ABSTRACT

The cells of many solid tumors have been found to contain supernumerary centrosomes, a condition known as centrosome amplification. Centrosome amplification, accompanied by the overexpression of an associated kinase, Aurora A (AurA), has been implicated in mechanisms leading to mitotic spindle aberrations, aneuploidy, and genomic instability. Using a well-established rat mammary model favorable for experimental carcinogenesis, we analyzed centrosome amplification as a cellular marker for early stages of transformation and its regulation by the kinase ratAurA. Parity or treatment with estrogen and progesterone conferred resistance to tumorigenesis, as well as to overexpression of ratAurA and to centrosome amplification. ratAurA, cloned from a rat mammary gland cDNA library, is a bona fide Ser/Thr kinase, and sequence comparison demonstrated high homology to members of the entire AurA kinase family. Using immunocytochemical localization with confocal microscopy, we found ratAurA to be localized at the centrosome in normal and neoplastic tissues of the rat mammary gland. Normal ductal epithelium and stromal cells displayed an expected complement of one to two centrosomes/cell, whereas comparable cells in methylxanthine-treated animals displayed significantly elevated centrosome numbers. In tumors, 46% of cells showed more than two centrosomes/cell, and ratAurA expression levels coincided with higher centrosome numbers. Both centrosome numbers and ratAurA expression were permanently elevated. Centrosome amplification was found to occur at a very early, premalignant stage prior to detectable lesions after treatment with methylxanthine, a condition that was not detected in mammary glands of rats made refractory to the carcinogen via pregnancy or estrogen and progesterone treatment. Our results indicate that hormones influence kinase expression, and progesterone had the major effect on ratAurA expression levels. Cumulatively, these results suggest that ratAurA overexpression and centrosome amplification were linked to tumor development and progression and may serve as early markers in tumorigenesis.

INTRODUCTION

One consistent result in the epidemiology of human breast cancer is the inverse relationship of risk and early full-term pregnancy. Observations that the same reproductive endocrine events control mammary development and influence breast cancer risk support the idea that mammary gland development and mammary gland carcinogenesis are fundamentally related. Parity results in persistent changes in the mammary gland that confer resistance to tumorigenesis (1). One hypothesis proposed to explain the parity effect is that hormonal stimulation by estrogen and progesterone induce a differential switch in specific stem cell populations that result in progeny with persistent changes in intracellular pathways governing proliferation and response to carcinogens (1–7). Resistance has also been suggested to be attributed to a greater degree of differentiation and reduced cell proliferation of the mammary epithelium (8, 9), combined with a modulation of estrogen and progesterone receptor level expression (10). In addition, resistance has been suggested to be caused by persistent changes in systemic factors, such as reduction in the circulating levels of prolactin (11, 12), growth hormones (6), or concomitant changes in receptor levels, such as estrogen receptor, progesterone receptor, or epidermal growth factor receptor (6), leading to low levels of proliferation and subsequently a weak carcinogenic response. Hormonal changes predict persistent differences in signal transduction pathways, cell cycle regulators, transcription factors, and other yet unknown regulatory molecules between the parous involuted gland and the mature gland (13). Molecular changes are manifested in cellular responses that differentiate these two states. Some known responses are a block in carcinogen-induced proliferation and a block in proliferation of estrogen receptor-positive cells (13). We hypothesize that one of the cellular responses involves a block in carcinogen-induced centrosome amplification.

Centrosomes are tiny cellular organelles that nucleate microtubule growth and organize the mitotic spindle for segregating chromosomes into daughter cells. As microtubule-organizing centers, centrosomes also play an important role in many microtubule-mediated processes, such as establishing cell shape and cell polarity, processes essential for epithelial gland organization (14, 15). Centrosomes also coordinate numerous intracellular activities, in part by providing a site enriched for regulatory molecules, including those that control cell cycle progression, centrosome and spindle function, and cell cycle checkpoints (16–19).

