

Research

Cyclin D₁ expression during rat mammary tumor development and its potential role in the resistance of the Copenhagen rat

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Received: 7 June 1999
Revisions requested: 13 July 1999
Revisions received: 10 August 1999
Accepted: 6 September 1999
Published: 7 October 1999
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Important note about how to cite this article

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Korkola JE, Wood GA, Archer MC: Cyclin D₁ expression during rat mammary tumour development and its potential role in the resistance of the Copenhagen rat [peer-reviewed research]. <http://breast-cancer-research.com/vol1no1/07oct99/research/3>

Abstract

Background: Resistance to mammary tumorigenesis in Copenhagen rats is associated with loss of early preneoplastic lesions known as intraductal proliferations. The cause of this disappearance, however, is unknown.

Results: There were no differences in the numbers of lesions in mammary whole-mounts prepared from Copenhagen or Wistar–Furth rats at 20 or 30 days after *N*-methyl-*N*-nitrosourea treatment, but at 37 days there were significantly fewer lesions in Copenhagen glands. Furthermore, lesions in Copenhagen glands were exclusively intraductal proliferations, whereas in Wistar–Furth glands more advanced lesions were also present. Immunohistochemical staining showed frequent

cyclin D₁ overexpression in Wistar–Furth lesions at 37 days, but not in Copenhagen lesions. There were, however, no differences in p16^{INK4a} protein expression, bromodeoxyuridine labeling and apoptotic indices, or mast cell infiltration between Copenhagen and Wistar–Furth lesions at any time.

Conclusions: Overexpression of cyclin D₁ in preneoplastic lesions may be important in the development of mammary tumors in susceptible rats, although this overexpression does not appear to cause significant changes in cell kinetics. Furthermore, the low levels of cyclin D₁ expression in Copenhagen intraductal proliferations may play a role in the resistance of these rats to mammary tumorigenesis.

Keywords: Copenhagen rat, cyclin D₁, intraductal proliferations, mammary gland, preneoplastic lesions

Introduction

Most strains of rats develop multiple mammary tumors when initiated with chemicals or radiation. Several strains, however, are resistant to mammary tumorigenesis induced by both of these means. The Copenhagen rat is the best characterized of these strains [1,2], although the mechanism of resistance is still unknown. Recently, linkage analysis has identified at least four loci that modify mammary tumorigenesis in the Copenhagen rat, but the genes have yet to be cloned [3].

In order to characterize the phenotype associated with resistance, we recently examined mammary whole-mounts from both Copenhagen and susceptible Wistar–Furth rats at various times after treatment with the mammary carcinogen *N*-methyl-*N*-nitrosourea (MNU) [4]. At 15 days

after MNU treatment, we found that both strains had developed the earliest detectable preneoplastic lesions, known as intraductal proliferations (IDPs). The majority of IDPs from both strains contained activating mutations in the *Ha-ras* oncogene, a common alteration in MNU-induced rat mammary adenocarcinomas [5]. By 45 days after MNU treatment, in addition to IDPs more advanced lesions such as ductal carcinomas *in situ* (DCIS) and adenocarcinomas were detectable in the glands from Wistar–Furth rats. In contrast, the IDPs from Copenhagen rats failed to progress and instead declined in number, such that by 60 days after MNU treatment the glands were essentially free of lesions.

To investigate a potential mechanism that could explain the failure of the Copenhagen IDPs to progress and their

subsequent disappearance, we have examined the expression of cyclin D₁ within IDPs and other lesions from Copenhagen and Wistar–Furth rats. Cyclin D₁ has been shown to be important in the transition from the G₁ to the S phase of the cell cycle, and perturbations in this control point can lead to neoplastic transformation [6]. Indeed, cyclin D₁ is frequently overexpressed in both human [7] and rat mammary tumors [8], and is thought to be an important factor in their development. This notion was strengthened by studies that showed that mice engineered to overexpress cyclin D₁ in their mammary glands develop hyperplastic lesions and eventually mammary carcinomas [9]. Overexpression of cyclin D₁ may be an important event in determining whether preneoplastic lesions go on to develop into malignant or benign lesions in humans and is of particular relevance to the present study [10].

