



Cyclin D1 overexpression combined with *N*-nitrosomethylbenzylamine increases dysplasia and cellular proliferation in murine esophageal squamous epithelium

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We previously described the oral-esophageal tissue-specific expression of cyclin D1 with the Epstein–Barr virus ED-L2 promoter in transgenic mice, and resulting dysplasia. Given the evidence for an interplay between environmental and genetic factors in esophageal squamous carcinogenesis, the aim of this study was to determine the potential cooperation of the nitrosamine compound *N*-nitrosomethylbenzylamine (NMBA), an esophageal specific carcinogen, in the cyclin D1 transgenic mice. NMBA was first demonstrated to induce dysplasia in two strains of inbred mice, C57BL/6 and FVB/N. Subcutaneous NMBA was then administered to wild type and transgenic mice beginning at 4 weeks of age. Mice were monitored for the duration of the study for general appearance, activity and weight, and were euthanized at 12 and 15 months. Histopathologic analysis revealed increased severity of dysplasia in cyclin D1 mice treated with NMBA compared with treated age-matched wild-type mice and untreated mice. There was also increased proliferating cell nuclear antigen (PCNA) expression in the esophagi of NMBA treated cyclin D1 mice. Taken together, these findings suggest that a genetic alteration, specifically cyclin D1 overexpression and a chemical carcinogen, NMBA, may cooperate to increase the severity of esophageal squamous dysplasia, a prominent precursor to carcinoma.

Keywords: cyclin D1; *N*-nitrosomethylbenzylamine; esophageal squamous dysplasia; transgenic mice

Introduction

Esophageal squamous cell carcinoma (ESCC) is a common malignancy worldwide with considerable geographic variation. A number of environmental factors, namely tobacco, alcohol, high dietary salt content, vitamin and mineral deficiencies and chemical carcinogens (Walker *et al.*, 1979; Li *et al.*, 1986) have been implicated as contributing to a hyperproliferative state and promoting malignant transformation. The

largest single group of chemical carcinogens are the *N*-nitroso compounds, sources of which include diet, tobacco products, and metabolites in the gut. Trace amounts of these compounds (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosomethylbenzylamine (NMBA) and *N*-1-methylacetyl-*N*-3-methylbutyl nitrosamine) have been identified, for example, in cornbread and pickled vegetables in Linxian County, China (Morse and Stoner, 1993). In the stomach, *N*-nitroso compounds are formed by reactions with nitrites and amines both of which are widely distributed in foodstuffs and in the environment.

N-nitroso compounds are not carcinogenic *per se*; they require *in vivo* transformation and production of intermediate reactive products that can lead to the formation of alkylating electrophiles (Antrup and Stoner, 1982). These agents interact with DNA to form stable adducts such as alkylated purine and pyrimidine bases (Doniger *et al.*, 1985; Fong *et al.*, 1979; Van Bentham *et al.*, 1992). Metabolic studies of explant cultures of esophageal tissues have shown that the human and rat esophagi are fully capable of converting nitrosamines into metabolites that alkylate DNA at the N⁷ and O⁶ positions of guanine (Antrup and Stoner, 1982; Harris *et al.*, 1979). O⁶-methylguanine persists, accumulates and leads to DNA mutations as previously demonstrated in esophageal squamous papillomas in nitrosamine treated rats (Siglin *et al.*, 1996).

An understanding of the molecular pathogenesis of esophageal squamous cell carcinoma can be appreciated to a great extent through animal models. A well-established rat model for esophageal squamous carcinogenesis has been widely studied. The appropriate dose, route of administration, and treatment schedule for the nitrosamine NMBA have been established (Siglin *et al.*, 1996), leading to the potent induction of esophageal squamous papilloma and carcinoma in rats (Barch *et al.*, 1986; Craddock and Driver, 1987; Druckrey *et al.*, 1963; Hodgson *et al.*, 1982; Lijinsky *et al.*, 1982; Stinson *et al.*, 1978). Mechanistically, abnormal expression of many genes has been implicated in the formation of tumors in this model. For example, NMBA induces *Ha-ras* codon 12 mutations in rat esophageal papillomas (Wang *et al.*, 1990). More recently, alterations in transforming growth factor alpha and epidermal growth factor

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Received 5 March 1998; revised 20 July 1998; accepted 21 July 1998

receptor expression have been documented in rat esophageal papillomas (OS Wang *et al.*, 1996a) as well as cyclin D1 and cyclin E overexpression (OS Wang *et al.*, 1996b), mutational Ha-*ras* activation (Wang *et al.*, 1990) and p53 mutation (D Wang *et al.*, 1996). A second group recently confirmed the overexpression of cyclin D1 in an NMBA rat model (Youssef *et al.*, 1997). Another mechanism recently described includes reduced intercellular gap junctional communication in esophageal squamous epithelial cells obtained from rats treated with NMBA (Garber *et al.*, 1997).

