examined tumors. We also found several genes that showed decreased expression in carcinomas. Most of these (*e.g., Ca3*, *Cd36* and *Acsl1*) were adipocyte-associated genes and may merely reflect the adipocyte-rich composition of normal mammary tissue. Nevertheless, a previous study has shown that the non-coding RNA *Mg1*, which is encoded by the *RGD:727910* locus, is associated with hormone-induced protection against mammary cancer development in rats.[37] Our result is the first to show that this gene product is down-regulated in mammary carcinomas and supports the possibility that this non-coding RNA is a tumor suppressor.

In summary, our results indicate that spontaneous and radiogenic rat mammary cancers are distinguishable based on global and specific gene expression patterns, even though most gene expression changes were common to both cancers. The data indicate that spontaneous and radiogenic mammary cancer development involves distinct molecular and cellular mechanisms. When applied to human cancers, the distinction between radiogenic and spontaneous cancers will be helpful in assessing the risk of cancer from low doses of radiation.

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Radiation-Induced Mammary Carcinogenesis in Rodent Models: What's Different from Chemical Carcinogenesis?

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Mammary/Carcinogenesis/Animal model/Chemical/Rodent.

Ionizing radiation is one of a few well-characterized etiologic factors of human breast cancer. Laboratory rodents serve as useful experimental models for investigating dose responses and mechanisms of cancer development. Using these models, a lot of information has been accumulated about mammary gland cancer, which can be induced by both chemical carcinogens and radiation. In this review, we first list some experimental rodent models of breast cancer induction. We then focus on several topics that are important in understanding the mechanisms and risk modification of breast cancer development, and compare radiation and chemical carcinogenesis models. We will focus on the pathology and natural history of cancer development in these models, genetic changes observed in induced cancers, indirect effects of carcinogens, and finally risk modification by reproductive factors and age at exposure to the carcinogens. In addition, we summarize the knowledge available on mammary stem/progenitor cells as a potential target of carcinogens. Comparison of chemical and radiation carcinogenesis models on these topics indicates certain similarities, but it also indicates clear differences in several important aspects, such as genetic alterations of induced cancers and modification of susceptibility by age and reproductive factors. Identification of the target cell type and relevant translational research for human risk management may be among the important issues that are addressed by radiation carcinogenesis models.

INTRODUCTION

Ionizing radiation is one of the few well-characterized etiologic factors of human breast cancer. Epidemiologic studies on Japanese atomic bomb survivors and on clinically irradiated patients established that female breast is among the most susceptible organs to radiation-induced cancers.[1–4)]

Animal models of radiation carcinogenesis have many advantages in providing information about radiation-associated human cancer risk. First, they are the only measure for estimating radiation-associated cancer risk when epidemiologic evidence is lacking. For example, animal experiments are essential to estimate the carcinogenic effect of neutron radiation and nuclear fuel materials.[5,6)] Second, since randomized human studies are impossible, animal experiments provide complementary information where epidemiologic studies suffer from biases and confounding factors. For instance, in the studies on Japanese atomic bomb survivors, estimation of the modifying effect of age at exposure on radiation-associated cancer risk is complicated by the chronologic changes in the background cancer incidence in some cancers; thus, cancer incidence data of the childhood exposure population are compared to the most recent incidence data, while data of the adulthood exposure group are compared to old data.[4)] Third, mechanistic understandings are essential for interpretation of epidemiologic observations, and this is provided by animal models, which are usually well defined with respect to genetic and environmental conditions.

Rodent models play an important role in understanding the natural history, mechanism, and modifying factors of breast cancer development. As discussed elsewhere,[7)] since human breast cancer is heterogeneous at the morphological, genetic and molecular levels, any given animal model could not mimic the spectrum of human breast cancers, and animal models could mimic, at best, major subsets and pathways. In

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this respect, rodent models provide a variety of breast cancer types, as reviewed below, and can be used for numerous types of studies including assays of putative oncogenic and chemopreventative agents, pathogenesis due to specific treatment regimens or genetic alterations, etc. Two classes of rodent models have been used frequently: genetically modified mouse models and chemically induced carcinogenesis models. The radiation-induced model, nevertheless, is potentially very important, since radiation exposure is one of the very few epidemiologically proven etiologic factors of human breast cancer.[7–9] As reviewed here, animal models of radiation carcinogenesis sometimes gives us observations that do not hold in other models. We focus on the difference between radiation- and chemically induced mammary carcinogenesis, with an emphasis on rat models, and attempt to clarify the future role of animal models of radiation-induced mammary cancer.

MODELS OF RADIATION-INDUCED BREAST CANCER

Reported models of radiation-induced mammary carcinogenesis in rodents are summarized in Table 1. The rat is a widely used model to study the risk and mechanism of breast carcinogenesis because rat mammary cancers are comparable to human breast cancers in many respects, such as high frequency of hormone dependence and, pathologically, progression from ductal hyperplasia and ductal carcinoma in situ.[9–11] More than fifty years have passed since induction of mammary cancer by a single X-ray irradiation of the Sprague-Dawley rat was first reported.[12] Until recently, the Sprague-Dawley rat,[13–19] as well as several Wistar-related strains (Wistar Furth, WAG, WM, and Lewis)[20–23] have been used in most studies. Although the use of other strains such as the F344 strain has been documented,[24–26] comparative studies have revealed that the Sprague-Dawley strain is most susceptible to radiation-induced mammary carcinogenesis.[26–29] The ACI rat is a unique model in which mammary cancer is induced by estrogen treatment, which can synergize with cancer induction by ionizing radiation.[30]