In addition to cyclin D₁, we also chose to examine expression of the p16^{INK4a} protein in the lesions, because it is a specific inhibitor of the cyclin D₁–cdk4 complex that drives the transition from G₁ to S in the cell cycle [11]. Expression of p16^{INK4a} in normal cells is thought to lead to a growth arrest [11]. In order to relate changes in the expression of these genes to changes in cell kinetics within the lesions, we used bromodeoxyuridine to label cells during the S phase as a measure of the proliferative index and counted apoptotic cells based on morphology to estimate cell loss. Finally, we stained lesions for mast cells, because they have been implicated in promoting the growth of IDPs [12].

Materials and methods

Animals and carcinogen treatment

Copenhagen and Wistar–Furth rats (6–7 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, Indiana, USA), maintained on a 12 h light/dark cycle, fed Harlan Teklad rat chow (6% fat; Harlan Teklad, Madison, Wisconsin, USA), and were given free access to water. After 1 week of acclimatization the rats were given an intraperitoneal injection of 50mg/kg MNU dissolved in acidified normal saline.

Bromodeoxyuridine treatment and mammary whole-mount preparation

At 20, 30, and 37 days after MNU treatment, five rats from each strain selected randomly were given an intraperitoneal injection of 50mg/kg bromodeoxyuridine (Boehringer, Laval, Canada) dissolved in phosphate-buffered saline. Three hours later, they were killed and mammary whole-mounts prepared, using the technique we described previously [4].

Paraffin embedding, staining, and immunohistochemistry

Putative lesions in the whole-mounts were microdissected from the glands, cleared in xylenes, processed through three changes of paraffin wax, and then embedded in paraffin wax (Fisher, Whitby, Canada) for sectioning.

Sections (4µm thick) were placed on poly-L-lysine (Sigma, St Louis, Missouri, USA) coated slides and stained with hematoxylin and eosin. Positive identification of IDPs, DCIS, and adenocarcinomas was based on the criteria we used previously [4]. Serial sections from confirmed lesions were then used for cyclin D₁, p16^{INK4a}, and bromodeoxyuridine immunohistochemistry using established techniques [13–15]. Anti-cyclin D₁ and anti-p16^{INK4a} antibodies were obtained from Santa Cruz (Santa Cruz, California, USA) and anti-bromodeoxyuridine antibodies from Boehringer. Archival rat mammary tumor tissue was used as a positive control for cyclin D₁, because its overexpression has been reported in these tumors [8]. The levels of cyclin D₁ in the stained samples were scored as negative (–), low (+), or high (++), based on number of positive cells in the lesion as well as staining intensity. Because the measurement of staining intensity was somewhat subjective, the coded samples were also scored independently by a second individual, with identical results. The percentage of cyclin D₁-positive cells was determined as the number of positive cells divided by total cell number in a lesion.

The bromodeoxyuridine labeling index was determined by the number of bromodeoxyuridine-positive cells divided by total cells in a lesion. Small intestine from bromodeoxyuridine-treated rats or livers from partially hepatectomized rats were used as positive controls for staining.

For all immunohistochemistry, the specificity of the staining was ensured by replacing the primary antibody with 1% normal sheep serum. In all cases, no staining was observed.

For determination of apoptotic indices, bromodeoxyuridine-stained sections were also scored for apoptotic cells based on their morphology (pyknotic nuclei, cell shrinkage) [16]. Because this is a subjective method, samples were scored independently by two individuals, with similar results.

For staining of mast cells, samples were deparaffinized in xylenes, rehydrated through acetone and water, stained in 0.025% toluidine blue (Sigma) for 30 s. Slides were washed in distilled water, dehydrated in acetone, cleared in xylenes, and mounted using Permount (Fisher). Mast cells were counted per high power (400×) field of view around the lesion.

