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Molecular and Endocrine Biomarkers in Non-Involved Breast: Relevance to Cancer Chemoprevention

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Abstract The animal models for chemoprevention of breast cancer have provided important experimental systems to evaluate the efficacy of tumor suppression by dietary macro- and micronutrients. In the initiation/promotion cascade, early occurring premalignant changes constitute less extensively examined aspects of disease progression. Molecular, endocrine and cellular biomarkers may provide clinically relevant endpoints for prevention of breast cancer that focus on downregulation of preneoplastic transformation. *In vitro* models derived from non-involved murine and human mammary tissues are utilized to identify molecular, endocrine and cellular markers that are perturbed in response to such diverse initiators as viruses and chemical carcinogens. This upregulation was manifested as persistent Ras p21-GTP binding, altered C16α/C2 hydroxylation of estradiol, and hyperplasia preceding tumorigenesis. Prototypic chemopreventive agents such as n-3 polyunsaturated fatty acids, retinoids, and indole-3-carbinol were capable of downregulating all of the preneoplastic markers perturbed by initiators. Experimental modulation of these biomarkers in murine and human mammary tissue prior to the expression of a fully transformed tumorigenic phenotype is suggestive of their potential clinical application in chemopreventive intervention for breast cancer.

Key words: chemoprevention, *in vitro* models, estradiol metabolism, intermediate biomarker, mammary preneoplasia, Ras p21

Breast cancer is a multifactorial disease whose development is profoundly influenced by genetic, environmental and endocrine factors. The American Cancer Society has estimated that in 1991 approximately 175,000 new cases will be diagnosed and about 44,500 will die of the disease in the United States [1]. Only about 25% of the cases present conventional risk factors that include: i) family and/or personal history of the disease; ii) endocrine changes such as early menarche, late pregnancy, and nulliparity; and iii) pathological abnormalities such as fibrocystic disease and proliferative breast lesions [2]. Thus, in a vast majority of breast cancer patients, there is no apparent disposition to the disease.

The high incidence of breast cancer combined with a lack of definitive evidence for its etiology and pathogenesis have prompted the development of reliable model systems that can provide fundamental information about the causative agents and risk factors, and facilitate meaningful evaluation of prevention strategies.

The commonly used laboratory models for breast cancer include the carcinogen-induced mammary cancer in rat and mouse, spontaneous mammary cancer in mouse, and the transplantable mammary tumor in mouse [3–5]. All these in vivo models have provided strong support to the concept that, independent of the nature of the initiating event, development of experimental breast cancer is modifiable by

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exogenous manipulations (e.g., dietary interventions) and therefore is preventable. In these models, changes in tumor incidence, multiplicity, and latency have been the primary quantitative parameters. Since the major quantitative endpoint in the conventional in vivo animal models is overt cancer, experimental modulation of tumor growth reflects therapeutic rather than preventive efficacy. An equally important, but less extensively examined aspect, is efficacious intervention leading to primary prevention, i.e., suppression of first cancer. Such intervention is possible by experimental down-regulation of preneoplastic transformation that leads to the appearance of first cancer. Breast cancer develops through several distinct steps: initiation (mutagenesis of the target cell), promotion (immortalization and preneoplastic transformation), and progression (emergence of tumorigenic and metastatic phenotype). In the murine mammary system ductal hyperplasia and hyperplastic alveolar nodules are the recognized preneoplastic lesions that are at a greater risk for tumorigenic transformation than is the normal mammary epithelium [3–6]. In humans, mammary terminal duct lobular units (TDLU) with atypical hyperplasia are considered as high risk lesions for the development of breast cancer [7,8]. In principle, effective intervention of mutagenic perturbation of the mammary epithelial cell, acquisition of growth advantage of "mutant" cell, and formation of morphologically aberrant cellular lesions may provide alternative means for chemoprevention of breast cancer.

Efforts in several laboratories have been focused on developing reliable in vitro models where the above mentioned critical events in preneoplastic transformation can be examined directly on the target tissue at molecular, metabolic, endocrine and cellular levels. To this end, we have undertaken comparative studies on in vitro models derived from murine and human mammary tissue, with the goal of identifying common and unique sequences of events in initiation and promotion that are associated with tumorigenic transformation of mammary tissue from the two sources [6,9–16].

