Methylation Profile and Amplification of Proto-Oncogenes in Rat Pancreas Induced with Phytoestrogens (43842)

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Abstract. **Specific gene hypermethylation has been shown in DNA from neonatal rats exposed to the phytoestrogens, coumestrol, and equol. The pancreas is an organ in which estrogen receptors have been shown to be present. Studies have correlated the development of acute pancreatitis with rising levels of human estrogen binding proteins. Neonatal rats were dosed with 10 or 100 μg of coumestrol or equol on postnatal day (PND) 1–10. The animals were sacrificed at Day 15. The pancreas was excised and pancreatic acinar cells isolated for molecular analysis. DNA was isolated from the cells by lysis in TEN-9 buffer supplemented with proteinase K and 0.1% SDS. High molecular weight (HMW) DNA was digested with the methylated DNA specific restriction enzymes, Hpa II and Msp I, for determination of methylation profiles. Both coumestrol and equol at high doses caused hypermethylation of the c-H-ras proto-oncogene. No hypermethylation or hypomethylation was observed in the proto-oncogenes, c-*myc* or c-*fos*. Methylation is thought to be an epigenetic mechanism involved in the activation (hypomethylation) or inactivation (hypermethylation) of cellular genes which are known to play a role in carcinogenesis. Epidemiology studies have shown that equol may have anti-carcinogenic effects on some hormone-dependent cancers. Additional studies are needed to further understand the role of phytoestrogens and methylation in relation to pancreatic disorders.**

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Phytoestrogens, or plant-derived estrogens, occur in an abundance of foods, including fruits, seeds, rice, beans, and soybean products (1, 2). These compounds are known to have a variety of characteristics. Studies have shown that a number of these compounds have low estrogenic, antiestrogenic, anticarcinogenic, antifungal, and antiviral activity (3). Equol is an isoflavonic phytoestrogen, and coumestrol is a member of the coumestan series. Equol is also a well known metabolite of formononetin which is metabolized by the intestinal microflora. The pancreas, which is not known to be responsive to estrogens, possesses estrogen receptors (4). The effects of phytoestrogens on the exocrine pancreas are currently not known. However, increased levels of human estrogen binding proteins have been associated with acute pancreatitis (5). Pancreatitis and various alterations in pancreatic functions have been reported to occur with estrogen therapy in the exocrine pancreas (6). There is no experimental evidence that directly suggests that estrogens might play a role in the pathogenesis of pancreatitis or pancreatic carcinogenesis.

This study examined the mechanism by which phytoestrogens exert their effects on the exocrine pancreas. The present study examined the effects of coumestrol and equol on the methylation profile of specific proto-oncogenes (c-Ha-ras, c-*fos*, and c-*myc*). Methylation is a mechanism thought to inactivate cellular genes, while demethylation may activate them. Hypomethylation of H-ras has been shown to play a role in liver (7) and pancreatic carcinogenesis (8). The ras gene family has been shown to be asso-

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ciated with morphological and transformational changes in the pancreas (9, 10). The H-ras gene product, p21, is known to function as a G-protein, thus playing a role in the signal transduction pathway. Alteration of the signal transduction pathway often leads to generation of second messengers in the cell such as diacylglycerol (DAG), calcium (Ca^{++}), and inositol triphosphate (IP_3). These second messengers can exert additional changes in cell functions. Alterations in the signal transduction pathway has been implicated in the initiation stage of carcinogenesis (11). Understanding the mechanism of action of phytoestrogens on the exocrine pancreas will enhance our knowledge in their potential use as anticarcinogenic therapeutics.

Materials and Methods

Animals, Treatment, and Cell Culture. Fourteen neonatal Sprague-Dawley rats were injected with 10 or 100 μg of coumestrol or equol purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA) on postnatal day (PND) 1–10 (Medlock *et al.*, this volume). These animals, obtained from the Division of Developmental Toxicology, were offspring of date-mated rats from the NCTR breeding colony. The animals were culled according to sex and the females randomly distributed to foster dams within 24 hr of birth. The animals were sacrificed at Day 15 and their pancreata excised.

Pancreatic acinar cells were isolated for DNA analysis and cell culture. Pancreatic acinar cells were separated from islet cells by centrifugation. The cells were cultured on positively charged Primaria dishes (Becton-Dickinson) and maintained in Williams' E medium supplemented with 10% fetal bovine serum, 2 m*M* glutamine, and 1% penicillin-streptomycin antibiotic (GIBCO) (8).