Statistical analyses

For comparison of numbers of lesions, bromodeoxyuridine-labeling indices, and apoptotic indices at 20, 30, and 37 days after MNU treatment, *t*-tests using Bonferroni's correction were used. The data were also analyzed by square root transformation followed by *t*-tests using Bonferroni's correction. For comparison of cyclin D₁ staining in Copenhagen and Wistar–Furth IDPs, a χ^2 test was used, with the groups being IDPs that do not overexpress cyclin D₁ (– or +) and

IDPs that do overexpress cyclin D₁ (++)). For comparison of percentages of cyclin D₁-positive cells in Copenhagen and Wistar–Furth IDPs at day 37, a one-tailed *t*-test was used.

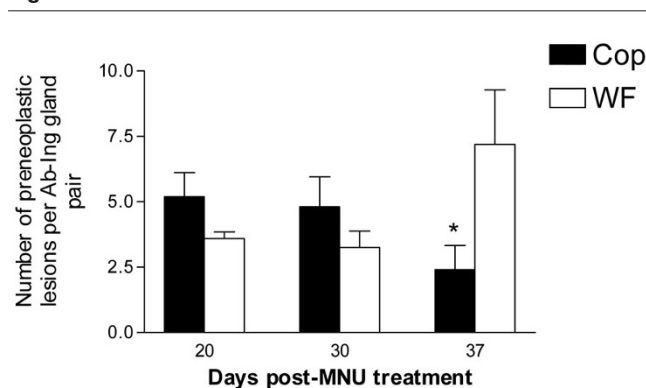
Results

To examine the expression of cyclin D₁ and p16^{INK4a} proteins within lesions from Copenhagen and Wistar–Furth rats, mammary whole-mounts were prepared at 20, 30, and 37 days after MNU treatment. In order to estimate proliferative indices within the lesions, all rats were administered bromodeoxyuridine before killing. As expected, at 20 and 30 days after MNU treatment the number of lesions in Copenhagen rats was not different from that in Wistar–Furth rats (Fig. 1). By 37 days after MNU, however, there were significantly fewer lesions in the glands of Copenhagen rats (Fig. 1). Furthermore, we observed only IDPs in the glands of Copenhagen rats, whereas more advanced lesions such as ductal carcinomas *in situ* (DCIS) and small, nonpalpable tumors were also present in the glands of Wistar–Furth rats at 37 days; this is consistent with our previous results [4]. Figure 2 shows the same region of the inguinal mammary gland from typical whole-mounts from a Wistar–Furth and a Copenhagen rat, demonstrating the striking difference in development of lesions in the glands at 37 days.

We determined cyclin D₁ expression immunohistochemically in sections from lesions (Fig. 3a–c). The staining levels were characterized as negative (–), low (+), or high (++)). We observed no staining in either Wistar–Furth or Copenhagen IDPs at 20 or 30 days after MNU treatment, or in any normal mammary tissues. At 37 days, however, there was cyclin D₁ staining in 10 out of 17 Wistar–Furth IDPs, with six of these showing high levels of expression (overexpression), as shown in Fig. 3b. In contrast, only three out of nine IDPs from Copenhagen rats showed any cyclin D₁ staining, with all of these being at a low level (Fig. 3a). A χ^2 analysis showed that overexpression of cyclin D₁ was significantly higher in Wistar–Furth IDPs than in Copenhagen IDPs ($P < 0.05$). Furthermore, we stained the few advanced lesions present in Wistar–Furth glands at 37 days for cyclin D₁, and observed overexpression in four out of five DCIS and in all of three nonpalpable adenocarcinomas (Fig. 3c); this is in good agreement with the published observations that approximately 80% of rat mammary tumors overexpress cyclin D₁ [8,17].

As an additional measure, we determined the mean percentage of cells that expressed cyclin D₁ within lesions at 37 days. We found that $5.9 \pm 3.4\%$ of cells within Copenhagen IDPs stained for cyclin D₁, compared with $17.5 \pm 4.0\%$ for Wistar–Furth IDPs, $22.6 \pm 7.5\%$ for Wistar–Furth DCIS, and $32.1 \pm 2.1\%$ for Wistar–Furth adenocarcinomas (all values are means \pm standard error of the mean). Statistical analysis of Copenhagen and Wistar–Furth IDPs by *t*-test showed that the percentage of cells

Figure 1



Number of preneoplastic lesions in the mammary glands of treated Copenhagen (Cop) and Wistar–Furth (WF) rats at 20, 30, and 37 days after *N*-methyl-*N*-nitrosourea treatment. * $P < 0.05$, versus Wistar–Furth rats.