Centrosome defects have been suggested to play a role during neoplastic transformation and tumor progression by increasing the incidence of multipolar mitoses and related spindle abnormalities that lead to chromosomal segregation abnormalities and aneuploidy (20–23). Defects in centrosome number, organization, and behavior constitute a condition defined as centrosome amplification (22, 24) characteristic of some breast cancers and solid tumors in general (25–30). Although the underlying mechanisms for the formation of abnormal centrosomes are not clear, several possibilities have been proposed and implicated in the development of cancer such as alterations of checkpoint controls initiating multiple rounds of centrosome replication within a single cell cycle and failure of cytokinesis, cell fusion, and cell cycle arrest in S-phase uncoupling DNA replication from centrosome duplication (reviewed in Ref. 25).

Although a direct link between defects in the mitotic apparatus and aneuploidy in cancer has yet to be fully elucidated, there is a growing list of molecular components and regulatory processes, making the mitotic machinery a prime target for tumorigenesis (27). One of the candidates is the BTAK,3 a member of the Aurora/Ipl-like family (31), discovered recently in human cells (32) and renamed AurA (33). AurA is a centrosome-associated regulatory molecule and oncogene, the overexpression of which leads to centrosome amplification, transformation, and aneuploidy (28, 32). Much has been reported on AurA in cultured cells, but relatively few studies have focused on the mechanisms of centrosome amplification in vivo.

We analyzed the kinase AurA and centrosome amplification during...
tumorigenesis in a well-characterized rat mammary tumor model involving mammary glands from virgin and parous animals exposed to MNU (1). This rat model for mammary carcinogenesis provides an excellent biological model for human breast cancer and facilitates studies of molecular events leading to mammary tumorigenesis (2).

Our studies indicate that the intrinsic differences between the parous and virgin gland influence the expression of the kinase AurA, thereby protecting mammary epithelial cells from centrosome amplification and associated events leading to tumor formation and progression. On the basis of these observations, we propose that AurA expression and centrosome amplification play a key role during tumor development and progression, and both could provide a powerful tool for the assessment of prognostic factors in breast cancer.

MATERIALS AND METHODS

Animals. Female WF rats, 35 days of age, were purchased from Harlan (Indianapolis, IN). The WF rat is an inbred strain of rats sensitive to both MNU- and 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis (34). The animals were maintained in a clean facility in the Center for Comparative Medicine at Baylor College of Medicine with unrestricted access to food and water under conditions of a 12/12-h light-dark cycle.

Tumorigenesis Studies. MNU was prepared as described by Sivaraman et al. (1). Studies on pregnancy-induced resistance to the carcinogen MNU as well as the design for the experimental regimen to mimic pregnancy were performed as described previously (1). The experimental regimen for hormone treatment and carcinogenesis studies are summarized in Fig. 1.

Northern Analyses. The abdominal mammary glands (gland #4) were dissected using standard surgical procedures and frozen in liquid nitrogen for RNA extraction. Total RNA and poly(A+) RNA were purified using Triazol (Life Technologies, Inc., Bethesda, MD) and a mRNA purification kit (Amersham Pharmacia, Piscataway, NJ) according to the manufacturers’ recommendations. For Northern analysis, 5 μg of poly(A+) RNA was resolved in formaldehyde-agarose gels, transferred to Hybond N nylon membrane (Amersham, Piscataway, NJ), and probed with radioactively labeled cDNAs. An S12 ribosomal protein cDNA was used as a probe to normalize for mRNA loading. The blot was exposed to a PhosphorImager, and the results were quantitated using the software Image-Quant (Molecular Dynamics). The data are plotted in arbitrary relative volume, units/μg RNA normalized for S12 expression.

RESULTS

Centrosome Amplification Correlates with Tumorigenesis in Rat Mammary Glands. To determine a possible correlation of centrosome amplification and tumor development, a well-defined model of mammary carcinogenesis in rats was used (1). Mammary carcinomas were induced by the chemical carcinogen MNU (see Fig. 1), and rats developed tumors 3–6 months after treatment. To induce refractoriness to breast cancer, parous animals and animals treated with estrogen plus progesterone were treated with carcinogen after a period of involution for 28 days (see Fig. 1). Earlier studies have demonstrated that hormones effectively induce a refractory state to carcinogen-induced tumorigenesis in rats (1, 2, 5, 6).