There is compelling evidence for the contribution of genetic alterations to the molecular pathogenesis of squamous cell carcinomas such as those originating from skin, head and neck, and esophagus. These mechanisms involve activation of oncogenes and inactivation of tumor suppressor genes. A genetic susceptibility to squamous skin papilloma progression in mice has already been established (Stern *et al.*, 1995) and early overexpression of cyclin D1 probably plays a role in skin carcinogenesis (Bianchi *et al.*, 1993; Mitsunaga *et al.*, 1995; Robles and Conti, 1995). Perhaps the most critical genetic alteration in esophageal squamous carcinoma is the activation of cyclin D1 through gene amplification (Jiang *et al.*, 1992, 1993; Nakagawa *et al.*, 1995; Sheyn *et al.*, 1997) and transcriptional regulation through mitogenic stimuli such as transforming growth factor alpha (Yan *et al.*, 1997). To develop a genetic model of esophageal squamous carcinogenesis, we successfully targeted cyclin D1 to the esophageal squamous epithelium using the Epstein-Barr virus ED-L2 promoter (Nakagawa *et al.*, 1997). In this cyclin D1 overexpressing transgenic mouse, a dysplastic esophageal squamous phenotype is associated with cellular abnormalities which may ultimately predispose to the formation of cancer if combined with other factors (Mueller *et al.*, 1997). These abnormalities include increased cellular proliferation, epidermal growth factor receptor overexpression and p53 overexpression.

To determine the possible combinatorial effects of NMBA and cyclin D1 overexpression, we administered NMBA to cyclin D1 transgenic mice. This provides the first direct experimental testing of the hypothesis that environmental and genetic factors cooperate in the molecular pathogenesis of esophageal squamous cell carcinoma. Since previous studies with this carcinogen have been performed using rat models, we first determined if subcutaneously administered NMBA results in dysplasia in the C57BL/6 and FVB/N inbred strains of mice. Based on these results, we designed an experiment to examine the effects of NMBA and cyclin D1 overexpression in FVB/N

mice. Although previous work has suggested that abnormalities in the expression of cyclin D1 play a role in the pathogenesis of esophageal squamous carcinoma, this model has the advantage of testing experimentally whether overexpression of the cyclin D1 oncogene induces an acceleration of processes initiated by a known esophageal carcinogen, NMBA.

Results

NMBA causes dysplasia in wild-type FVB/N and C57BL/6 mice at a rate that varies according to genetic background but independent of dosing schedule

Prior to the study of NMBA's effects in transgenic mice, we established the efficacy of NMBA in inducing dysplastic changes in the esophageal squamous epithelium of wild-type mice. Extensive studies in rats led us to examine the effects of subcutaneously administered NMBA in two mouse strains, one of which (FVB/N) had an identical genetic background with our previously generated cyclin D1 transgenic mice. To maximize the possibility of inducing dysplastic changes in mice with NMBA, several NMBA administration schedules were chosen including those which had been previously shown to be effective in inducing neoplasia in rats. The parameters of NMBA administration are summarized in Table 1.

Both the FVB/N and C57BL/6 strains of inbred mice tolerated NMBA administration and continued to gain weight and remain active throughout the study. There were no significant weight differences between the two strains. There were also no external signs of malignancy noted by weekly examination. Representative hematoxylin and eosin stained sections of the tongue, hypopharynx, glandular stomach and proximal duodenum were made and the histologic features were similar to untreated animals. The squamous forestomach from treated mice demonstrated mild proliferative changes at 8 months such as cellular crowding in the basal layer (data not shown). There was no dysplasia, papilloma formation or malignancy.

At three months of age, mild proliferative changes in the form of esophageal basal cell hyperplasia were observed only in the treated C57BL/6 mice (data not shown). At 5 months of age, histologic changes included nuclear atypia and increased mitoses in the basal layer as well as the presence of immature cells migrating toward the lumen (data not shown). Proliferative changes included increased mitotic figures. There was evidence of decreased maturation sequence throughout the squamous epithelium coupled with disorganization of the keratin layer and decreased numbers of

Table 1 NMBA treatment parameters for mouse strain comparison (FVB/N and C57BL/6) and cyclin D1 transgenic experiments. Mice in each study were administered different dosages of subcutaneous NMBA for varying durations and autopsied at the timepoints indicated

Variable	Strain comparison study	D1 transgenic study
Strain, genotype	FVB/N-/-, C57BL6-/-	FVB/N-/-, FVB/N-D1
NMBA dose (mg/kg weight)	0, 0.5, 1.0	0, 1.0
Treatment duration (week)	5, 12	5, 15
Injection frequency (/week)	1, 3	1, 3
Endpoints (month)	3, 5, 8	9, 12, 15

keratohyaline granules. These histologic changes were more evident in C57BL/6 than in FVB/N mice.