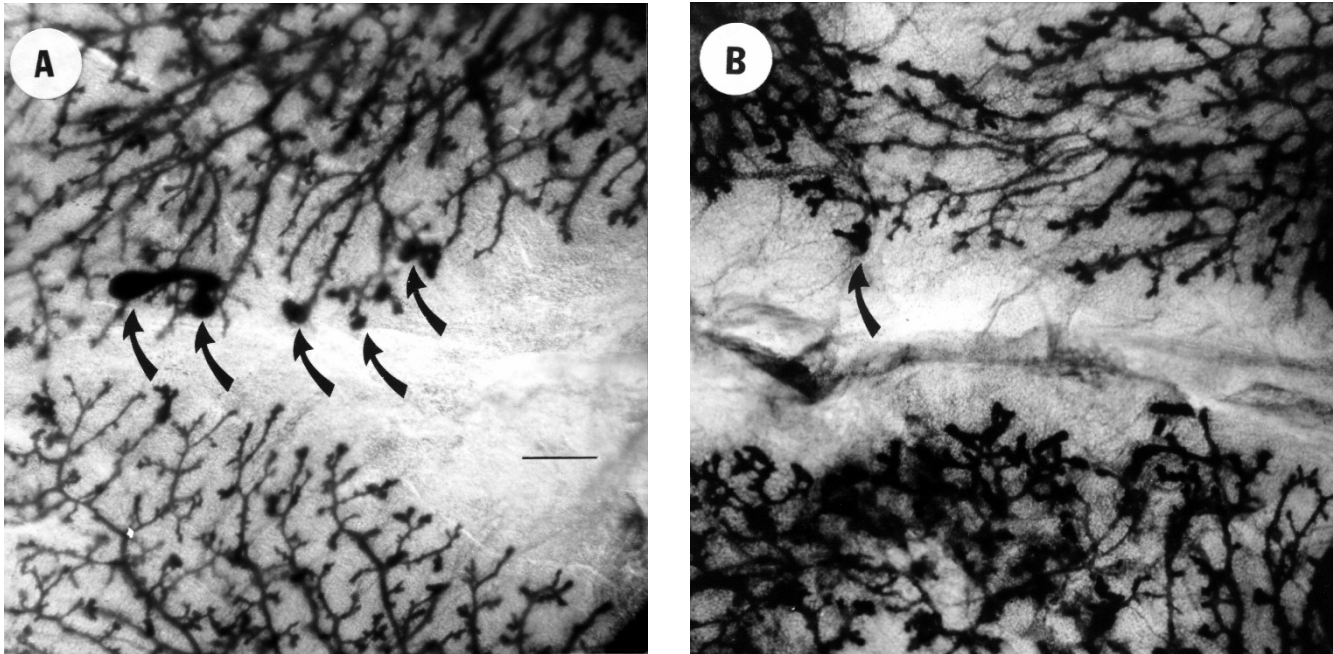
that expressed cyclin D₁ was significantly higher in the Wistar–Furth lesions ($P < 0.05$). It should be noted that it was not possible to perform Western analysis to confirm the cyclin D₁ expression levels. Suspected lesions must be microdissected from the whole-mounts, then embedded and sectioned to confirm their identity, leaving insufficient tissue for Western analysis.

Next, we determined p16^{INK4a} expression in the sections by immunohistochemistry (Fig. 3d–f). We observed similar levels of staining for this protein in all of the samples from both strains, including normal mammary tissue, IDPs, DCIS, and tumors.