This review discusses the development of in vitro models, identification of specific biomarkers that are associated with initiation and/or promotion of preneoplastic transformation in the models, and validation of the biomarkers as relevant endpoints for chemoprevention of preneoplastic transformation.

IN VITRO MODELS

A major technical limitation of whole animal studies in the area of organsite carcinogenesis has been the limited utility of the experimental system to delineate the effects of carcinogens and/or modulators directly at the target tissue. Systemic and humoral factors are known to indirectly modify target tissue response to experimental manipulations [2–4]. As an alternative approach, in vitro models have been developed from non-involved target tissue to examine the effects of diverse initiators and modulators of carcinogenesis [6,9,10,13,17]. Mammary explant cultures retain the cellular heterogeneity that is intrinsic to the organ, and thus maintain epithelial-stromal interactions that are critical for proper epithelial morphogenesis and for overall responsiveness of the tissue to endocrine, autocrine or paracrine influences.

For the experiments utilizing the explant culture model, entire mammary glands from mice or intact TDLU from human mammary tissue are cultured in a chemically defined, serum-free medium that is supplemented with mammotropic hormones such as estrogen, progesterone, insulin, prolactin and hydrocortisone. Depending upon this hormonal stimulation, cell proliferation and cytodifferentiation can be induced in the epithelial component [6,9–13,15,17].

INTERMEDIATE BIOMARKERS FOR TRANSFORMATION

These constitute a series of molecular, metabolic, endocrine and cellular endpoints such as genotoxic damage, DNA repair, mutagenic perturbation, oncogene expression, metabolism of carcinogens, hormones and xenobiotics and hyperproliferation/hyperplasia. In the development of cancer these biomarkers may be perturbed during phases of initiation and/or promotion leading to preneoplastic transformation [9,10,13,16–18]. Since these endpoints are detectable in non-involved tissue well before the

TABLE I. Intermediate Biomarkers for Mammary Cell Transformation

Phase of Transformation	Type of Biomarker	Endpoints
Initiation	Molecular	DNA damage, DNA repair, mutagenesis, oncogene expression
	Metabolic	Carcinogen-DNA adduct formation, DNA repair enzymes
Promotion	Metabolic	Metabolism of mammotropic hormones
	Cellular	Hyperproliferation/ hyperplasia, anchorage-independent growth

appearance of overt cancer, they are referred to as intermediate biomarkers.

A systematic identification of endpoints that are associated with initiation/promotion of preneoplastic transformation should provide a means to accurately quantify the extent of transformation in the non-involved target tissue prior to the appearance of tumor. Furthermore, effective downregulation of the events perturbed during transformation will provide definitive evidence for successful chemoprevention. The intermediate biomarkers listed in Table 1 are specific to one or more critical events in initiation and promotion of mammary cell transformation at molecular, metabolic, endocrine and cellular levels.

In our investigations with the in vitro models of mammary explant culture, we have chosen to monitor the alterations in Ras p21-GTP binding, estradiol biotransformation and hyperproliferation as molecular, endocrine and cellular intermediate biomarkers, respectively.

MOLECULAR BIOMARKER

Ras expression plays a fundamental role in signal transduction and mitogenesis. The oncogene product Ras p21 noncovalently binds and hydrolyses guanine nucleotide, influencing positive and negative regulation of transduction of mitogenic signal to promote growth [19]. Elevation and/or persistence of GTP binding activity of Ras p21 may be a manifestation of a continuous mitogenic signal resulting in unrestrained growth. This could occur via deregulated expression of normal proto-oncogene, or via expression of point mutated oncogene, both of which are known to cause tumorigenic transformation [17,19,20,37,39,40]. We have measured the alterations in GTP binding of endogenous Ras p21 by determining the extent of GTP bound to the G proteins, and confirming the specific Ras p21 binding by immunoprecipitation [12,26,30].