At 8 months of age, further progression of the proliferative changes were observed in both NMBA treated strains. There was crowding of basal cells with prominent mitotic figures. Definitive mild dysplastic changes were notable at this time. Representative sections of esophagi demonstrating mild dysplasia from both NMBA treated strains at 8 months of age are shown in Figure 1. An esophageal section from an untreated wild type mouse is also shown for comparison. Figure 2 summarizes the development of histologic abnormalities in this initial experiment.

For each strain, comparable histologic changes were identified regardless of the NMBA treatment regimen administered, indicating the absence of a dose-response relationship over the dosing range used. Between strains, however, the rate of development and degree of dysplastic change differed with the C57BL/6 being more susceptible to the effects of NMBA at an earlier age. Unlike in rats treated with NMBA at similar dosages, no gross papillomas or cancers were observed in the two strains of mice.

NMBA increases the severity of esophageal squamous dysplasia in FVB/N–cyclin D1 overexpressing transgenic mice

To compare the effects of NMBA administration and genetic alteration in the form of overexpression of cyclin D1, we designed a second NMBA treatment protocol. Table 1 summarizes the NMBA treatment parameters chosen for use in this study. NMBA was

administered in the higher dose amount (1 mg/kg) tolerated by the mice in the initial experiment. In addition to a short intensive exposure (injections three times weekly for 5 weeks), a ‘chronic’ administration protocol was given to different groups of mice (injections once weekly for 15 weeks). The duration of chronic exposure was increased from 12–15 weeks based on work in rats showing this schedule to be more effective in inducing neoplastic changes (unpublished observations, G Stoner). Additional FVB/N wild type and cyclin D1 control mice were not treated with NMBA. Four mice were included in each treatment group to allow for statistical comparisons in the analysis of the histologic changes.

We previously reported that cyclin D1 transgenic mice develop nuclear atypia by 6 months, mild dysplasia by 12 months and severe dysplasia by 18–20 months (Nakagawa *et al.*, 1997; Mueller *et al.*, 1997). As noted above, NMBA administration causes mild dysplasia beginning at 8 months of age in FVB/N and C57BL/6 mice. Two later time points (12 and 15 months) were selected for examination of esophagi of the different groups of mice. These were chosen to represent the time at which severe dysplasia would not normally have developed in mice with either factor (cyclin D1 or NMBA) alone. Additional NMBA treated and untreated wild type and transgenic mice were followed in parallel to each arm of the study. These mice were examined at 9 months of age (data not shown) to monitor the development of dysplasia and to determine whether other unforeseen effects of NMBA and cyclin D1 had occurred. At this time point, mild dysplasia was evident in NMBA treated wild type and untreated cyclin D1 mice, confirming our previous results. The NMBA treated transgenic mice had also developed dysplasia by this time point which was not different in severity. Weight, general appearance and level of activity did not differ between the groups.

Administration of NMBA to all groups of mice led to an increased severity of squamous dysplasia. Untreated FVB/N did not develop esophageal abnormalities. The development of dysplasia did not vary between the two different NMBA treatment regimens. Histologic scores were determined by two different examiners and assigned to each pathologic specimen in a blinded fashion. Scores were assigned according to

