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I. Biological and Clinical Rationale for a Combined Approach to Breast Cancer

EXPRESSION AND MODULATION OF ESTROGEN RECEPTORS IN HUMAN BREAST CANCER

ITALO NENCI
Istituto di Anatomia e Istologia Patologica, Università di Ferrara, Ferrara 44100, Italy

Summary—Estrogen receptors have been traced at the tissue level in premalignant and malignant changes of human mammary gland by an immunoperoxidase technique using a monoclonal antibody against the receptor protein. Proliferative premalignant cells show an increased and homogeneous expression of the estrogen receptor by this technique; it seems that a constitutive expression of the receptor may play a role in the promotional carcinogenetic process. Moreover, infiltrating tumors show a striking heterogeneity in the cellular expression of estrogen receptor, which seems related to differentiation level.

INTRODUCTION

It seems reasonable to anticipate that further advances in cancer control may depend not only on the discovery of new highly effective therapeutic agents, but on the development of new concepts concerning the tumor biology. The present period will be remembered in the history of the endocrine biology of human breast cancer as that of the great breakthrough. As with many other scientific advances, new tools and new thoughts now make possible vast inroads into this field. Among these, the hybridoma technology is revolutionizing also this area of tumor biology, since monoclonal antibodies against hormones and hormone receptors may provide information not otherwise achievable and may be fundamental in fully understanding some relevant events subserving the hormonal control of tumor growth.

The results (to be published in full) upon which the present discussion is based have been obtained thanks to a mouse monoclonal antibody against the estrogen receptor protein (kindly provided by Dr Indu Parikh, Wellcome Research Labs, Research Triangle Park, N.C., U.S.A.). This antibody exhibits a very high affinity for all molecular forms of the estrogen receptor, and cross-reacts with the estrogen receptor from rat and human tissues [1, 2]. When traced with the bridged avidin–biotin immunoperoxidase technique, this antibody proved to be ideally suited for the specific visualization *in situ* of both occupied and free estrogen receptors in cell and tissue preparations. The displayed positivity is surely due to the estrogen receptor; the pre-absorption of the receptor antibody with the purified estrogen receptor protein (kindly provided by Dr Giovanni Alfredo Puca, Institute of General Pathology, University of Naples, Italy) prevents any positivity.

ESTROGEN RECEPTOR EXPRESSION AND TUMOR PROMOTION

When tracing estrogen receptors in normal human mammary tissue, a predominant nuclear localization of the receptor is clearly evident in epithelial cells of both the lobules and the ducts. The cell nucleus appears to be the predominant receptor localization also in target tissues of normal experimental animals; however, after ovariectomy the receptor is traced chiefly at the cytoplasmic level; and 2 h after the injection of estradiol in these spayed animals, the nucleus reappears as the predominant site of receptor localization. These results reflect the classical two-step cytoplasm-to-nucleus model of the steroid receptor mechanism [3]: it appears that all the synthesized receptor molecules are continuously translocated from the cytoplasm into the nucleus in normal target (e.g. mammary) cells exposed to physiological hormone levels.

In contrast with the normal tissue, when looking into proliferative changes of mammary tissue, it can be easily observed that a cytoplasmic positivity for the receptor is often apparent besides the nuclear localization. Such an abnormal presence of cytoplasmic receptor is very apparent in some hyperplastic and in more advanced pre-malignant and malignant lesions. We suggest that the appearance of cytoplasmic estrogen receptors in proliferative changes may be the result of an increased and continuous synthesis of the receptor protein which is translocated into the nucleus only in part, according to the peripheral estrogen levels.

This abnormal amount of estrogen receptors which we have found in hyperplastic, preneoplastic and neoplastic breast lesions deserves some comments which blend into the general subject of "preneoplasia". Carcinogenesis is believed to occur in at least two major steps: initiation, followed by promotion. Promotion is the process whereby focal proliferations develop on initiated tissue, one or more of which may act as a precursor for subsequent steps in the carcinogenic process [4–6].