ENDOCRINE BIOMARKER

The well documented endocrine responsiveness of the mammary tissue has prompted several investigations to examine the influence of such mammotropic hormones as estradiol, progesterone, prolactin, and glucocorticoids on the process of proliferation, cytodifferentiation and neoplastic transformation [3–6,8,10,13,16, 36,38]. Experimental as well as clinical in vivo studies on the relative extent of estradiol biotransformation have demonstrated significant elevation of the 16α-hydroxylation pathway in mouse strains that differ in relative risk for developing mammary tumors [21], human subjects at elevated breast cancer risk [22], and patients with clinically identifiable breast cancer [23,24].

In an effort to identify an endocrine biomarker capable of measuring relative risk for developing breast cancer, previous studies have

focused on the biological effects of various intermediate metabolites generated during metabolic biotransformation of the natural estrogen, 17β-estradiol (E2). The two metabolites 16α-hydroxyestrone (16α-OHE_1) and 2-hydroxyestrone (2-OHE_1) possess estrogenic and antiestrogenic properties, respectively. These metabolites are generated via the cytochrome P450-dependent exclusive hydroxylations at C16α or C2 positions of E2. We have measured the alterations in estrogen metabolism using a radiometric assay that involves exposure to stereospecifically labeled estradiol and measurement of $^3H_20$ formed due to a stoichiometric tritium exchange from the ligand [20,21,24,28].

CELLULAR BIOMARKER

One of the most frequently documented cellular intermediate biomarkers having increased risk for tumorigenic conversion is atypical hyperproliferation and/or hyperplasia [7,8,10,11,13,20,26,29,30]. These cellular changes have been detected by the mammary fat pad transplantation assay [6,10,11,13]. Studies with non-transformed target tissue can specifically recognize the preneoplastic and neoplastic transformation induced by diverse initiators such as metabolism-dependent carcinogens, metabolism-independent mutagens, transforming retrovirus, and cellular oncogenes [10,11–13,15,20,26,29,30], all of which are tumorigenic in the rodent model [3–5,10,13].

INDUCTION OF PRENEOPLASTIC TRANSFORMATION

In an effort to systemically characterize the process of preneoplastic transformation, we have measured the upregulation of molecular, endocrine, metabolic and cellular biomarkers in the non-involved rodent and human mammary tissue maintained in vitro as explant culture (Table 2). It was interesting to observe that both murine as well as human mammary tissue respond to the carcinogenic insult by exhibiting upregulation in GTP binding of Ras p21, the molecular marker. Chemical carcinogens that produce tumorigenic transformation of the mammary epithelium are also known to induce point mutations in Ras genes at codons 12, 13, or 61 [17,19,31]. Although elevated levels of the Ras mRNA and protein have been detected in primary human tumors [32,33,37], activation of the Ras oncogene by point mutation has rarely been observed [32]. This raises the possibility that downregulated expression of Ras proto-oncogene, rather than amplification of point mutated Ras oncogene, may be associated with progression of human breast cancer. In accord, our studies utilizing the Ras p21-GTP binding assay have shown specific perturbation of this molecular biomarker in response to chemical carcinogens, as well as to mammary tumor virus [12,26,30].

Our recent studies on non-involved mammary tissue of murine and human origin have examined the perturbation of the endocrine biomarker by measuring E2 metabolism via 2-hydroxylation and 16α-hydroxylation pathways [25,26, 28]. It is noteworthy that similar to our observations on Ras p21-GTP binding, murine as well as human mammary tissue exhibit increased amounts of 16α-OHE_1 after carcinogenic initiation [17,20,26].

Comparative studies with "low risk" mammoplasty specimens and "high risk" mastectomy specimens revealed higher constitutive levels of the two biomarkers in the latter. These TDLU were also hyper-responsive to carcinogenic insult [12,17], as seen by a higher extent of Ras p21-GTP binding and increased 16α-OHE_1 formation. Altered metabolism of estradiol at the target tissue may be a critical event influencing cell proliferation, neoplastic transformation or both within the organ. A specific increase in estradiol 16α-hydroxylation in the target tissue well before the appearance of cancer may thus constitute a useful endocrine biomarker of cancer risk [21,23,24]. While the incidence of a cellular marker (preneoplastic hyperplasia) in mouse tissues corresponded with the perturbed expression of molecular and endocrine markers [15,20,26,30], a similar correlation in the human tissues remains to be demonstrated.