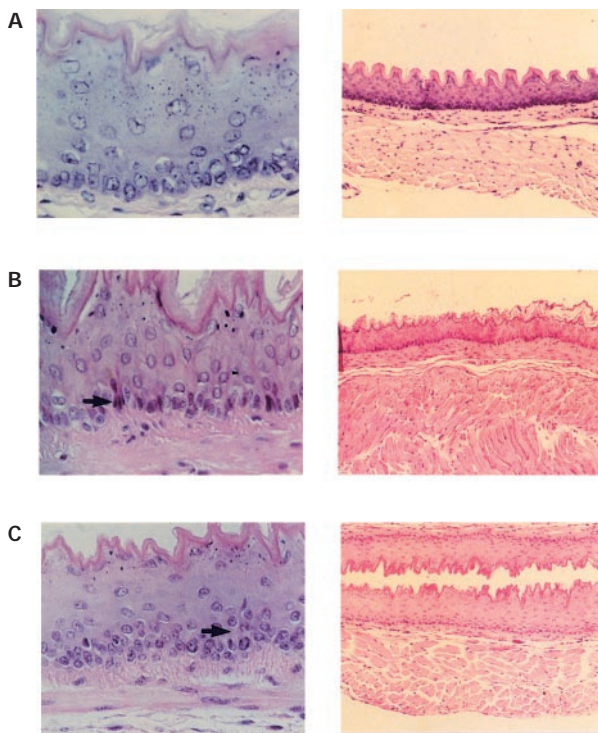


Figure 1 Hematoxylin and eosin stained sections (left, 320 ×; right, 100 ×) of esophagi from 8 month old mice. (a) Untreated wild-type FVB/N (same as untreated wild-type C57Bl/6, not shown); (b) NMBA treated C57BL/6, demonstrating mild dysplasia characterized by nuclear atypia (arrow); (c) NMBA treated FVB/N, demonstrating mild dysplasia characterized by nuclear atypia and cellular crowding in the basal layer (arrow)

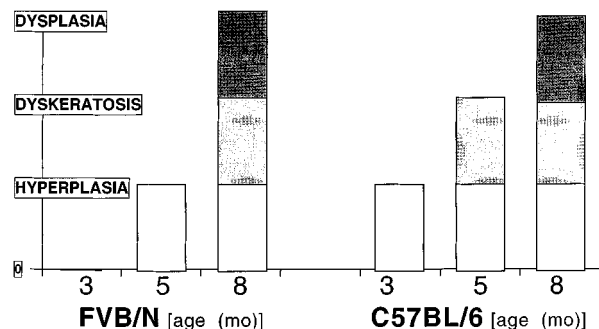


Figure 2 Schematic representation of histologic findings in the esophagi from FVB/N and C57BL/6 strains of inbred mice treated with NMBA. Hyperplastic and dyskeratotic changes developed earlier in C57BL/6 mice, while dysplasia was present in both strains at 8 months of age

the most severe degree of dysplasia in a specimen with normal-0, mild dysplasia-1, moderate dysplasia-2, severe dysplasia-3, carcinoma-4. The esophageal histologic scores for the different groups are shown in Table 2, calculated by adding the proximal and distal esophageal scores. At both time points, the esophageal histologic score was statistically significantly higher in mice with the cyclin D1 transgene treated with NMBA according to either schedule (Table 2). In addition, the histology score was higher at 15 months than at 12 months.

Since the cyclin D1 transgene is also expressed in the tongue and forestomach (Nakagawa *et al.*, 1997) a total score was also calculated by adding the histologic scores of the tongue, proximal and distal esophagus, and squamous forestomach. The total score was also highest in the NMBA treated cyclin D1 transgenic mice (data not shown). Untreated cyclin D1 mice developed less severe dysplasia by the same time points, and wild type mice showed no abnormalities. The rate at which these abnormalities developed was not determined, although it was known from previous studies that NMBA treated wild type mice and untreated cyclin D1 transgenic mice developed mild dysplastic changes as early as 8 months.

Representative examples of dysplasia in the proximal esophagus from 12 month old mice treated with NMBA for 15 weeks are shown in Figure 3. The highest grade of dysplasia is seen in the cyclin D1 NMBA treated mice. In these mice, hyperplasia, nuclear atypia and increased mitoses are evident in the basal layer. Disorganization of the keratin layer and decreased numbers of keratohyaline granules are also prominent at 12 months of age. The esophagus from a 12 month old wild type FVB/N mouse treated with NMBA for 15 weeks shows mild dysplasia which has not progressed from that seen in similar mice at 8 months of age. The distal esophagus from cyclin D1 NMBA treated mice also shows a higher degree of dysplasia compared with distal esophagi from mice in the other treatment groups (Figure 4).

Cyclin D1 and NMBA cooperatively increase the proliferative index of esophageal squamous cells

To independently quantitate the results of the histologic analysis, proliferating cell nuclear antigen (PCNA) assays were performed on proximal and distal

Table 2 Histology scores from NMBA treated cyclin D1 and FVB/N wild type mice. Mean histology scores were calculated for each of the treatment groups based on criteria outlined in the Materials and methods. Comparisons of genotype, treatment duration, and age at examination of esophagi were made utilizing the general linear model procedure. *P* values are shown for each comparison

Genotype	Duration	Endpoint	Mean histology score
FVB/N-D1	5	12	2.00 ± 0.82
FVB/N-D1	5	15	2.50 ± 0.58
FVB/N-D1	15	12	1.75 ± 0.50
FVB/N-D1	15	15	2.75 ± 0.50
FVB/N-/-	5	12	1.00 ± 0.82
FVB/N-/-	5	15	1.75 ± 0.50
FVB/N-/-	15	12	1.25 ± 0.50
FVB/N-/-	15	15	1.75 ± 0.96

Genotype (FVB/N-/- vs D1) *P* = 0.0011; Treatment duration (5 vs 15 week) *P* = 0.7812; Endpoint (12 vs 15 month) *P* = 0.0045