The mouse provides an indispensable model system in which the effects of gene manipulation can be studied in vivo. Although mouse mammary tumors do have some dissimilarities from human breast cancers, such as the low frequency of hormone dependence and the progression of carcinoma predominantly from alveolar hyperplasia,[9–11] they provide a valuable route for genetic experimentation. BALB/c mice have been used frequently in radiation carcinogenesis studies.[31] The BALB/c strain is known to harbor a unique functional polymorphism of the *Prkdc* (DNA-dependent protein kinase catalytic subunit) gene.[32] Heterozygous mutant mice of the tumor suppressor gene *Tp53*, in the genetic background of BALB/c, develop mammary cancer at a low frequency and the incidence increases after radiation exposure.[33,34] The induction is further increased by hemizygosity for *Atm*.[35] Double heterozygous mice for *Brca1* and *Tp53* also develop mammary cancer after irradiation.[36] Mutant mouse strains for *Apc* (adenomatous polyposis coli) show increased incidence of mammary tumors after irradiation, though these tumors show different histopathologic characteristics from human breast carcinoma.[37,38]

Table 1. Rodent models of radiation-induced mammary carcinogenesis

Species	Strain/genotype	Type of radiation
Rat	Sprague-Dawley	Photon, neutron, heavy ions, ^{238}Pu
	Wistar (Wistar-Furth, WAG, WM, Lewis)	Photon, neutron, ^{238}Pu
	ACI	Photon, neutron
Mouse	BALB/c	Photon, neutron
	BALB/c *Tp53*$^{+/-}$	Photon
	BALB/c *Tp53*$^{+/-}$ *Atm*$^{+/-}$	Photon
	Brca1$^{+/-}$ *Tp53*$^{+/-}$ (strain unspecified)	Photon
	C57BL/6 *Apc*$^{Min/+}$, *Apc*$^{1638N/+}$	Photon

In addition to sparsely ionizing (low linear energy transfer [LET]) radiations such as photon (X- and γ-ray) radiation, induction of mammary cancers is observed after administration of densely ionizing (high LET) particle radiations including neutrons and heavy ions. Rat and mouse models have provided important information on the risk of neutrons[5,39–41] and heavy ion radiations including neon, iron, and carbon ions.[29,42,43] These studies have indicated the high relative biologic effectiveness of high-LET radiation for mammary cancer induction. Mammary cancer development following administration of plutonium has also been documented.[44]

To briefly summarize chemically induced mammary carcinogenesis models, BALB/c, FVB, and other strains of mice are frequently used for mammary cancer induction by two classes of carcinogens: polyaromatic hydrocarbons (methylcholanthrene, 1,2:5,6-dibenzanthracene, and 7,12-dimethylbenz(a)anthracene [DMBA]) and alkylating agents (1-methyl-1-nitrosourea [MNU], 1-ethyl-1-nitrosourea [ENU], and urethane).[8] Rat mammary cancers are usually induced by a single, high-dosage treatment with DMBA or MNU.[45] Also used recently is a protocol in which rats are treated repeatedly with heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a representative food-borne carcinogen.[46] Sprague-Dawley and F344 rats are among the most frequently used rat strains in these studies.

PATHOLOGY AND NATURAL HISTORY

Extensive descriptions of the pathology of radiation- and chemically induced rodent mammary tumors have been documented in several excellent reviews.[45,47,48] Very briefly, development of benign mammary tumors such as fibroadenoma is prominent after irradiation of rats, although significant development of adenocarcinoma (malignant tumor) is observed.[49] In comparison, chemical carcinogens tend to induce mainly adenocarcinoma.[45] To date, no pathologic differences between chemically induced and radiation-induced adenocarcinomas have been documented.[45]

Temporal histopathologic changes leading to the development of carcinoma in the chemical carcinogenesis model have been extensively studied.[9] The mammary gland consists of epithelial tissue of the mammary ducts embedded in fatty stromal tissue (Fig. 1A). In the course of normal organogenesis, the mammary gland rapidly grows during and after puberty, when mammary ducts elongate and bifurcate to fill the subcutaneous mammary fat pad (Fig. 1B). This is achieved by a controlled balance of cell proliferation and death within the terminal end bud (TEB), the club-shaped structure at the growing ductal terminus, which is present from prepubertal to postpubertal stages (Fig. 1C).[50] Differentiated structures such as terminal ducts and alveolar buds are formed along the duct (Fig. 1C) and, as the gland attains full development, TEBs also regress and differentiate into one of these structures.[51] Many lines of evidence suggest that the TEB is the major target structure of chemical carcinogens. After treatment of rats with chemical carcinogens, TEBs show pathological changes such as delayed regression, high proliferation index, and consequent development of hyperplastic and premalignant ductal lesions.[52–54]

Information regarding radiation-induced carcinogenesis is relatively limited. In mice and rats, pyknotic nuclear aberration and suppressed cell proliferation have been observed in TEBs at 6–24 hours after irradiation, which return to normal levels by 1.5–3 days.[55,56] Delayed regression of TEBs, with sustained cell proliferation, and development of ductal hyperplasia are seen 4–8 weeks after irradiation of rats (Fig. 2).[55] These changes are similar to those observed after stimulation with chemical carcinogens, as mentioned above. Hyperplastic alveolar nodules (HANs) are induced by X-irradiation in rats,[57] though they are not considered to be precursor lesions of rat mammary carcinoma in chemical induction models.[9] Thus, evidence suggests that the pathogenesis of radiation-induced mammary carcinogenesis may be largely similar to that of chemical carcinogenesis.

