioate oligonucleotides (14). A 15-mer c-myc sequence-specific antisense phosphorothioate oligonucleotide at 10 μM/L inhibited the estrogen-induced c-myc protein synthesis by >90% in MCF-7 cells. It was similarly effective in suppressing c-myc protein in the MDA-MB-231 cells. A 15-mer sense oligonucleotide used as the control was without effect on c-myc protein synthesis. As an additional control, we used a 15-mer pS2 sequence-specific antisense phosphorothioate oligonucleotide, which blocked estrogen-induced pS2 protein production by ~80%.

The c-myc-specific antisense oligonucleotide blocked not only the estrogen-induced cell growth in MCF-7 but also the autonomous growth of the hormone-insensitive MDA-MB-231 cells. Neither the c-myc-specific sense oligonucleotide nor the pS2-specific antisense oligonucleotide affected the growth of these cell lines. These results lend further support to the notion that c-myc expression alone is sufficient to provide the necessary growth stimulus to both hormone-responsive and non-responsive breast cancer cells. Because the growth of the MCF-7 cells was not impeded, despite the >80% blockade of pS2 synthesis by the pS2-specific antisense oligonucleotide, we conclude that pS2 is not essential for growth of breast cancer.

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References