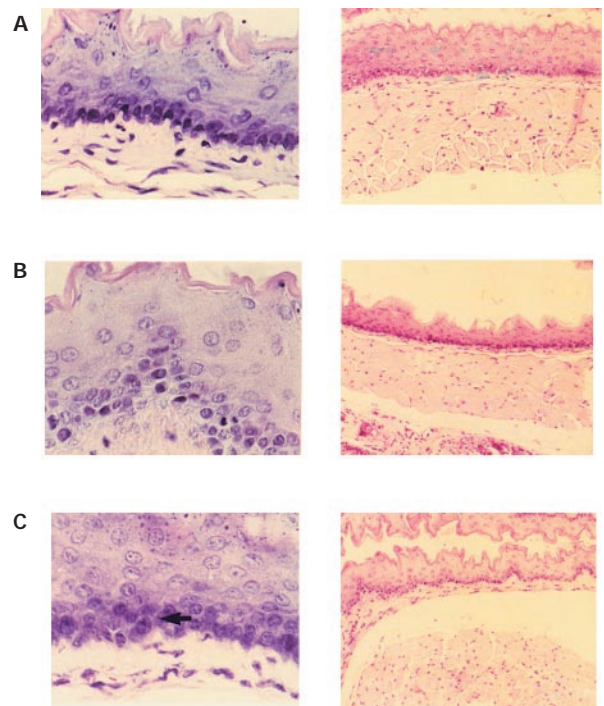


Figure 3 Hematoxylin and eosin stained sections (left, 320 ×; right, 100 ×) of proximal esophagi from 12 month old mice; (a) Untreated FVB/N, demonstrating normal histology; (b) NMBA treated FVB/N demonstrating mild dysplasia; (c) NMBA treated FVB/N-cyclin D1 demonstrating features of moderate-severe dysplasia including nuclear enlargement and hyperchromasia (arrow) and persistence of dysplastic cells into the suprabasal, intermediate and superficial layers

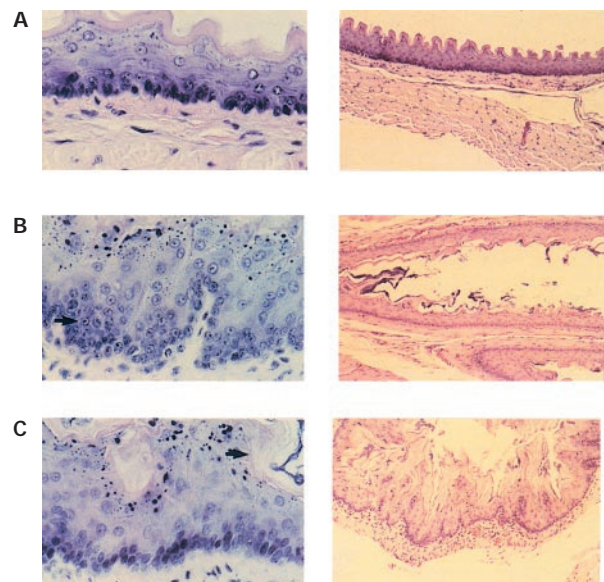


Figure 4 Hematoxylin and eosin stained sections (left, 320 ×; right, 100 ×) of distal esophagi from 12 month old mice; (a) Untreated FVB/N with normal distal esophageal histology; (b) NMBA treated FVB/N demonstrating mild dysplasia with abnormal nuclear morphology. Additionally there are large nucleoli (arrow); (c) NMBA treated FVB/N-cyclin D1 with moderate to severe dysplasia, including crowding and hyperchromasia of cells in the basal layer. This section also demonstrates prominent disorganization of the superficial keratin layer (arrow)

esophageal specimens from all mice. A labeling index (described in methods section) was calculated for each specimen, allowing for comparison between the groups. These data are summarized in Table 5. Sections were numerically coded and scores were calculated in a blinded fashion. The degree of histologic abnormality correlated with increased PCNA expression in comparisons between wild type and cyclin D1 treated and untreated mice. Thus, PCNA scores were highest in the NMBA treated cyclin D1 mice.

Histologic scores were higher at the 15 month time point than at 12 months. The PCNA scores, however, remained constant or decreased as the mice aged (Table 3). Representative PCNA stained esophageal

sections from NMBA treated cyclin D1 transgenic mice are shown in Figure 5, and demonstrate increased PCNA staining in the basal and suprabasal layers. Untreated age matched wild type control sections are included for comparison and demonstrated a typical pattern of staining in the basal layer.