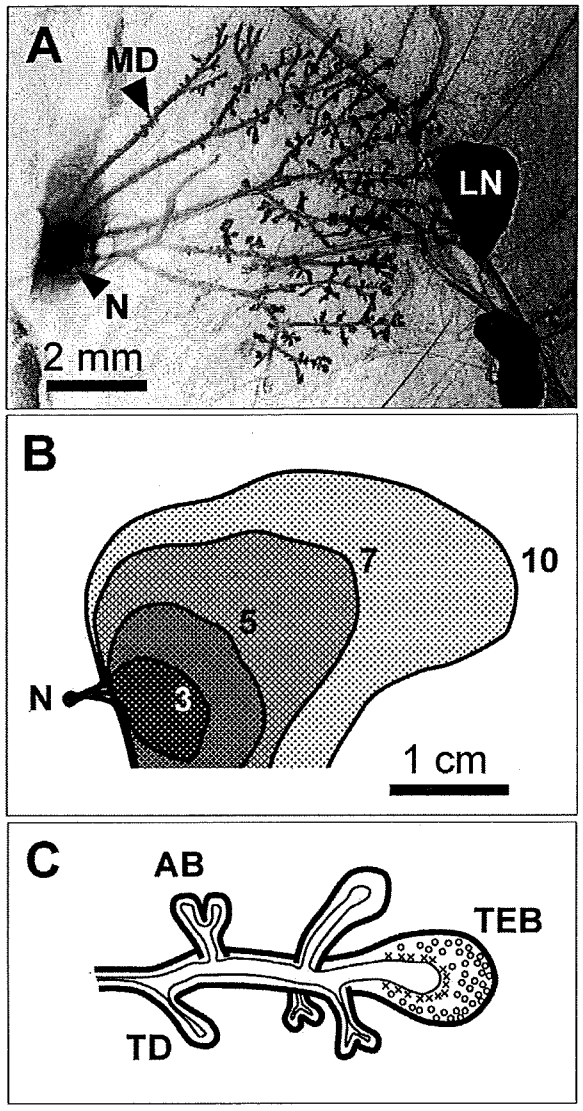

Fig. 1 Normal mammary gland development in the rat. (A) A photomicrograph of a whole-mount preparation of the rat mammary gland at 3 weeks of age. *LN*, lymph node; *MD*, mammary duct; *N*, nipple. (B) A schematic drawing depicting the area occupied by the mammary epithelial tissue during pre- and postpubertal development in the rat. Numbers indicate age in weeks. (C) A schematic illustration of structures of the mammary epithelial tissue around the postpubertal period. *AB*, alveolar bud; *TD*, terminal duct; *TEB*, terminal end bud. Regions of proliferating (*circles*) and apoptotic cells (*crosses*) in TEB are indicated.

GENETIC CHANGES IN CANCER

One characteristic property of ionizing radiation is that it produces DNA double strand breaks, in addition to other oxidative damage; as a consequence, deletions and discontinuous loss of heterozygosity (LOH) are a signature of the mutagenic action of radiation.[58] On the other hand, carcinogenic chemicals used to induce mammary cancer generally generate adducts to DNA and result in base substitutions and small deletions.[59]

Base substitution mutations (especially at codons 12 and 13) in the H-*ras* proto-oncogene are frequently seen in

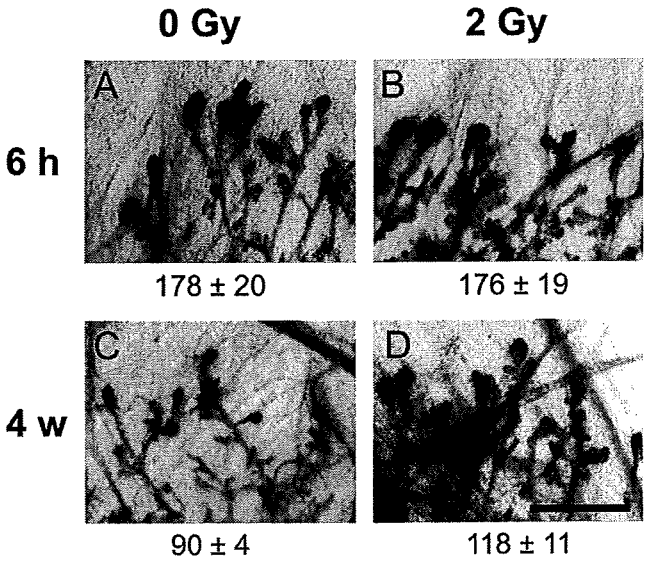

Fig. 2. Delayed regression of the terminal end bud (TEB) after irradiation. Rats were either untreated (*0 Gy*) or X-irradiated (*2 Gy*) at 7 weeks of age and whole-mount preparations of the mammary gland were prepared at 6 hours (*upper panels*) and 4 weeks (*lower panels*) after irradiation. *Bar*, 1 mm. Numbers below each panel indicate mean diameters of TEBs (μm; mean ± S.D., $n = 3$). Modified based on the data in Imaoka *et al.*[55]

MNU-induced rat mammary cancer[60] and to a lesser extent in PhIP-induced ones[61–63] but not in DMBA-induced cancers.[64] Rat mammary cancers induced by γ-rays and heavy ions harbor no mutation of H-*ras*,[29,65] as expected from their preferential induction of deletions and LOH. H-*ras* mutation is thus unlikely to be a causative event in radiation-induced rat mammary carcinogenesis.