DNA Isolation, Digestion, and Methylation Studies. High molecular weight (HMW) DNA was isolated from primary and secondary cultures (Passage 3) of pancreatic acinar cells (8). Cells were lysed in TEN-9 buffer (Tris pH 9, EDTA, and NaCl) containing 0.1% SDS and 500 μg/ml of proteinase K (7). After treatment with DNase-free RNase, the DNA was extracted twice with phenol and once in chloroform. After recovery in ethanol, the degrees of methylation of the cellular homologs of the oncogenes, c-H-Ras, c-*fos*, and c-*myc* in the DNA samples from each dose group were analyzed by using enzymes which detect CpG methylation sites on DNA. The restriction enzymes, Hpa II and Msp I, were used to differentiate between methylated and unmethylated sequences in HMW DNA from each control and experimental group. DNA was also digested with the restriction enzyme, Hind III for specific H-Ras fragment amplification. All restriction enzymes were obtained from Bethesda Research Laboratories. After digestion, the DNA was subjected to electrophoresis and transferred to Hybond nylon filter for DNA hybridization. The filters were probed with cDNA [^{32}P]labeled H-Ras, *myc*, or *fos* probes (Clontech Laboratories). The filters were prehybridized and hybridized under stringent conditions (25 m*M* $KHPO_4$, 5× SSC, 5× Denhardt's, 50% formamide, 1% SDS) at 42°C for 18 hr. The filters were washed and subjected to Betascope analysis or autoradiography. For gene amplification, the DNA was applied at 2 μg/slot on N-bond nylon filters using a slot-blot apparatus. The slot-blot was prehybridized and hybridized with the above [^{32}P]labeled c-DNA probes.

Results

In order to determine overall methylation profiles of DNA from both the control and experimental groups, digested DNA was observed in ethidium bromide stained gels. Figure 1 represents an ethidium bromide stained gel with DNA samples digested with Hpa II and Msp I. Lane 3, 5, 7, 9, and 11 demonstrate Msp I digestions and Lane 2, 4, 6, 8, and 10 represent Hpa II digestions. The most significant data in this gel appears in Lane 6, from an animal given a high dose of equol and digested with Hpa II which generated a more tightly condensed band than the control in Lane 2. Thus, at the highest dose of equol, the DNA is hy-

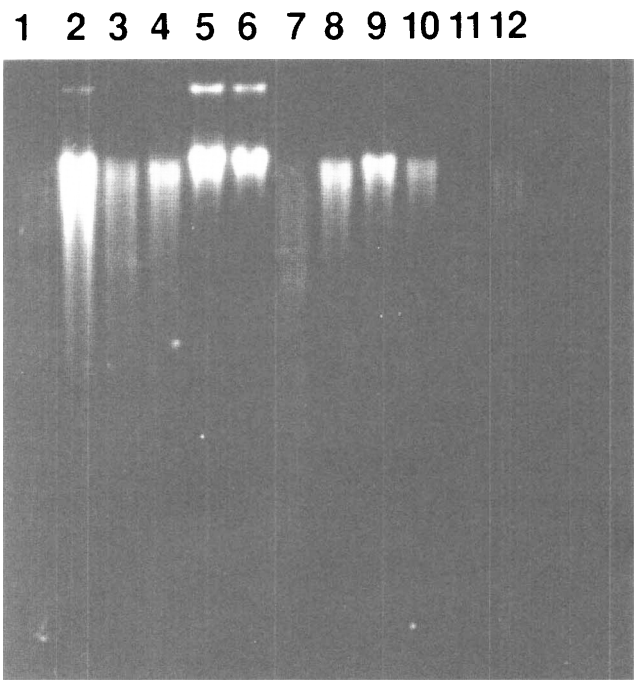

Figure 1. A micrograph of an ethidium bromide-stained agarose gel with DNA samples digested with Msp I or Hpa II methylase. Hpa II digests (Lane 2, 4, 6, 8, and 10) demonstrated that equol at the high dose (Lane 6) showed a hypermethylated overall profile compared with the control (Lane 2). It was also noted that at the high dose of equol, Msp I did not cut the DNA (Lane 5).

permethylated. Lane 5, which is from the high dose group of equol and was digested with Msp I, shows a tight high molecular weight band. Msp I cuts CpG sites whether a methyl group is present or not. This condensed band may represent a highly coiled DNA molecule which could interfere with the action of the enzyme, Msp I.

Figure 2 illustrates an autoradiograph of the methylation profile of the c-H-ras gene. Lanes 1, 3, 5, 7, 9, and 11 represent Hpa II digestions and Lane 2, 4, 6, 8, and 10 represent Msp I digestions. Lane 1 and 2 are control samples of DNA. Lane 6 (high-dose equol) and 10 (high-dose coumestrol) show increased fragments with Msp I that are not present in the Hpa II lanes. This increase represents more hypermethylated sites in the DNA samples. Lane 5 and 9 demonstrated hypermethylated DNA in the Hpa II digested DNA samples when compared with Lane 1 (control). Additionally, we examined the methylation profiles of c-*myc* and c-*fos*, and found no evidence of hypomethylation or hypermethylation when compared with the control (data not shown).