None of the mice developed squamous cell carcinoma of the esophagus during the study. Cancer of this type has been induced in mice by repeated oral gavage of a solution of nitrosamine and ethanol (Odeleye *et al.*, 1992a,b). This regimen was avoided in favor of subcutaneous NMBA which induces the development of dysplasia and which is dosed with higher accuracy and reproducibility.

Discussion

Esophageal squamous cell carcinoma (ESCC) remains a prevalent form of cancer worldwide despite recent advances in the understanding of the epidemiology of this tumor. Associated environmental and genetic factors have been identified. Abnormalities in cyclin D1 expression (Jiang *et al.*, 1992, 1993; Nakagawa *et al.*, 1995; Sheyn *et al.*, 1997) and exposure to nitrosamines (Barch *et al.*, 1986; Craddock and Driver, 1987; Druckrey *et al.*, 1963; Hodgson *et al.*, 1982; Lijinsky *et al.*, 1982; Stinson *et al.*, 1978) appear to be particularly important in its pathogenesis. Nevertheless, it has been difficult to merge these two factors into a unified model of disease progression. The goal of this study was to determine whether cyclin D1 and NMBA cooperate in the development and progression of squamous dysplasia.

We have shown previously that mice overexpressing the cyclin D1 oncogene develop dysplasia that increases in severity throughout their lifespan of 24 months (Nakagawa *et al.*, 1997). In the first part of this study, we drew on extensive experimental work with rats using the nitrosamine NMBA to design a protocol that would induce similar changes in mice. Similar to the histologic changes observed in the esophagi of cyclin D1 transgenic mice, NMBA treated wild type mice with two different genetic backgrounds developed dysplasia by 8 months of age. There was no difference observed in the NMBA doses employed in this study, although further analysis with varying combinations of duration of exposure and dose would be required to conclusively establish a dose-response relationship or absence thereof. Of note, higher NMBA doses have been reported to be toxic in rats (unpublished observations, G Stoner) and were avoided in this study.

The combination of cyclin D1 and NMBA in the FVB/N background resulted in increased dysplastic severity when compared with histologic changes observed in NMBA treated FVB/N mice and untreated cyclin D1 mice. The increased severity of dysplasia was accompanied by a statistically significant increase in PCNA staining in the treated cyclin D1 mice. The mechanism for the slight decrease in PCNA index within a each treatment group at the 15 month time point is uncertain. This observation does, however, reflect our previous similar observation in cyclin D1 transgenic mice (Mueller *et al.*, 1997)

The findings in this study suggest an additive effect of cyclin D1 and NMBA. It is possible that both

Table 3 PCNA scores from NMBA treated cyclin D1 and FVB/N wild type mice. PCNA scores were calculated for each of the treatment groups, based on criteria outlined in the Materials and methods section. Comparisons were made for the variables shown below, using the general linear model procedure. *P* values are shown for each comparison

Genotype	Duration	Endpoint	Mean histology score
FVB/N-D1	5	12	59.1±2.4
FVB/N-D1	5	15	50.9±3.3
FVB/N-D1	15	12	61.8±5.5
FVB/N-D1	15	15	59.3±2.9
FVB/N-/-	5	12	41.1±2.5
FVB/N-/-	5	15	37.8±1.3
FVB/N-/-	15	12	40.0±2.4
FVB/N-/-	15	15	35.5±3.0

Genotype (FVB/N-/- vs D1) *P*=0.0001; Treatment duration (5 vs 15 week) *P*=0.1658; Endpoint (12 vs 15 month) *P*=0.0011

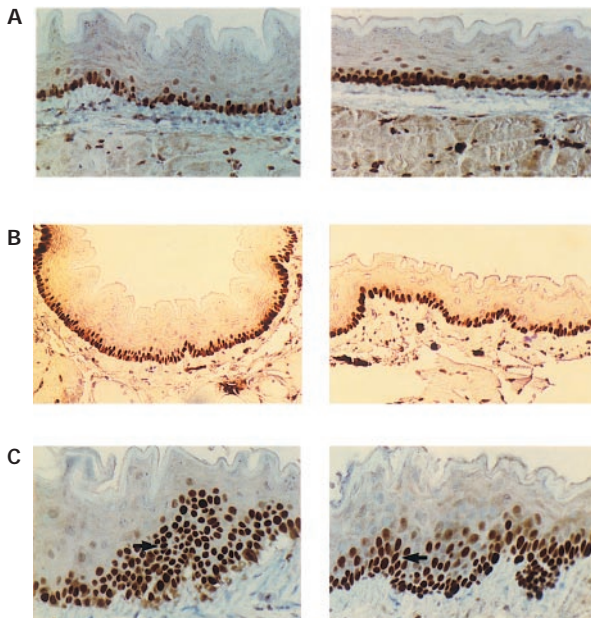


Figure 5 PCNA stained sections of esophagi from 12 month old NMBA treated FVB/N-cyclin D1 and untreated wild type FVB/N mice (left, proximal esophagus; right, distal esophagus). (a) Esophagus from wild type mouse demonstrates normal proliferative pattern in the basal layer; (b) Esophagus from untreated cyclin D1 transgenic mouse demonstrates expansion of the proliferative zone into the suprabasal region; (c) Esophagus from NMBA treated cyclin D1 transgenic mouse shows markedly increased PCNA staining reflecting expansion of the proliferative zone in the basal and suprabasal layers (arrow)