EXPERIMENTAL MODULATION OF PRENEOPLASTIC TRANSFORMATION

In vivo experiments with animal models have demonstrated that mammary tumorigenesis can be modulated by dietary intervention. The type,

TABLE II. Transformation-Associated Perturbation of Intermediate Biomarker in Mammary Explant Cultures[a]

Experimental System	Intermediate Biomarker			
	RAS p21-GTP	16α-OHE_1	2-OHE_1	Hyperplasia
Mouse				
C57/BL	+	+	++	–
BALB/c-DMSO[b]	2–3×↑	nt	nt	5–8×↑
BALB/c-NMU	2–3×↑	8–10×↑	decreased	8–10×↑
BALB/c-DMBA	2–5×↑	3–5×↑	nt	8–10×↑
Human				
TDLU-LR[c]	+	+	++	–
TDLU-LR/BP	2–4×↑	2–4×↑	nt	nt
TDLU-HR	2–4×↑	2–4×↑	++	nt
TDLU-HR/BP	5–8×↑	5–8×↑	decreased	nt
TDLU-HR/NMU	8–10×↑	nt	nt	nt

[a] Summarized from References #9,11,15,17,26,30,34.
[b] DMSO: dimethylsulfoxide; NMU: N-nitroso-N-methylurea; DMBA: 7,12-dimethylbenz-(a)anthracene; ↑: increased relative to the appropriate control; nt: not tested.
[c] TDLU-LR: terminal duct lobular units from non-cancerous mammoplasty; BP: benzo(a)pyrene; TDLU-HR: terminal duct lobular units from cancerous mastectomy; LR: low risk (absence of cancer); HR: high risk (presence of cancer).

amount, and total calorie content of dietary fat appear to be major determinants in altering tumor growth. In general, diets rich in polyunsaturated fatty acids of n-6 family (n-6 PUFA) promote the development of experimental mammary tumors, whereas those rich in n-3 family (n-3 PUFA), saturated fatty acids, and calorie restricted diets suppress tumor incidence and multiplicity. Similarly, hyperalimentation with retinoids and administration of antiestrogenic agents are also known to suppress tumor development [2–6,9]. These conventional animal models for experimental mammary carcinogenesis have thus provided important leads in understanding the mechanism(s) for dietary modulation of mammary tumors. In contrast, very little definitive information is available as regards the influence of dietary agents on early occurring initiational and promotional events leading to preneoplastic transformation. This information may play a critical role in studies evaluating chemopreventive efficacy of clinically relevant dietary interventions.

The evidence for dietary modulation of human breast cancer, however, is largely derived from epidemiologic studies [2,5]. Conventional western European and American diets are rich in n-6 PUFA, while the conventional oriental and Japanese diets are rich in n-3 PUFA. These dietary habits have been correlated with breast cancer incidence in the respective countries. Given the multistep progression of mammary tumors, it is now important to examine whether dietary agents identified as important in epidemiologic studies are also effective in inhibiting, retarding and/or suppressing early occurring initiational and promotional events of preneoplastic transformation.

In previous studies we have utilized the murine mammary explant culture system to

TABLE III. Experimental Modulation of Transformation-Associated Biomarkers in Mammary Explant Culture by Chemopreventive Agents[a]

Experimental System[b]	Chemopreventive Agent[c]	Intermediate Biomarker		
		RAS p21-GTP	16α-OHE$_1$	Hyperplasia
Mouse				
RIII	LNA	5–10×↑	nt	2–5×↑
	ARA	5–10×↑	nt	2–5×↑
	EPA	2–5×↓	nt	2–5×↓
	STA	nt	nt	2–5×↓
BALB/c-DMBA	LNA	2–5×↑	2–5×↑	2–3×↑
	ARA	2–5×↑	2–5×↑	2–3×↑
	EPA	2×↓	2×↓	2–3×↓
	DHA	2×↓	2×↓	2–3×↓
Human				
TDLU-HR	LNA	2–5×↑	4×↑	nt
	EPA	2–3×↓	2×↓	nt
	I3C	nt	2–5×↓	nt
TDLU/BP	LNA	2×↑	2–5×↑	nt
	EPA	4×↓	2×↓	nt

[a] Summarized from References #6,9,15,17,26,30,34.