To quantify the number of centrosomes in mammary epithelial and stromal cells of normal and tumor-bearing rats, frozen tissue sections were immunostained for centrosomes using a human antiserum to γ-tubulin (a gift from J. B. Rattner, The University of Calgary, Calgary, Alberta, Canada), and nuclei were stained with propidium iodide. Confocal microscopy was essential for the accurate detection and assessment of the number of centrosomes/nucleus within multiple layers of mammary tissue. As shown in Fig. 2, A and B, in stained sections of normal tissue 104 days of age, one or two centrosomes were seen adjacent to the nuclei, whereas multiple centrosomes, often in clusters or linear arrays, were detected in sections of tumors (Fig. 2, C and D). In our preparations, individual centrosomes were rea-
sonably uniform in size and distribution around nuclei, unlike those reported in human mammary tumors (24, 30). Centrosome profiles varied in mammary gland cells from rats that had been treated with MNU at 97 and 104 days and sacrificed months later (Fig. 2D). In groups A–D, abnormal centrosome numbers were present in 9–11% of cells (Fig. 2D); groups E–H represent tumor samples with abnormal centrosome numbers present in 28–46% of cells (Fig. 2D). Histological sections indicated that samples A–D displayed a normal glandular morphology, sample E with 28% abnormal centrosomes displayed atypical ductal hyperplasia, samples F and G with 43 and 46% abnormal centrosomes displayed adenocarcinoma, and sample H with 45% abnormal centrosomes was ductal carcinoma in situ. Thus, the extent of abnormal centrosome numbers correlated with the neoplastic state and the carcinogenic status of the mammary tissues (two tailed t test, P < 0.13). The nonproliferating stromal cells in both normal glands and tumors displayed mostly single centrosomes/cell as expected.

To determine when during tumorigenesis centrosome abnormalities, including centrosome amplification, begin to occur, glands of animals treated with MNU as early as 40 days after treatment were examined (Fig. 3, A and B; Table 1). Centrosome counts had shown a normal profile in the majority of the cells (Fig. 2, A and B). However, tissue from two animals showed as much as 27% increase in cells with greater than two centrosomes/cell, indicating that small foci of cells with centrosome amplification may exist within epithelial cells, even at this early stage (Fig. 3, A and B). Such alterations were not detected in non-carcinogen-treated animals (Fig. 2, A and B).

As mentioned previously, parity confers resistance to chemical carcinogen-induced mammary tumors in WF rats. Parous rats, or rats that received estrogen/progesterone treatment prior to carcinogen, exhibited tumor incidences of only 10% (1). To determine whether pregnancy or estrogen/progesterone treatment prior to carcinogen also prevented centrosome amplification, counts were made in glands of animals that were either parous or treated with estrogen and progesterone prior to carcinogen challenge. As shown in Fig. 3, C–E, in both groups the majority of cells contained one or two centrosomes/cell and showed only 6–11% elevation of centrosomes compared with 5% in AMVs (Table 1).

These experiments confirmed not only that centrosome amplification in vivo generally corresponded to tumor development, but amplification, as shown in Fig. 2D, might also be a useful indicator of hyperplasia in mammary gland and an early marker for progression to malignancy. Hormone treatment or pregnancy, known to be protective against mammary tumors, also prevented centrosome amplification (Table 1).

**The Kinase ratAurA Is a Homologue to AurA/BTAK.** The first evidence that a Ser/Thr kinase might be responsible for centrosome amplification came from studies by Zhou et al. (28). Overexpression of the kinase AurA/BTAK could induce centrosome amplification and aneuploidy in transiently transfected NIH 3T3 cells and in human breast carcinoma MCF-10 cells (28). To evaluate the kinase disposition and role in rat mammary tumorigenesis, the homologue to AurA/BTAK was cloned. A composite sequence from the cDNA clone is presented in Fig. 4. The predicted ratAurA polypeptide contains 397 amino acid residues with a calculated molecular mass of 44 kDa. The sequence homology to known members of the AurA family is high;
the identity of ratAurA to AurA/BTAK is 85%, and the identity to the mouse orthologue, IAK-1, is 96%. The consensus motifs, i.e., R-R-T-L and R-R-I-S, and residues, i.e., R-D-I-K-P-E-N, and G-T-L-D-Y-Q-P-, shown in Fig. 4, strongly suggest that ratAurA is a bona fide serine/threonine kinase (37) containing two sequences that match the protein kinase A consensus phosphorylation site (Fig. 4).