LOH is one of the mechanisms through which tumor suppressor genes are inactivated in cancer cells. Searching for LOH regions in cancer, therefore, is generally a promising strategy to discover important tumor suppressor genes. Several LOH regions have been found in PhIP-induced rat mammary cancers,[66,67] whereas LOH is a rare event in DMBA-, MNU-, and radiation-induced rat mammary cancers.[20,68] The low frequency of LOH in radiation-induced mammary cancer is surprising considering the ability of ionizing radiation to induce LOH in other studies.[58,69,70] Comparative genomic hybridization is a powerful tool to detect amplification and deletion of chromosomal regions and has been used to study PhIP- and DMBA-induced rat mammary cancers.[71] This analysis has revealed that amplification of several chromosomal regions is characteristic to PhIP-induced cancer, whereas such copy number aberrations are absent in DMBA-induced cancers.[71] Studies on radiation-induced cancer are awaited.

Comprehensive gene expression profiling using microarrays is a warranted strategy to dissect important gene expression changes. Rat mammary cancers induced by ionizing radiation, PhIP, DMBA, MNU, and other chemical carcinogens, as well as spontaneously arising mammary cancers, have been analyzed in this manner.[72–77] Many of these studies have questioned if cancers of different etiological origins exhibit different gene expression patterns. The results indicate the existence of some differences in gene expression patterns; however, the expression of most genes seems to be similar between cancers of different etiological origins.

Epigenetic events such as alteration of DNA methylation at CpG islands have not been extensively studied in rodent mammary cancer models, and remain an open and promising area of research. Taken together, evidence so far suggests that the genetic alterations may be different between radiation and chemical carcinogenesis models, but the resulting alterations in gene expression are largely similar.

INDIRECT EFFECTS OF CARCINOGENS

Although direct genetic alteration is believed to be the principal role of ionizing radiation in carcinogenesis, evidence suggests the existence of some other effects. An in vitro study has suggested the effect of irradiated human mammary fibroblasts on co-cultured non-irradiated mammary epithelial cells to disrupt normal morphogenesis of epithelial ducts.[78] An in vivo study has revealed that irradiated mouse mammary stroma has the ability to transform a non-tumorigenic mammary cell line to a tumorigenic state upon transplantation of the cells into stroma.[79] Stroma-derived transforming growth factor β (TGFβ) may be involved in mediating such indirect effects of ionizing radiation. Evidence indicates that irradiation induces chronic activation of TGFβ in the fatty stroma of the mouse mammary gland,[80,81] and may cause remodeling of the stromal extracellular matrix.[81] Activated TGFβ may also translocate to the epithelial tissue[80,81] and mediate p53-dependent radiation response and epithelial-mesenchymal transition.[82,83] In chemical carcinogenesis, a similar indirect (stroma-mediated) effect of MNU has been documented in a rat experiment in which non-treated mammary epithelium gives rise to cancer after being transplanted into the mammary stromal fat of MNU-treated rats.[84] In contrast, the stroma is not a major target in DMBA-mediated tumorigenesis of mouse mammary preneoplastic cells.[85] These opposing lines of evidence may indicate differences in the mode of action of the carcinogens, differences between species (mouse vs. rat), or differences in experimental design. Because more information is currently available for radiation carcinogenesis models, future comparative studies using chemical induction models could offer important clues to unveiling a more complete picture of such indirect carcinogenic effects.

RISK MODIFICATION BY REPRODUCTIVE FACTORS

Human breast cancer risk is positively associated with obesity (high body mass index) as well as reproduction-related risk factors such as early age at menarche, late age at first full-term pregnancy, and late age at menopause, all of which are related to a prolonged period of endogenous estrogen exposure.[86] A study of atomic bomb survivors indicated that many of these factors, as well as estrogen use, modify the radiation-associated risk of breast cancer in women.[87] Estrogen-related modification of mammary cancer induction is thus an important issue in animal models.

Estrogen receptor (ER) expression of tumors is associated with the estrogen responsiveness of tumors. Whereas non-ovariectomized rats develop ER-positive mammary cancers after irradiation, prepubertally ovariectomized rats, irradiated at adulthood, develop ER-negative mammary cancer, albeit at a low incidence.[88,89] Similarly, most mammary carcinomas that develop in prepubertally MNU-treated rats are ER-positive, whereas ER-negative carcinoma develops at a low incidence when the rats are ovariectomized shortly after MNU treatment.[90,91] Most DMBA-, MNU-, and radiation-induced rat mammary cancers undergo regression after ovariectomy and are thus ovary dependent.[92–94] These results are consistent in that the estrogen/ER signaling plays pivotal roles in promotion/progression and maintenance of most radiation- and chemically induced rat mammary cancers.

Regarding the risk modification by parity, pregnancy reduces the incidence of chemically induced rat mammary cancer whether carcinogen is administered before, during, or after pregnancy.[95–97] This protective effect of parity may be due to pregnancy-associated changes in the systemic hormonal environment,[98,99] the mammary gland content of hormone-responsive cells,[95] and the initial responsiveness of the mammary cells to carcinogens.[100,101] In contrast, pregnancy after irradiation does not affect the incidence of rat mammary cancer.[102] The incidence of mammary cancer in rats irradiated during pregnancy, lactating, or post-lactating stages is no different from that in age-matched virgins.[103] When virgin, pregnant, and lactating rats are irradiated and then subjected to long-term estrogen treatment (*i.e.*, identical promotional environment), the lactating rat is by far the most susceptible, while the virgin is most resistant.[104] Further studies such as transplantation experiments may be needed to distinguish between these putative initiation- and hormone-related effects of parity on radiation carcinogenesis.