As far as experimental mammary tumors are concerned, they require a protracted promotional process and the rate of production is invariably deter-

mined by endocrinological factors: the endocrine products acting as promoters, rather than initiators, of cancer development. This seems to be true also for human breast cancer since its development depends on having sustained endocrinological support. In spite of extensive investigation, this support has not been shown to be dependent on abnormalities of hormonal secretory patterns, circulating hormone concentrations or peripheral hormone metabolism. On the contrary, the problem appears to lie centrally in an abnormal response of initiated mammary epithelial cells to an essentially normal endocrine environment. Cellular changes relevant to malignant behavior of cells will be those that will enable the cells to survive and multiply in an environment that imposes an efficient growth control on normal tissue components: the acquisition by cells of receptors for growth-promoting stimuli can be envisaged as a way in which initiated cells could escape the growth control signal [7]. Since the hormones exert their effects on target tissues via specific receptors, it seems conceivable that the changes in hormone responsiveness may result from variations in the number of functionally active hormone receptors. The high levels of estrogen receptors detected by our monoclonal antibody in hyperplastic, preneoplastic and cancer cells may enable these cells to proliferate in an environment with a relatively normal concentration of circulating hormones. In this context, the quantitative changes of hormonal receptors in cancer cells could reflect the transition of cellular properties to certain developmental stages of their normal counterparts.

In conclusion, since preneoplastic lesions and the incipient tumors of the human mammary gland appear to be "turned on" for receptor production, the promotional process may be associated with a strong induction of the estrogen receptor system in the initiated cells of an otherwise resting gland. If confirmed, this concept could provide new diagnostic and therapeutic means for preventing the accomplishment of the carcinogenic process. The immunohistochemical recognition of strongly receptor-induced cells could provide the premorphological identification of potentially tumorigenic foci. Moreover, the selective suppression of these foci could be attempted on a rational basis by interfering with the estrogen receptor synthesis and/or function.

ESTROGEN RECEPTOR MODULATION AND CELL HETEROGENEITY

Some interesting results have been obtained also when this kind of immunomorphological approach was focused on the problem of predicting the endocrine responsiveness of the established breast cancer.

The profile of receptor phenotypes displayed by neoplastic cells within a given tumor is often complex. Tumors displaying homogeneous cell types—that is, all cells exhibiting the estrogen receptor or not—are rarely seen. As previously reported [8], one of the most significant outcomes of the morphologically oriented approach is the very apparent heterogeneity of tumor cells as far as the estrogen receptor positivity, in both the cytoplasm and the nucleus, is concerned: most of the tumors have proved to be composed of mixed receptor positive and negative cell populations with various intermediate degrees. Interestingly enough, it seems that tumors of recent origin are not less heterogeneous than the well-established ones: breast cancer appears to be a very complex entity from its first detectable expression.

The existence of cellular heterogeneity in tumors adds a new biological dimension to the kinetics of tumor cell populations and may have important implications for our understanding of the natural history of tumors and their response to treatment.

Biological significance

Several circumstances may be envisaged to explain the tumor cell heterogeneity. First, one possibility is that tumors could have a multicellular origin; so that the heterogeneous hormonal properties of tumor cells could primarily be dependent on the plurality of stem cells and the diverse cell properties would reflect their diverse parentage (polyclonal origin).

Alternatively, the heterogeneity could be attributed to the stepwise emergence and selection of variant cells of monoclonal origin [9]; in fact, it is possible that breast cancers become heterogeneous by spontaneous mutation, and that biological selection is responsible for the changes in the character of cancer cell populations with time (clonal selection).

There is however some evidence that these simple models may not be valid. In fact, whether tumors have a unicellular or multicellular origin, what is not at all clear is how these variants continue to populate the neoplasm overlife. One might expect that mixtures of cancer cell populations which differ in such important properties as hormone dependence, would be subjected to strong selection pressure *in vivo*, rapidly producing an overgrowth by one population. Yet heterogeneity is maintained even in large tumors, when selection pressures have presumably had ample time to eliminate many variants. Thus, the hypothesis has to provide a model to explain how tumor heterogeneity is maintained.