**ratAurA Expression in Rat Mammary Gland Correlates with Tumor Development.** To examine ratAurA gene expression in the various treatment regimens (see Fig. 1), Northern analyses of several RNAs were carried out as presented in Fig. 5. A 440-bp fragment of ratAurA, comprising part of the kinase region (n625–n1066), was used as a probe. Comparing the mRNA expression level of different tissues indicated that, in rat mammary tumors and in tissue of MNU-treated rats (40 days after treatment), the ratAurA mRNA levels were significantly elevated compared with control (AMVs). Estrogen/progesterone treatment prior to MNU protected the rats from higher ratAurA expression levels. In tissue from pregnant animals, kinase levels were almost undetectable. Collectively, the expression levels in the various regimens correlated positively with the centrosome profiles as shown above (Figs. 2 and 3; two-sided t test, P < 0.023). Carcinogen treatment resulted in higher ratAurA mRNA expression levels, which corresponded to centrosome amplification and tumor progression (Fig. 2, C and D). Centrosome amplification correlated to mRNA expression levels of ratAurA, suggesting a role of ratAurA during tumorigenesis (Figs. 2, 3, and 5).

**ratAurA Expression Levels Are Influenced by Hormones.** The refractory effect to carcinogenesis brought about by hormone treatment strongly implied a specific influence on ratAurA gene expression (Fig. 5). As shown in Fig. 5, tissue from animals treated with estrogen and progesterone prior to MNU displayed lower ratAurA mRNA expression levels compared with glands from MNU-treated animals and mammary tumors. How such long-lasting intrinsic differences influence ratAurA mRNA levels remains to be elucidated.

To determine whether hormones influence ratAurA mRNA levels directly, the ratAurA mRNA expression levels in tissue from pregnant animals were measured. ratAurA mRNA expression levels were observed to change at different stages of pregnancy. Early in pregnancy (6 days), ratAurA mRNA levels were elevated (comparable with the levels measured after 6 days of estrogen/progesterone treatment shown in Fig. 6), but in late pregnancy (18 days) and during lactation (10 days), the levels decreased markedly and were comparable with basal ratAurA levels observed in AMVs (comparable with the levels shown in Fig. 6). It is known that the levels of cell proliferation in the mammary gland change during pregnancy and are influenced by the hormones progesterone, estrogen, and prolactin. During early pregnancy, proliferation levels are the highest but decrease at the end of pregnancy, so the levels were almost undetectable. Collectively, the expression levels in the various regimens correlated positively with the centrosome profiles as shown above (Figs. 2 and 3; two-sided t test, P < 0.023). Carcinogen treatment resulted in higher ratAurA mRNA expression levels, which corresponded to centrosome amplification and tumor progression (Fig. 2, C and D). Centrosome amplification correlated to mRNA expression levels of ratAurA, suggesting a role of ratAurA during tumorigenesis (Figs. 2, 3, and 5).
pregnancy and at the beginning of lactation. Thus, the two events, tissue proliferation and levels of ratAurA kinase expression, appear to be linked, as might be anticipated if the kinase is required for centrosome function during the cell cycle. To test which hormone(s) might be linked, as might be anticipated if the kinase is required for centrosome function, animals were treated

As shown in Fig. 6, a second transcript of 8 kb in size was detected with the ratAurA probe. Under the hormonal influence of estrogen, progesterone, and prolactin, the expression levels of the 8-kb transcript were altered, and estrogen had the major influence on the levels of this transcript. Genomic library screens and Northern analyses performed using sense and antisense probes of ratAurA (data not shown) indicate that the second transcript represents a processed pseudogene, which has also been described for AurA/aurora2 (32).