RISK MODIFICATION BY AGE AT EXPOSURE

One important issue in risk assessment of carcinogens is the modifying effect of age at time of exposure. A surprisingly high incidence of early-onset breast cancer is observed in atomic bomb survivors who were exposed in childhood.[105,106] Several studies have addressed this issue using both rat and $Apc^{Min/+}$ mouse models of radiation-induced mammary carcinogenesis.[21,27,38,94] Though the mechanism is not understood, the carcinogenic effect of prepubertal radiation exposure is small, the effects of postpubertal and adulthood exposures are larger, and the effect in old (> 60 weeks) animals is low (Fig. 3A).[21,27,38,94] This pattern is different from the narrow window of the susceptible period in chemically induced rat mammary carcinogenesis (Fig. 3B). Since the high susceptibility of postpubertal rats to DMBA does not exactly coincide with the number of TEBs or their proliferation index, this window of susceptibility is understood in association with the transitional differentiation state of TEBs into alveolar buds around this age.[107] In addition, the low level of DMBA-DNA adducts in prepubertal rats[108] may

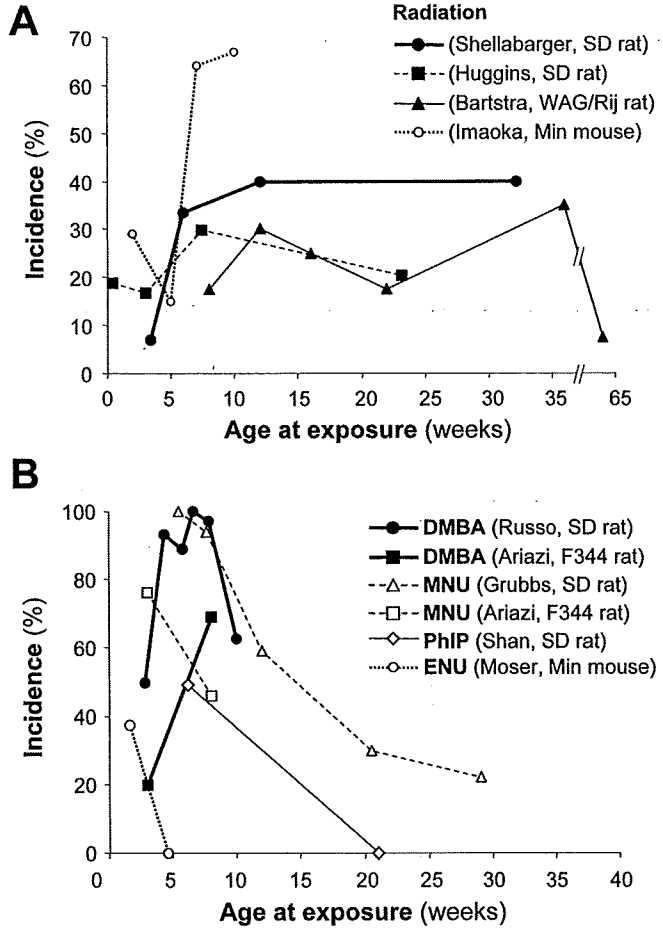

Fig. 3. Dependence on age at exposure of mammary tumor induction with ionizing radiation and chemical carcinogens in rodent models. (A) Radiation carcinogenesis data from literature.[21,27,38,94] (B) Chemical carcinogenesis data from literature.[107,109,110,112,113]

indicate weak metabolic activation of DMBA into its carcinogenic form in immature mammary gland. Administration of MNU to prepubertal rats is more effective in inducing mammary cancer than that to postpubertal rats, and the susceptibility decreases with age thereafter (Fig. 3B).[109,110] This age dependence of MNU is explained by deficiency of a DNA repair enzyme in immature rat mammary epithelial cells.[111] A similar age dependence is observed for ENU-induced mammary carcinogenesis of $Apc^{Min/+}$ mice (Fig. 3B).[112] The carcinogenic effect of PhIP is higher in pubertal rats than in mature animals (Fig. 3B).[113] This modifying effect of age does not correlate with either PhIP-DNA adduct levels or PhIP-induced mutation frequency, but is only explained by age-dependent gene expression changes induced by PhIP administration.[113] These studies suggest that, in mammary cancer induction, the modifying effect of age at exposure and its underlying mechanism are largely dependent on the carcinogen species.

MAMMARY TARGET CELLS OF CARCINOGENS

The mammary epithelium contains small fractions of stem cells (which are able to reconstitute the whole gland) and progenitor cells (with partial regenerative potency) plus a large population of differentiated cells.[114,115] The existence of mammary stem cells was first suggested by the complete regeneration of mammary gland after inoculation of isolated mammary epithelial cells into subcutaneous fat pads of syngeneic mice.[116] The regenerative activity of the rat mammary stem/progenitor cells is measured by inoculating dispersed mammary epithelial cells into the mammary fat pad of other rats and analyzing any resulting formation of colonies.[114] The unit of colony formation therein is termed a clonogen and is thought to represent individual stem and/or progenitor cells. In fact, these colonies contain cells that produce morphologically different (i.e., ductal and alveolar) colonies upon subsequent transplantation, indicating the stem cell–like property of clonogenic cells.[117] A significant number of clonogens survive after irradiation at a carcinogenic dose, and cancer initiation is calculated to occur frequently in surviving clonogens.[118–120] These observations suggest that mammary stem/progenitor cells that survive radiation exposure may initiate cancer development. Indeed, recent studies have identified a population of mouse mammary stem cells ($CD49f^{high}/CD24^{med}$ or $CD29^{high}/CD24^{+}$)[121,122] that are sensitive to a high dose (4 Gy) of radiation,[123] whereas a putative progenitor cell population ($CD29^{-}/CD24^{-}/Sca\text{-}1^{high}$) is radioresistant and enriched 24 hours after irradiation.[123]

The generative feature of the TEB, producing all mammary epithelial structures, strongly suggests the existence of stem cells in the TEB; however, the fact that transplantation of any portion of the gland parenchyma generates a complete gland also indicates their existence throughout the gland.[124] As mentioned above, mammary carcinogenesis induced by acute postpubertal stimulation is likely to originate mainly from TEBs. Because the TEB consists of proliferating, undifferentiated cells,[50] the target cell type of carcinogens may be stem or progenitor cells, which are abundant in this structure. However, since TEBs disappear once the postpubertal mammary gland development is complete, they do not seem to be important in spontaneous mammary cancer development in non-treated animals or in cancer induction by long-term or adulthood carcinogenic treatment. A careful observation of MNU-induced premalignant lesions that are disseminated throughout the gland[9] also implies that some cells outside TEBs are involved in cancer initiation.