contribute to increased cell proliferation, either through direct or indirect mechanisms. It is interesting to note that differences in rat versus mouse esophagi are present after NMBA treatment. In particular, rat esophagi develop dysplasia, papillomas and eventually may manifest cancer when treated with NMBA. While the biochemical basis for this interspecies difference is not known, it may reflect a difference in the metabolism of NMBA (Labuc and Archer 1982; Stoner *et al.*, unpublished observations).

The absence of cancer in the NMBA treated cyclin D1 mice supports the notion that several factors may be required to recapitulate squamous cancer. It has long been recognized that a combination of two or more factors is needed to recapitulate squamous skin cancer. For example, a combination of 7,12-dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is required to induce skin cancer in mice (reviewed in Balmain and Brown, 1988). In contrast, when cyclin D1 is targeted to the skin by the keratin 5 promoter, the resulting phenotype is one of increased cellular proliferation and basal cell hyperplasia but without cancer (Robles and Conti 1995). Hyperplasia, dysplasia and papillomatosis develop in the skin when the early region of human papillomavirus 16 (HPV 16) is targeted to this site with the keratin 14 enhancer/promoter (Albert *et al.*, 1994). These HPV 16 mice also do not develop skin cancer. A pattern emerging from these studies of the skin is that an oncogene, either cellular or viral, is not sufficient by itself to induce a squamous epithelial cell cancer. For cancer to develop, additional factors, either genetic or environmental, are necessary in this tissue and cell type.

Determination of the proper combination of genetic and environmental factors necessary to induce a squamous cancer is of paramount importance. In our model, cyclin D1 together with NMBA increase the severity of dysplasia, the prominent precursor of cancer. Additional factors may be required to promote cancer. Likely possibilities include p53 inactivation, Ha-*ras* activation, epidermal growth factor receptor overexpression, and other chemical carcinogens. Absence of p53 in genetically engineered mice (Donehower *et al.*, 1992) has not been shown to initiate or promote the development of skin cancer by DMBA/TPA, but has been shown to enhance the progression of these tumors (Kemp *et al.*, 1993). Ha-*ras* mutations appear to be critical in the DMBA/TPA model of skin cancer (Quintanilla *et al.*, 1986; Brown *et al.*, 1990). However, these mutations are not present in skin cancers induced with other carcinogens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or methylcholanthrene suggesting that other genes are targets (Balmain *et al.*, 1988; Brown *et al.*, 1990).

The combination of cyclin D1 overexpression and NMBA provides new insights into the molecular pathogenesis of ESCC. Establishment of this murine model provides a basis for study of the underlying genetic mechanisms which lead to environmentally induced and genetically predisposed ESCC. It is conceivable that cooperation between cyclin D1 and NMBA is mediated through cell cycle abnormalities occurring in G1 and S phases. We are hopeful that further studies will reveal novel information about the relative importance of a variety of genetic alterations

known to exist in ESCC, and provide a framework for understanding the progression of changes necessary for the development of ESCC.

Materials and methods

Chemicals

NMBA with an estimated purity of >99% as determined by HPLC (NCI Chemical Carcinogen Repository) was used. It was diluted in 20% DMSO, divided into aliquots, and stored in a light protected container at -20°C. It was administered subcutaneously using a 27 gauge needle and the dose was determined with a weight based algorithm.

Mice

Wild type FVB/N and C57BL/6 mice were obtained from Taconic Labs. Generation of the cyclin D1 transgenic mice (FVB/N background) was described previously (Nakagawa *et al.*, 1997). The mice were housed in stainless steel cages in a climate controlled room (four per cage) with a 12 h light and dark cycle. Fresh tap water and standard mouse chow were provided *ad libitum*. The animal room was cleaned daily and cages were changed on a regular basis to ensure hygienic conditions. This study was approved by the Massachusetts General Hospital Animal Studies Committee.