ratAurA Localizes to Centrosomes and in Addition to a Cytosolic Pool. To determine the subcellular localization and function of ratAurA in proliferating cells, a polyclonal antibody was raised against the NH2-terminal peptide of ratAurA. Exponentially growing mouse mammary epithelial cells were fixed and probed with affinity-purified anti-ratAurA antibody and double stained with an antibody for γ-tubulin for centrosomes and 4',6-diamidino-2-phenylindole to detect nuclei. As shown in Fig. 7, ratAurA was first detected in late G1-S phase in the proximity of centrosomes at the time of duplication of centrosomes, as shown in Fig. 7. ratAurA remained associated with the centrosomes during their migration around the nucleus in G2 and mitosis. It should be noted that the intensity of the ratAurA signal at the centrosome was significantly stronger during mitosis than in interphase, suggesting an increase in ratAurA mRNA translation coupled with recruitment to the centrosome.

In tissue sections of the rat mammary gland and tumors, ratAurA can be detected at centrosomes as well as shown in Fig. 8, in
alveolar buds of normal rat mammary glands, ratAurA localizes to centrosomes. In addition, the anti-ratAurA antibody detected a cytoplasmic pool, shown as a bright staining pattern in tissue of animals treated with progesterone for 6 days (Fig. 8B). In tumors, where the typical three-dimensional structures of ducts and lobuloalveoli are usually lost and replaced by sheet-like arrangements of nonpolarized cells, the staining pattern of ratAurA appears on the centrosomes as well (Fig. 8C). The cytoplasmic staining of ratAurA seemed to accumulate in certain foci (Fig. 8D).

**DISCUSSION**

Centrosome abnormalities are associated with chromosomal instability and defects in cell polarity in tumor cells. For example, human adenocarcinoma cells display a number of abnormalities, including an increase in centrosome number and volume, accumulation of excess pericentriolar matrix, extra centrioles, and aberrant phosphorylation of centrosome-associated proteins, that are believed to contribute to genetic instability and cancer (19, 30).

The data presented here implicate centrosomes in neoplastic outgrowth and tumor progression in breast cancer. A well-established carcinogen-induced rat mammary tumor model was applied, and results indicate that centrosome amplification occurred early during tumorigenesis, favoring growth advantage of cells and subsequent neoplastic outgrowth and tumor. Tumors were induced by treating animals with the chemical carcinogen MNU. Tumors appeared several months after treatment, and both early and later stages of tumor development correlated with the presence of centrosome amplification. As early as 40 days after treatment with MNU, foci were detected that showed elevated centrosome numbers, although the mammary glands showed no histological evidence for preneoplastic growth. Most cases of atypical ductal hyperplasia, ductal carcinoma in situ, or adenocarcinoma showed an increase of up to 46% of epithelial cells with higher centrosome numbers, suggesting that centrosome amplification and dysfunction causes modifications correlating with cellular and glandular disorganization, creating cells that are predisposed to additional changes that lead to tumor development.

Uniquely and importantly for our understanding of the mechanisms of hormone-induced protection to breast cancer, hormonal treatment of animals prior to carcinogen induced refractoriness to carcinogenesis and protected them from centrosome amplification, supporting our hypothesis that changes induced by parity block the carcinogen-induced centrosome amplification. This result in combination with previously reported hormone-induced block in carcinogen-induced proliferation, block in proliferation of estrogen receptor-positive cells, and increase in p53 accumulation and functional activity indicate that there are multiple and persistent changes that occur as a result of hormone exposure. We hypothesize that these alterations act in concert to prevent carcinogen-induced initiation and/or progression of mammary cells.