Cancer development in humans may be a result of accumulation of mutations over a long period of time. This assumption strongly suggests that the tissue stem/progenitor cell, which has a long lifespan, is the cell type from which cancer arises. Detection of long-term label-retaining cells is one criterion to indicate the existence of long-lived cells in vivo. In the mouse mammary gland, radiolabeled thymidine, administered to virgin mice, is retained in a subset of cells for more than 6 weeks (even after pregnancy).[125–127] A double-labeling experiment has suggested that the radiolabeled DNA molecules are retained in the cells through preferential retention of the template DNA strands within the long-lived mother cells during chromosomal segregation.[125] Long-lived (9 weeks), label-retaining cells of the mouse mammary gland contain enriched $Sca\text{-}1^{+}$ cells, a putative progenitor cell population.[128] In experimental carcinogenesis, fractionation and protraction of irradiation is a unique method to induce mutations selectively in long-lived cells. Protracted irradiation over a period of 16–28 weeks is known to induce rat mammary cancer at a level comparable to that after a single irradiation at the same total dose.[129,130] This observation indicates that the long-lived cell population is an important target cell type in radiation carcinogenesis, even if one takes into account the repair of damage after each fractionated irradiation.

In the rat model of mammary cancer induction by a single postpubertal irradiation, a short-term estrogen treatment during a period around irradiation increases the incidence of mammary cancer in rats[24,28,131] and treatment with an ER antagonist reduces the incidence.[15] Incidence of radiation-induced rat mammary cancer is also decreased by temporary hormonal ablation by ovariectomy prior to irradiation, followed by chronic estrogen supplementation after irradiation.[88,89] This reduction is recovered by estrogen treatment immediately after ovariectomy, but not by progesterone or prolactin. These lines of evidence suggest the involvement of estrogen-responsive cells in cancer initiation. Interestingly, in rats treated chronically with estrogen, protraction of irradiation over a period greater than 8 weeks diminishes the radiation-associated mammary cancer risk that is otherwise evident at the same total dose.[130] This estrogen-dependent protraction effect is explained by damage repair

in estrogen-responsive cells or, more attractively, the high susceptibility of estrogen-responsive target cells with a lifespan less than 8 weeks. Indeed, steroid hormone receptor expression is absent in mouse mammary stem cells isolated by surface markers and in long-lived (9 weeks) label-retaining cells,[128,132,133] whereas ER-expressing cells are enriched in label-retaining (7 weeks) cells of estrogen-stimulated mice.[126]

Taken together, it is postulated that, upon acute high-dose irradiation, most mammary stem cells may be killed and progenitor cells with induced mutations may proliferate thereafter. These progenitor cells may have a long life (in nature or by taking the evacuated stem cell niche), accumulate further mutations, and finally give rise to cancer. During repeated low-dose exposures, both stem and progenitor cells may survive and accumulate mutations. Estrogen may increase the number of estrogen-responsive progenitor cells that can be targeted by carcinogens. This working hypothesis must be challenged by further investigation. Identification of the target cell type has an important meaning for risk assessment, in that an infinite lifespan of the target cell would permit accumulation of mutations over the life time of the individual, and necessitate long-term management of exposure history.

PERSPECTIVE AND CONCLUSION

Animal models of radiation carcinogenesis are valuable tools to study underlying mechanisms of relevant human carcinogenesis. Among recent progresses in breast cancer research is the finding that human breast cancers are subdivided into the following five subtypes based on characteristic gene expression: luminal A, luminal B, HER2 (human epidermal growth factor receptor 2)–positive, normal-like, and basal-like.[134] Human breast cancer of the basal-like subtype shows triple negativity for ER, progesterone receptor, and HER2, but is positive for expression of basal markers such as cytokeratin 5/6.[134] It is noteworthy that mammary gland stem cells of mice show basal-like phenotypes.[132] Also interesting is the observation that many of the human breast cancers that develop after radiation therapy are associated with basal-like phenotypes.[135] Recently established mouse models of basal-like breast cancer[136–138] and yet-to-be-established carcinogenic induction models of these subtypes would provide valuable information on the mechanism underlying development of carcinogen-induced breast cancer and the relevance of the stem cell population as a target of carcinogens.

The evidence reviewed herein indicates that radiation and chemical carcinogenesis models of mammary cancer share certain characteristics, although some differences do exist (Table 2). TEB may be the major origin of premalignant lesions that progress into cancer in both radiation and chemical models, at least after postpubertal acute carcinogenic stimulation. The cancers in these models exhibit mostly similar gene expression profiles, albeit with some differences,

Table 2. Comparison of radiation and chemical models of mammary carcinogenesis*

Feature	Radiation	Chemical
Epidemiology	Abundant	Scarce
Pathology	Adenocarcinoma, fibroadenoma	Mainly adenocarcinoma
Genetic changes		
H-*ras* mutation	No	Yes (MNU, PhIP); no (DMBA)
LOH	Rare	Frequent (PhIP); rare (DMBA, MNU)
Transcriptome	Changed	Changed
Indirect effect	Possible	Possible
Risk modification		
Estrogen	Increases risk	Increases risk
Parity	Not protective	Protective
Susceptible age	Postpubertal	Postpubertal (DMBA); prepubertal (MNU); pubertal (PhIP)
Target structure	TEB, non-TEB	TEB (DMBA, MNU), non-TEB (MNU)