After an acclimation period, 4 week old male FVB/N wild type and cyclin D1 transgenic and male C57BL/6 mice received subcutaneous injections (right lower abdominal mid-axillary region) of NMBA in 20% DMSO in a dose according to weight. Animals were divided into groups to receive NMBA according to the schedules shown in Table 1 and 3. Mice were euthanized at the ages shown in Table 1 and 3. A previous study (Siglin *et al.*, 1996) determined that subcutaneous administration of 20% DMSO in distilled water has no esophageal effects; thus, vehicle control mice were not included in the present studies. Mice were monitored daily and weight was recorded weekly.

Genotyping cyclin D1 transgenic mice

One hundred and twenty mice derived from a single founder line previously shown to overexpress cyclin D1 in the esophageal squamous epithelium were analysed for transgene incorporation. This was performed using Southern blot analysis of *Pst*I digested genomic DNA, with a random primed human cyclin D1 cDNA (1.4 kb) radiolabeled probe. Genomic DNA was extracted and purified from mouse tails as previously described (Nakagawa *et al.*, 1997). Ten μ g of genomic tail DNA was digested with *Pst*I, fractionated with an 0.9% agarose gel, transferred onto a Hybond-N membrane and hybridized (Rapid hybridization kit, Amersham) with a random-primed α -³²P-dCTP-labeled human cyclin D1 cDNA probe (Megaprime labeling kit, Amersham).

Histology and immunohistochemistry

After CO₂ asphyxiation, an autopsy was performed and the tissues were examined under magnification for any gross abnormalities. The esophagus was excised, opened longitudinally and examined under magnification for the presence of any gross abnormality. The esophagi were then divided into two parts (proximal and distal), fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic evaluation. Other tissues, such as tongue, pharynx, trachea, stomach, liver and lung were examined as well. Histologic evaluation was performed by two of the authors (TDJ and

LRZ) both blinded to the experimental conditions. The following scoring system was established by our group to assess the relative severity of dysplastic change: 0-normal; 1-mild dysplasia; 2-moderate dysplasia; 3-severe dysplasia; 4-carcinoma. An esophageal dysplasia score was calculated by adding scores from the proximal and distal esophagus. In addition, a total dysplasia score was obtained by adding scores from tongue, proximal and distal esophagus, and forestomach

Immunohistochemical analysis was performed with the use of an antibody to proliferating cell nuclear antigen (PCNA antibody PC-10, Pharmingen) as described previously (Mueller *et al.*, 1997). Esophageal (proximal and distal) 3–5 μm tissue sections were dried at room temperature for 2 days. Then the sections were baked at 60°C in a microwave oven for 20 min, deparaffinized in xylene and rehydrated in alcohol and water. All slides were then microwave treated (800 W) in 10 mM sodium citrate (pH 6.0) at 93°C for 30 min. The slides were then cooled for 15 min and transferred to phosphate-buffered saline (PBS). After blocking with 1.5% horse serum in phosphate-buffered saline (PBS) for 15 min, the slides were incubated with the PCNA primary antibody at room temperature. After washing with PBS, slides were incubated for 30 min with a 1:200 dilution of the appropriate biotinylated secondary antibody, washed with PBS, and then incubated for 40 min with the avidin and biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Lab, Burlingame, CA, USA) at room temperature. After washing with PBS, the colorimetric reaction was obtained with 3,3-diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) and 0.1% H_2O_2 in 0.5 M TrisHCl buffer (pH 7.6). The sections were counterstained with hematoxylin, dehydrated and mounted in Permount (Fisher, Pittsburgh, PA, USA). Human tonsillar tissue

sections served as positive controls for the PCNA antibody. Normal horse serum was used as the primary antibody to serve as a negative control.

The mean percentage of PCNA staining for an individual tissue section was determined by counting the number of positively stained cells out of a total of 500 cells. Ten fields at 400 \times magnification were counted for each specimen.

Statistical analysis

Statistical analysis involved a three factor analysis of variance utilizing a general linear model procedure to evaluate comparisons between cyclin D1 and wild-type mice with and without NMBA treatment, the two different dosing schedules and the two time points at which tissues were examined histologically. Means and standard deviations were calculated, and $P < 0.05$ was considered to be statistically significant in the comparisons.

Acknowledgements

This work was supported by the ADHF/AGA Funderberg Awards (AR), an ACS Jr Faculty Research Award (AR), NIH DK53377 (AR), NIH P01 DE12467-01A1 (AR, RK), a Glaxo Wellcome Institute for Digestive Health Basic Research Award (TJ), an NIDDK K08 Award (TJ) and a Center for the Study of Inflammatory Bowel Disease Award from NIH 5P30DK 43357-08 (TJ). A. Mueller was supported by the Deutsche Forschungsgemeinschaft (DFG #1304-1-1). R. Odze was supported by the Stanley L. Robbins Research Fund from the Department of Pathology at Brigham and Women's Hospital.

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