To measure the proliferative index of IDPs from both strains we stained samples using an anti-bromodeoxyuridine antibody as shown in Fig. 3g–i. The labeling index was determined as the number of bromodeoxyuridine-positive cells divided by the total number of cells in the lesion. The labeling indices in the Copenhagen IDPs were not different from those in Wistar–Furth rats at either 20 or 30 days after MNU treatment when there was no cyclin D₁ overexpression in either strain (Fig. 4). At 37 days, when we observed high levels of cyclin D₁ staining in Wistar–Furth lesions but not in Copenhagen lesions, there were also no differences in the bromodeoxyuridine labeling indices between the two strains (Fig. 4). Furthermore, there was no significant correlation between labeling indices and cyclin D₁ expression levels in lesions from Wistar–Furth rats at this time.

In the same lesions that we determined the bromodeoxyuridine labeling indices, we also counted apoptotic cells based on their morphology. The apoptotic indices are shown in Fig. 5. There were no significant differences

Figure 2



Examples of mammary whole-mounts from (a) a Wistar-Furth rat and (b) a Copenhagen rat, both 37 days after *N*-methyl-*N*-nitrosourea treatment. Note the striking differences between the glands, with

multiple preneoplastic lesions present in the Wistar-Furth gland but only one putative lesion present in the Copenhagen gland, as indicated by the arrows. Bar = 1 mm.

between the Copenhagen and Wistar-Furth rats at 20, 30, or 37 days after MNU treatment.

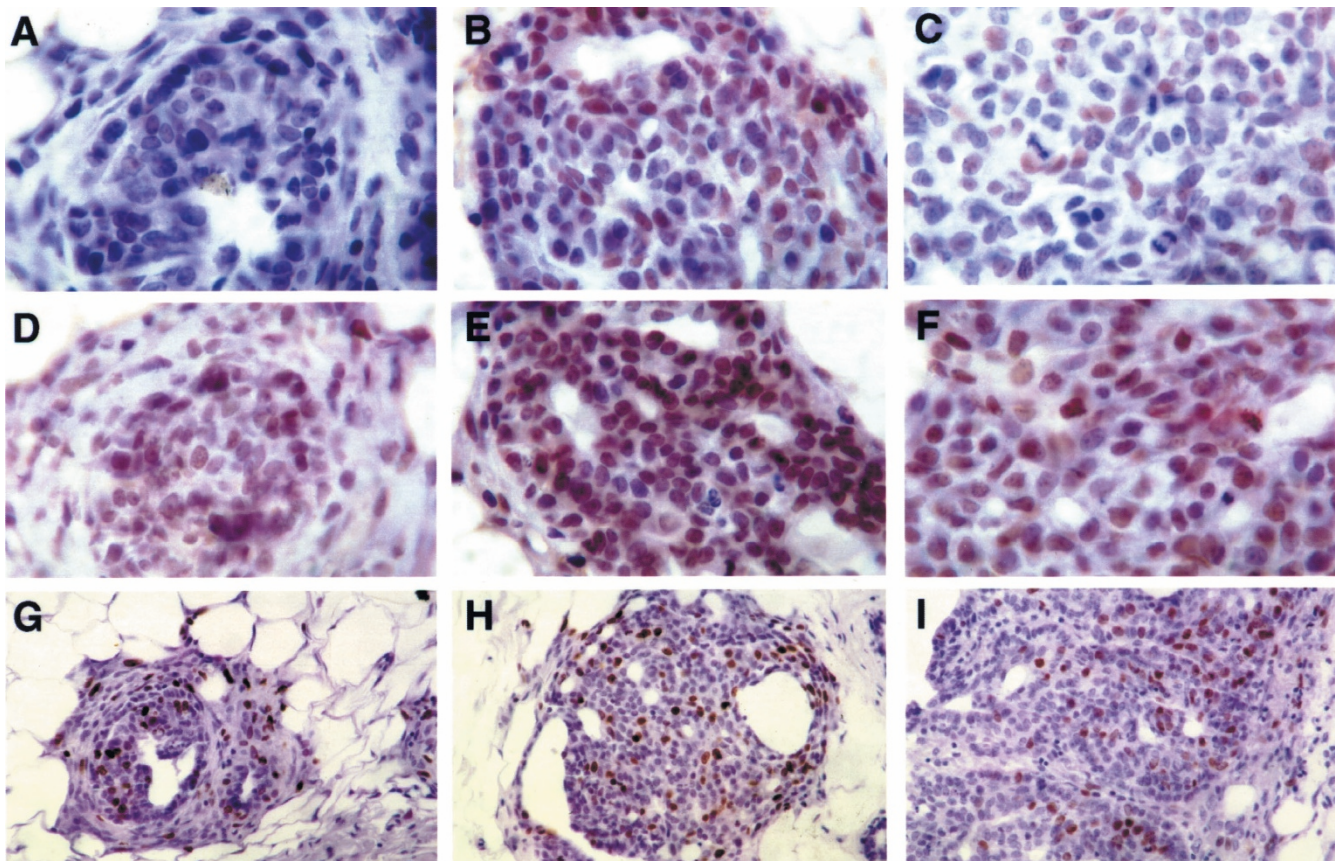
Toluidine blue, which stains mast cells metachromatically, was used to visualize these cells within sections. Samples were scored for the number of mast cells per high power field of view around each lesion. There were 3.6 ± 0.5 , 2.8 ± 0.3 , and 6.4 ± 0.7 mast cells around Copenhagen IDPs, and 4.3 ± 0.5 , 2.4 ± 0.5 , and 6.6 ± 0.8 mast cells around Wistar-Furth IDPs at 20, 30, and 37 days after MNU, respectively (all values are means \pm standard error of the mean). There were no significant differences in mast cell numbers between the two strains at any of the time points.