* Abbreviations. DMBA, 7,12-dimethylbenz(a)anthracene; LOH, loss of heterozygosity; MNU, 1-methyl-1-nitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; TEB, terminal end bud.

but they may have distinct genetic alterations, as observed in the characteristic occurrence of H-*ras* mutations in MNU-induced cancer and LOH regions in PhIP-induced cancer. The existence of indirect effects of carcinogens is suggested for both ionizing radiation and MNU models, though the identity of these indirect effects is uncertain. Estrogens may play a crucial role in the development of cancer in both radiation and chemical models, but the protective effect of parity seems weaker for radiation. Specifically, ionizing radiation may have a strong impact on cancer initiation in a lactating gland. Though only partial evidence is available, ionizing radiation and some chemical carcinogens such as MNU may target stem/progenitor cells located at the TEB or throughout the gland. We perceive that the animal model of radiation carcinogenesis will continue to play a crucial role in bridging results of in vivo animal experiments and observations from human studies and translate into a better understanding of mammary carcinogenesis.

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Combined Effect of Ionizing Radiation and *N*-Ethyl-*N*-Nitrosourea on Mutation Induction and Lymphoma Development

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Summary. Carcinogenesis in humans is thought to result from exposure to numerous environmental factors. Little is known, however, about how these different factors work in combination to cause cancer. Mouse thymic lymphoma is a good model for research on radiation and chemical carcinogenesis. We examined here the occurrence of thymic lymphoma and mutation induction following exposure to both X-rays and *N*-ethyl-*N*-nitrosourea (ENU) in B6C3F1 mice. Mice were exposed weekly to whole-body X-irradiation (0.2 or 1.0 Gy per each exposure) for 4 consecutive weeks, ENU (200 ppm) in the drinking water for 4 weeks, or X-irradiation followed by ENU treatment. The incidence of lymphoma after 0.2 and 1.0 Gy were 0% and 10%, respectively. ENU treatment induced lymphoma in 20% of exposed mice. When ENU was combined with 1.0 Gy, lymphoma incidence increased up to 94%, showing a synergistic effect. In contrast, combination of ENU with 0.2 Gy resulted in a decrease in lymphoma incidence, that is, an antagonistic effect. Mutant frequency of the reporter transgene *gpt* after ENU exposure alone increased by tenfold compared to untreated controls. Combined exposure of ENU with 0.2 Gy X-rays dramatically decreased mutant frequency. In contrast, 1.0 Gy X-rays combined with ENU further enhanced mutant frequency and accelerated clonal expansion of mutated cells. In conclusion, the mutagenic and carcinogenic effect of combined exposure of X-rays with ENU is dose dependent.

Key words Thymic lymphoma · Combined genotoxic effect · *N*-Ethyl-*N*-nitrosourea · Radiation · Clonal expansion

Introduction

Human beings are exposed to numerous natural and man-made agents that are potentially carcinogenic. Therefore, cancer risk by ionizing radiation should be assessed as a result of combined exposures with other agents, including tobacco,

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genotoxic and nongenotoxic chemicals, hormones, viruses, and metals. Alkylating agents are found in plants, food, cigarette smoke, fuel combustion products, and commonly used industrial solvents. Some alkylating agents are also used for cancer therapy in combination with ionizing radiation. However, available data on the combined effect are relatively few, especially on its mechanism. In the present study, we report the dose dependence of the mode of combined effect of radiation with *N*-ethyl-*N*-nitrosourea on mutation induction and lymphoma development.

Materials and Methods

Female B6C3F1 mice were exposed to whole-body X-irradiation at a weekly dose of 0.2 or 1.0 Gy for 4 consecutive weeks or to *N*-ethyl-*N*-nitrosourea (ENU) at 200 ppm in drinking water. The mice were also exposed to X-rays followed by ENU (Fig. 1). X-ray irradiation was performed at a dose rate of 0.7 Gy/min. The mice, which had symptoms of thymic lymphoma 2 to 4 months after exposure, were killed under anesthesia and autopsied. The B6C3F1 *gpt*-delta mice were similarly treated with both X-rays and ENU. After the 4th week of exposure, the thymus was analyzed for the frequency and spectrum of *gpt* mutation as described previously [1]. Recurrent mutations derived from the tissue of a single animal could be the result of clonal expansion that occurred early after mutagen treatment.

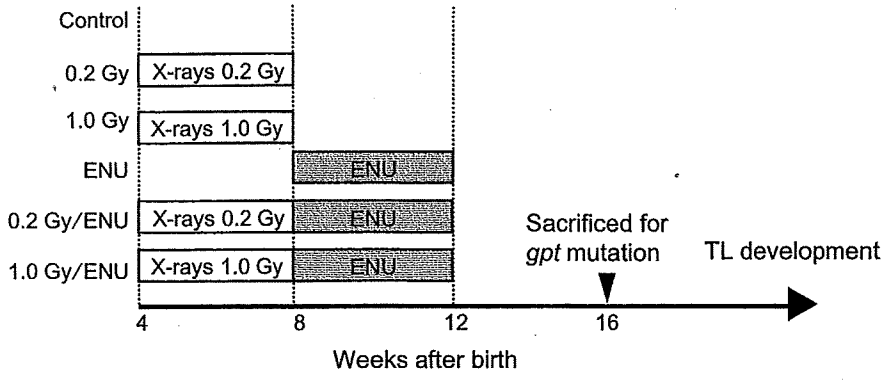

Fig. 1. Experimental design for thymic lymphomagenesis (TL) and *gpt* mutation analysis in mice treated with X-ray irradiation, *N*-ethyl-*N*-nitrosourea (ENU), or a combination of the two. Mice were exposed to X-rays weekly. ENU was administered at a concentration of 200 ppm in drinking water