Elucidation of the mechanisms by which centrosome changes occur leading to tumor formation involves the kinase AurA. This kinase is speculated to cause centrosome amplification if overexpressed (28). Our observations suggest a role of AurA during mammary tumorigenesis. We cloned the rat homologue to AurA, ratAurA, a bona fide Ser/Thr kinase. In our studies on tumorigenesis, ratAurA was linked to centrosome function, and overexpression correlated to centrosome amplification. In cells of rat mammary tumors, higher mRNA expression levels of ratAurA coincided with higher numbers of centrosomes and with tumor development and progression. We speculate that the chemical carcinogen MNU induces a deregulation of the ratAurA gene, leading to overexpression and subsequently to centrosome am-
plification and the initiation of tumorigenesis in rat mammary gland. A recent study on the mechanisms that regulate the transcription of the human AurA gene (38) identified E4TF1, which positively regulates the transcription of AurA. E4TF1 is a ubiquitously expressed Ets family protein. This family of proteins regulates >200 genes, including p53, Rb, FAS, and BRCA1; most of the gene products are key to the proper control of cellular proliferation, differentiation, and development and for malignant transformation. In addition, a tandem repressor element CDE/CHR was demonstrated to be essential for the G2-M-specific transcription of AurA. Mutations in this tandem repressor increased the transcriptional activity of AurA, leading to a loss of cell cycle regulation (38). These observations suggest that high-level expression of AurA in tumor cells might correlate with E4TF1 and/or alterations in the repressor element CDE/CHR responsible for higher transcriptional activity, leading to a loss of cell cycle regulation and tumor formation (38).

Our data also demonstrate higher ratAurA mRNA expression levels under the influence of progesterone, independent from centrosome amplification. These increased ratAurA expression levels are likely attributable to a secondary event associated with enhanced proliferation occurring in the tissue after progesterone treatment. Studies on the transcriptional mechanisms of the human AurA gene (38) implicated putative binding sites for the transcription factors E2F, Sp1, and Ets, likely ruling out a direct progesterone responsiveness of the ratAurA gene mediated through a progesterone response element. Immunolocalization studies with an antibody to ratAurA strongly indicated that the kinase has a direct function on the centrosome. The expression pattern for ratAurA is distinct, being localized to the separated, duplicated centrosomes throughout S- and G2 phases of the cell cycle and to the mitotic spindle of dividing cells. A potential role of ratAurA might be to stabilize microtubules, which could lead to centrosome separation and microtubule-mediated chromosome movements at mitosis. The cytoplasmic staining of ratAurA found in certain foci in the mammary gland after progesterone treatment and in tumor tissue indicate that ratAurA might also act on microtubule motor proteins that have been implicated in the process of centrosome separation and chromosome segregation. In fact, in human breast adenocarcinoma, centrosome amplification correlated with an increase in microtubule-nucleating capacity (24), suggesting that substrates for the kinase AurA may involve centrosomal proteins that interact with the mitotic machinery. Centrosomal proteins, similar to the family of TACCs (transforming acidic coiled-coil; Refs. 39, 40) known to interact with XMAP215 to regulate microtubule behavior and to stabilize centrosomal microtubules (41), are potential candidate substrates for AurA (42).

In addition to the function as a microtubule-organizing center, it is now evident that the centrosome is essential through its involvement in cell cycle progression (43, 44). The concentration of various enzymes and their substrates to the centrosome, including AurA, suggests that complex molecular interactions are occurring that are essential for the G1-S transition. It is still unclear whether centrosomes directly mediate the recruitment or concentration of molecules that are essential for the initiation of DNA synthesis (45–47) or
whether centrosome defects trigger checkpoints that monitor the completion of mitosis. It is apparent that the centrosome is actively participating in interdependent processes of the control of microtubule nucleation, the integration of its duplication into cell cycle progression, and the cell cycle checkpoint control (48).

The studies presented here will open up further avenues in an emerging field of cancer research that is focused on investigating how centrosome abnormalities lead to unequal chromosome segregation and increased genomic instability. Rat mammary carcinomas start to develop as diploid lesions with cytogenetically normal karyotypes; however, the existence of both diploid clones and abnormal clones bearing specific chromosomal rearrangements can be detected upon further progression (49). The ratAurA kinase might be one link between centrosome function and cell cycle progression. Investigating how increased expression levels together with centrosome amplification influence tumor formation and growth might provide a powerful tool for the assessment of prognostic factors in breast cancer.

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Centrosome Amplification and Overexpression of Aurora A Are Early Events in Rat Mammary Carcinogenesis

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