Discussion

Overexpression of cyclin D₁ has been reported in both human [7] and rat mammary tumors [8]. It has recently been shown [10] that cyclin D₁ overexpression might be a critical early event in human breast tumor development, because overexpression of this gene is common in early lesions that ultimately form malignant breast cancers, but not in those that form benign tumors. It is thought that rat mammary tumorigenesis occurs through the progression of the early IDPs to DCIS and eventually to adenocarcinomas [18]. Recently, cyclin D₁ expression has been investigated in normal mammary tissue, preneoplastic lesions, and tumors in a susceptible strain of rat [17]. The percentage of cyclin D₁-positive cells was shown to be very low (approx-

mately 2.4%) in normal mammary tissue. In IDPs, however, approximately 13.6% of cells were positive, and this value increased with each subsequent stage of tumorigenesis such that approximately 40% of cells within adenocarcinomas were positive. We reasoned that if cyclin D₁ overexpression is an early event that is necessary for tumorigenesis in the rat mammary gland, then differences in the expression of this gene in Wistar-Furth and Copenhagen rats could account for their different susceptibilities to mammary tumorigenesis. At 37 days after MNU treatment, when there were significantly more IDPs in Wistar-Furth than in Copenhagen glands, we observed cyclin D₁ overexpression only in Wistar-Furth IDPs. This overexpression was manifested as staining that was both more frequent and more intense than in IDPs from Copenhagen rats. We also found that the percentage of cyclin D₁-positive cells within Wistar-Furth IDPs was significantly higher than in Copenhagen IDPs at day 37. Furthermore, seven out of the eight DCIS and adenocarcinomas that had developed by this time in Wistar-Furth rats showed highly overexpressed cyclin D₁ relative to normal tissue. Both the DCIS and adenocarcinomas had higher percentages of cyclin D₁-positive cells than did IDPs, although the number of advanced lesions present at this time was too few to demonstrate this difference statistically. It should be noted that our values for Wistar-Furth lesions are in good agreement with those reported by Zhu *et al* [17]. Because cyclin D₁ protein levels are higher in DCIS and adenocarcinomas than in IDPs,