Results

Combined Effect of X-Rays and ENU on Thymic Lymphomagenesis

Repeated exposure to X-rays at 1.0 Gy per exposure (4.0 Gy in total) increased the incidence of thymic lymphomas to 10%, whereas X-rays at 0.2 Gy did not induce lymphomas at all. ENU at 200 ppm induced lymphomas at an incidence of 20%. Combined exposure of ENU with 1.0 Gy X-rays resulted in a dramatic increase in lymphoma incidence at 94%, indicating a synergy. When ENU was combined with low-dose X-rays (0.2 Gy), the incidence was significantly reduced compared to that of ENU treatment alone, suggesting a protective role of low-dose X-rays for ENU-induced lymphomagenesis.

Induction of Gpt Mutation after Combined Exposure

DNA mutations play a central role in carcinogenesis. The frequency and type of mutations that result from combined treatment may shed light on the molecular mechanism(s) underlying the carcinogenic effects of combined exposure to ENU and radiation. To delineate such mechanisms, we have examined the occurrence of mutations in thymic cells of B6C3F1 (*gpt*+/–) mice after combined exposure [2]. It was found that ENU increased mutant frequency by ten-fold relative to untreated controls (Fig. 2). The mutant frequency in mice exposed to 0.2 Gy or 1.0 Gy X-rays alone was, surprisingly, reduced compared to the control ($P < 0.05$). Exposure to high-dose X-rays (1.0 Gy) followed by ENU increased mutant frequency by three-fold relative to ENU alone and facilitated clonal expansion of mutated cells. When low-dose X-ray (0.2 Gy) was combined with ENU, mutant frequency was, unexpectedly, reduced, which was primarily the result of a decrease in G:C to A:T and

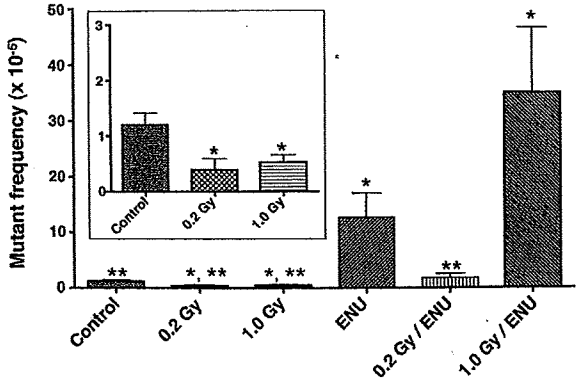

Fig. 2. Mutant frequency analysis of *gpt* recovered from thymus DNA from control, irradiated (0.2 Gy or 1.0 Gy), ENU-treated, and irradiated/ENU-treated mice. The *inset* shows an expanded scale for mutant frequency for the first three conditions. $*P < 0.05$, significantly different from control; $**P < 0.05$, significantly different from ENU. *Bars* represent mean ± SD

A:T to T:A mutations. In addition, clonality was drastically reduced compared with ENU alone (24.6% vs. 82.2%). The mode and mechanism of combined exposure clearly differs between low and high doses of radiation.

Discussion

We report here the dose dependency of the mode of thymic lymphomagenesis and mutagenesis after combined exposure to X-rays and ENU. It was shown that low-dose X-rays suppressed lymphoma induction by ENU whereas high-dose X-rays enhanced induction. In accord with this, low-dose X-rays reduced ENU-induced mutant frequency and clonal expansion of mutated cells and high-dose X-rays promoted both.

It is reported that preirradiation of X-rays decreases the incidence of brain tumors in rats exposed in utero [3]. This protective effect of preirradiation appears to correspond to the inductive effect of ionizing irradiation on O^6-alkylguanine alkyltransferase (ATase), which protects cells from G to A mutation. Induction of ATase by irradiation has been frequently observed in several tissues both in vitro and in vivo [4,5]. Our results, showing that 0.2 Gy X-rays combined with ENU decreased the G to A transition, suggest increased activity of ATase by radiation.

On the other hand, high-dose radiation can kill the target cells, thereby providing an environment for the surviving cells to expand. Irradiation of thymic epithelial cells enhances interleukin (IL)-7 production, and thymocytes at the preleukemic stage proliferate more vigorously in response to IL-7 [6,7]. We previously found frequent mutations of *Ikaros* in X-ray- and ENU-induced thymic lymphomas [8–10]. T cells with reduced or dominant-negative Ikaros activity, which may result from either a lack of or a point mutation in the zinc finger responsible for DNA binding, exhibit a greater proliferative response to IL-2 [11]. Taken together, these results suggest that high-dose radiation provides a thymic microenvironment ripe for the occurrence of prelymphoma cells, which harbor growth-advantageous mutations following ENU treatment.

In conclusion, low-dose (0.2 Gy) X-rays reduce not only the frequency of spontaneously occurring but also ENU-induced mutations, suggestive of an adaptive response. Low-dose X-rays also reduce the clonal expansion of cells following ENU treatment, whereas 1.0 Gy X-rays accelerate cell expansion. Thus, low- and high-dose radiation plays different roles in lymphomagenesis when combined with ENU exposure.

Conclusion

Combined exposure of carcinogens is a characteristic of ordinary human life. The dose of radiation is a critical factor to determine the mode of combined effect of radiation and ENU.

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