[b] RIII: mammary explant cultures from RIII strain; BALB/c-DMBA: mammary explant cultures treated with DMBA from BALB/c strain; TDLU-HR: human mammary terminal duct lobular unit from cancerous breast; TDLU/BP: TDLU explant cultures treated with benzo(a)pyrene; ↑: increased relative to the appropriate control; ↓: decreased relative to the appropriate control; nt: not tested.

[c] LNA: linoleic acid (C18:2,n-6); ARA: arachidonic acid (C20:4,n-6); EPA: eicosapentaenoic acid (C20:5,n-3); I3C: indole-3-carbinol.

examine whether selected fatty acids or retinoids can suppress preneoplastic hyperplasia that is induced by murine mammary tumor virus and chemical carcinogen [6,9,15,26,34]. The results presented in Table 3 summarize our studies on modulation of intermediate biomarkers in mouse and human mammary explant cultures by prototypic modulators. All the fatty acids tested were capable of modulating constitutive as well as the induced levels of molecular, endocrine and cellular biomarkers. Independent of the type of initiator (e.g., oncogenic retrovirus or chemical carcinogen) selected n-6 PUFA perturbed the biomarkers. In contrast, selected n-3 PUFA, saturated fatty acids, retinoid and indole-3-carbinol downregulated all the biomarkers examined. These observations are consistent with the documented modulation of tumorigenesis in vivo that is achieved by hyperalimentation [3,5].

It was interesting to note that n-6 and n-3 PUFA were similarly responsive in human

mammary explant cultures. The constitutive levels of Ras p21-GTP binding and 16α-OHE_1 formation in TDLU-high risk (HR) (from mastectomy specimen of cancerous breast, and therefore at high risk) were enhanced by linoleic acid (LNA), and suppressed by eicosapentaenoic acid (EPA). A similar trend in the modulation was also observed in TDLU-HR treated in culture with the chemical carcinogen benzo(a)-pyrene (BP).

The mechanisms underlying the modulation of Ras p21 binding and of alteration in estradiol metabolism by fatty acids in our in vitro systems are unclear at present. With regards to the GTP binding activity, it is possible that the fatty acids may differentially regulate the GTPase activating protein (GAP). Consistent with such a possibility are recent reports that have demonstrated altered GAP activity by mitogenic lipids [17]. The alteration in estradiol biotransformation by PUFAs may reflect their indirect effects on hormone-mediated cell proliferation, since these agents are potent mitogens for the mammary tissue [6,9,30,34,35]. The mitogenicity of estradiol is associated with the elevation of the 16α-hydroxylation pathway, leading to the generation of 16$\alpha$$OHE_1$ and persistent binding of this intermediate metabolite to the nuclear estrogen receptor [17,21,23, 36,38].

CLINICAL RELEVANCE OF LABORATORY STUDIES

Human breast cancer is considered a sporadic disease since less than 25% of women express conventional familial risk factors. Thus, there is a pressing need for more sensitive and specific markers that can effectively identify women at elevated risk for developing the disease. Once identified, these women could then be directed toward aggressive primary prevention programs, including dietary intervention.

Attempts to correlate amplification/overexpression of Ras with human breast cancer progression have not been very successful [17,19,32,33,37]. However, carcinogen induction of point mutations in Ras in non-involved rodent mammary cells [17,31,40], together with the fundamental role of the oncogene in signal transduction, warrants a systematic analysis of Ras expression in high risk non-involved human tissue to validate Ras expression as a clinically relevant molecular marker for risk assessment.

Endocrine responsiveness of human cancer is fundamental to adjuvant therapy, and more than half of the conventional risk factors are either directly or indirectly related to alteration in endocrine milieu. Our recent in vitro experiments on estradiol metabolism in the target tissue [17,20,25,26,28,30] suggest that constitutive and/or induced alterations in metabolic pathways leading to formation of 16α-OHE_1 and/or 2-OHE_1 may also play an important role in cell transformation. Thus, in vitro models may provide a system to examine the relationship between altered endocrine responsiveness during initiation of tumorigenic transformation.

In the context of preventive intervention, effective modulation of molecular, endocrine and cellular biomarkers of preneoplastic transformation by dietary components [2,9,13,15,17, 22,27] provide a potential application of the present in vitro model in chemopreventive strategies for human breast cancer.

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