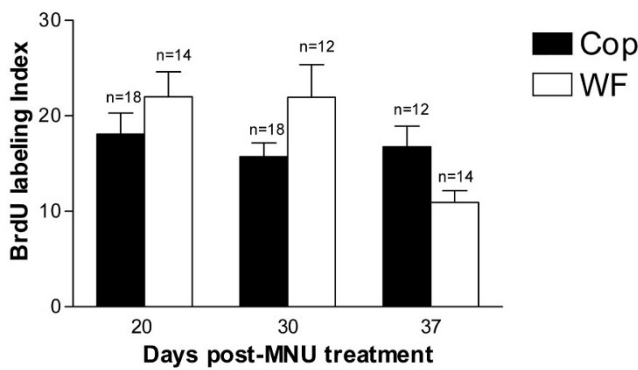
Figure 3



Immunohistochemical staining for (a–c) cyclin D₁, (d–f) p16^{INK4a}, and (g–i) bromodeoxyuridine. Positive staining cells appear brown, counter-stained negative cells appear purple. (a), (d), and (g) are serial sections of an intraductal proliferation (IDP) from a Copenhagen rat; (b), (e), and (h) are serial sections of a large IDP from a Wistar-Furth

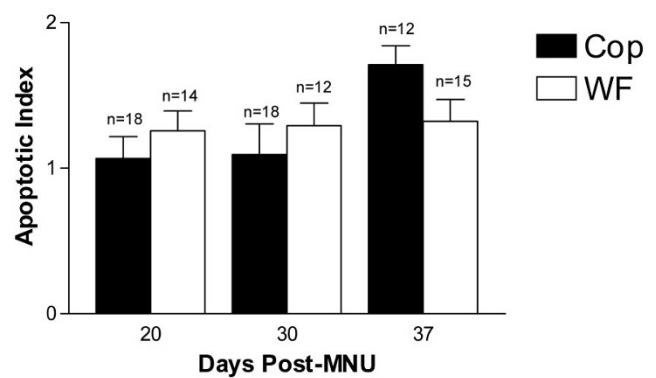
rat; and (c), (f), and (i) are serial sections of a tumor from a Wistar-Furth rat. Note the overexpression of cyclin D₁ in Wistar-Furth lesions (b and c) but not in the Copenhagen IDP (a). All lesions are from mammary glands of rats 37 days after MNU treatment. (a–f) 1000x magnification and (g–i) 400x magnification.

Figure 4



Bromodeoxyuridine (BrdU) labeling indices in Copenhagen (Cop) and Wistar-Furth (WF) rats at 20, 30, and 37 days after MNU treatment.

Figure 5



Apoptotic indices in Copenhagen (Cop) and Wistar-Furth (WF) lesions at 20, 30, and 37 days after MNU treatment.

overexpression of this gene might be important in the transition from precancerous to cancerous lesions. Furthermore,

the lack of cyclin D₁ overexpression in Copenhagen IDPs may play a role in their inability to progress to DCIS and

tumors, a notion supported by our observation of only a single DCIS in a total of 31 MNU-treated Copenhagen rats from this and our previous study [4].

Transition from the G₁ to S phase of the cell cycle is tightly regulated within cells. Activity of the cyclin D₁-cdk4 complex that drives this transition can be blocked by the p16^{INK4a} protein, leading to growth arrest [11]. In tumors, a sustained blockage induced by p16^{INK4a} may lead to apoptosis [19]. Loss of the G₁-S checkpoint control can occur by a variety of means, including loss of p16^{INK4a} or overexpression of cyclin D₁ [20]. Our staining showed that p16^{INK4a} was expressed in all samples, but, as described above, cyclin D₁ was overexpressed only in Wistar-Furth lesions at day 37. Therefore, we expected that the Wistar-Furth IDPs would have a higher labeling index or lower apoptotic index than those of Copenhagen rats. As expected, at 20 and 30 days after MNU treatment, when there were no differences in cyclin D₁ expression in IDPs or in number of IDPs between the two strains, we found no differences in the bromodeoxyuridine labeling or apoptotic indices. Surprisingly, at 37 days we found no significant difference in the bromodeoxyuridine labeling indices in IDPs from Copenhagen compared with Wistar-Furth rats, indicating that there was no correlation between cyclin D₁ overexpression and cell proliferation. Other studies have also found that cyclin D₁ overexpression does not correlate with the proliferation rate in rat mammary tumors [8] or in human tumors [10]. This indicates that cyclin D₁ overexpression may play other roles in tumorigenesis that are unrelated to the cell cycle. Indeed, it has been reported that cyclin D₁ can transactivate the estrogen receptor and influence genomic stability [21]. It has also been found that cyclin D₁ overexpression can inhibit apoptosis [21]. There was no difference, however, in the apoptotic indices at 37 days between the strains.