One way by which this could occur would be by continued stem cell differentiation [10, 11]: if one postulates the existence of tumorigenic progenitor cells capable of giving rise to phenotypically different variants, this would provide a model to explain how tumors may maintain continual cell heterogeneity. The idea that a heterogeneous neoplasm contains a stem cell population that produces phenotypically distinct tumor cells through a differentiation process is consistent with the basic tenets of developmental biology [12]. It is conceivable that different types of breast tumor cells may arise by a process mimicking

normal differentiation; the cell sensitivity to specific control signals (e.g. hormone receptors) being switched on and off according to the level of differentiation. The variable receptor expression in various cancer cells could thus be related to that of the developmental stages represented in the cancer. Studies of stem cell renewal tend to support the possibility that the immediate microenvironmental "niche" occupied by each stem cell may be paramount in controlling its committment to differentiation [13].

The stem cell hypothesis is not the only one which could explain how tumors remain heterogeneous in spite of apparent opportunities for selection. In fact, its seems noteworthy that variable receptor expression occurs randomly within primary infiltrating and metastatic breast carcinomas. The staining pattern by monoclonal receptor antibody indicates that cells which are morphologically and topographically identical may coexist simultaneously in at least two different states of functional differentiation; that is receptor positive and negative, respectively. This feature stresses that phenotypic specializations occurring within a neoplasm may be irrespective of the nature of the progenitor cells. In this respect, it seems worthy of note that multiple phenotypic expression is not confined to malignant cells, since the heterogeneity observed in breast tumors may be identified in normal breast tissue. Normal mammary structures are populated by receptor positive and negative cells which are intermingled, side by side, without any appreciable morphological or topographical differences. It may be suggested that the expression of particular determinants by morphologically identical cells may represent fluctuations in the functional status of these cells with time (phenotypic modulation) [14–16]. Hence, the variable receptor expression may define specific fluctuating cell subpopulations of normal breast and breast cancer: the reversible phenotypic drift being activated by environmental signals (or lack thereof).

Clinical significance

The picture that emerges from the evidence for cancer heterogeneity is that a neoplasm is a tissue almost bewildering in its complexity [17, 18]. The present most important clinical implications of intratumor receptor heterogeneity are those relevant to our approach to cancer therapy.

First of all, the implications of tumor cell heterogeneity (differential hormone sensitivity) seem to challenge the clinical value of conventional receptor assays [19], which are based on the assumption that the tumor is a fairly homogeneous collection of cells.

Similar considerations apply to efforts to develop combined modality therapies. The existence of tumor cell heterogeneity provides a new and compelling rationale for combination therapy (multiple targets). The apparently easiest way to proceed may exploit a mere combination of hormonal and cytotoxic agents: given the inherent heterogeneity, it is not surprising that combined endocrine and cytotoxic therapy is the approach that has the best chance of success (see other papers in this issue). An important challenge at present for medical oncologists is the rational selection of the most appropriate sequences and schedules. More updated approaches may try to exploit each single treatment to increase the sensitivity of tumor cells to the other one.

However, the effective control of neoplastic growth could depend upon our ability to manipulate tumor heterogeneity (induced phenotypic modulation). Methods of directing and controlling the differentiation of tumor cells could potentially limit heterogeneity through the conversion of entire populations of malignant cells to a single phenotype. One possibility would be to induce tumor cells to differentiate to stages more likely to express hormone receptors. It would be of considerable interest if differentiation could be channeled toward a particular cell status responsive to endocrine treatment: it is well-established that certain chemicals cause phenotypic changes in cancer cells that have been equated with differentiation events [20,21]. Hence, an approach deserving investigation would be the development of treatments which could switch on and off the estrogen receptor synthesis. The understanding of controlling mechanisms that affect the phenotypic expression of estrogen receptors in breast cancer promises to provide an exciting area for future research, especially as these may related to cellular responses to therapy.

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