Cyclin D₁ may provide a promotional stimulus for Wistar-Furth IDPs, but we were unable to detect an alteration in cell kinetics. It is possible, however, that small perturbations in the rates of cell loss and/or cell growth may occur that would be undetectable in short-term assays. Such changes could have profound effects over the long period of tumor development. Indeed, our observation that preneoplastic lesions disappear from the glands of Copenhagen rats indicates that cell loss may be occurring, although redifferentiation of preneoplastic cells to a more normal phenotype is also plausible, as we have previously hypothesized [4].

It has been postulated by Russo and Russo [12,22] that there are two populations of IDPs. Initiated plus promoted IDPs are able to form more advanced lesions such as DCIS and tumors, whereas the IDPs that are only initiated are unable to progress [12,22]. Those authors distinguished initiated plus promoted IDPs from initiated IDPs by the infiltration of mast cells, which are three times more abundant around the former. They postulated that these mast

cells may be involved in promoting the growth of lesions, by the secretion of either mitogenic or angiogenic factors. If mast cells are more abundant surrounding Wistar-Furth than Copenhagen IDPs, then secretion of mitogenic factors could lead to overexpression of cyclin D₁ in the former. We found, however, that there were no differences in the numbers of mast cells surrounding IDPs of the two strains at any time point. It seems unlikely, therefore, that mast cell infiltration plays a role in either cyclin D₁ overexpression or in the resistance of the Copenhagen rat.

It is unclear what mechanism is responsible for the overexpression of cyclin D₁ we have observed. It has been reported that the *ras* oncogene can induce expression of cyclin D₁ [23,24], but it is unlikely that this is involved, because we have previously shown [4] that similar percentages of Copenhagen and Wistar-Furth IDPs harbor mutant *Ha-ras* alleles. Recently, Tetsu and McCormick [25] have shown that expression of cyclin D₁ can also be regulated through the actions of transcription factors controlled by the β -catenin and adenomatous polyposis coli genes in colon carcinoma cells [25]. Those authors speculated that abnormal levels of β -catenin can contribute to the accumulation and overexpression of the cyclin D₁ protein and hence transformation. The β -catenin pathway, therefore, merits investigation in rat mammary tumorigenesis.

In conclusion, we measured several parameters that could potentially be involved in the resistance of the Copenhagen rat to mammary tumorigenesis. We found no differences in the number of lesions in Copenhagen compared with Wistar-Furth mammary glands at 20 or 30 days after MNU treatment, but at 37 days there were significantly fewer lesions in the Copenhagen glands. Furthermore, by this time advanced lesions such as DCIS and adenocarcinomas were present in Wistar-Furth glands, whereas no such lesions were observed in Copenhagen rats. Immunohistochemical staining of lesions from both strains indicated that cyclin D₁ was frequently overexpressed in Wistar-Furth lesions at 37 days, but not in Copenhagen lesions from the same time. Expression of p16^{INK4a} protein, bromodeoxyuridine labeling and apoptotic indices, and mast cell infiltration around lesions were not significantly different between the two strains at any time. These findings indicate that overexpression of cyclin D₁ might play a fundamental role in the progression of IDPs to DCIS and adenocarcinomas during rat mammary tumorigenesis. Furthermore, this gene might also play a role in the resistance of Copenhagen rats to MNU-induced mammary tumorigenesis.

Acknowledgements

The authors thank Dr Henry Thompson for discussions on bromodeoxyuridine staining of IDPs, Dr Alan Medline for help with identification and staining of mast cells, Dr Ross Cameron for help with photography, and Dr Laurie Stephen for her help with the animal work. This investigation was supported by a grant from the Canadian Breast Cancer Research Initiative. MCA is the recipient of a Natural Sciences and Engineering Research Council of Canada Industrial Research Chair and acknowledges support from the member companies of the Program in Food Safety (University of Toronto).

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