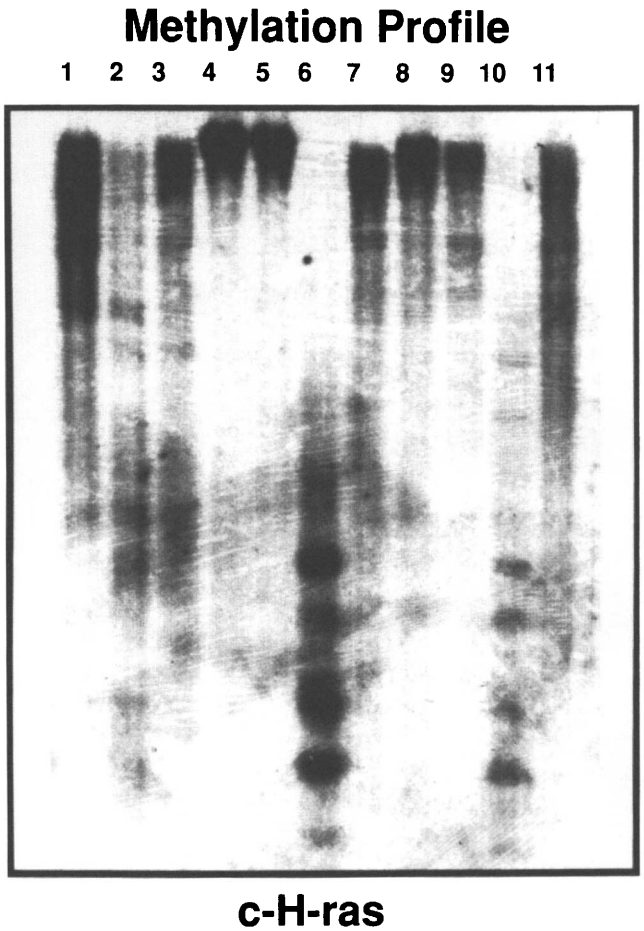

Figure 2. An autoradiograph of the methylation profile of the c-H-Ras gene. Lane 1, 3, 5, 7, 9, and 11 represent Hpa II digestions, and Lane 2, 4, 6, 8, and 10 represent Msp I digestions. Lane 1 and 2 are control samples of DNA. Lane 4 (high-dose equol) and Lane 8 (high-dose coumestrol) showed highly condensed HMW bands when cut with Msp I. Hpa II digestion demonstrated a hypermethylated profile of the H-Ras gene (Lane 3, 5, 7, 9, and 11) when compared with the control (Lane 1).

Examining gene amplification at specific sites (Fig. 3) in the DNA from both the control and experimental groups revealed significant differences in the high-dose group. The control samples show two Hind III sites in the c-H-ras gene. In the low dose of equol (Lane 3) and coumestrol (Lane 5 and 7), two similar Hind III sites are observed. However, again in the high-dose groups of equol (Lane 4) and coumestrol (Lane 6), the Hind III sites are not present. Overall amplification of the c-H-Ras gene in the DNA samples from the low-dose groups of equol and coumestrol are shown in Figure 4. Coumestrol decreased in gene amplification when compared with the control.

Discussion

Methylation is an epigenetic mechanism thought to play a role in gene activation and inactivation. Hypomethylation has been associated with the activation of specific genes that are known to play a role in carcinogenesis (11). Methylation is also thought to play a role in the condensation of the DNA molecule. Hypersensitive sites, which are detected by DNase I digestion, have been associated with regions of the DNA

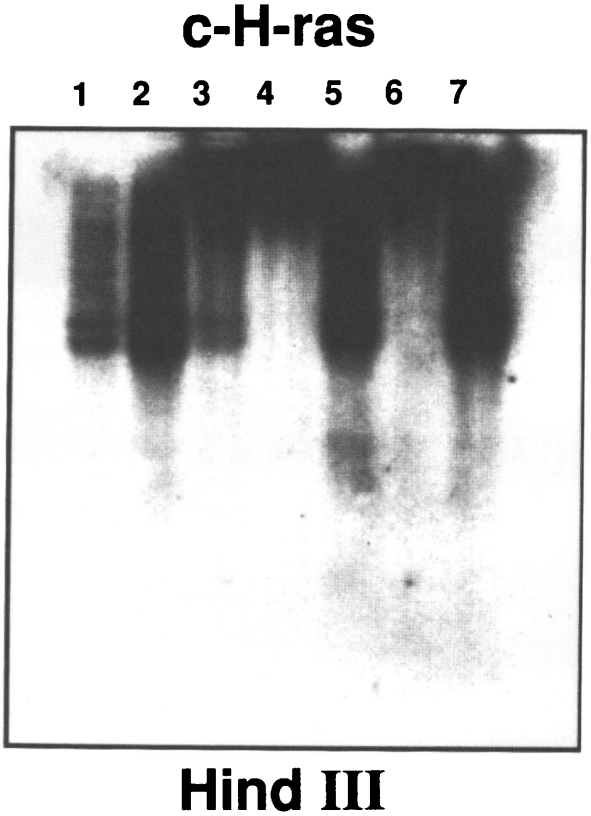

Figure 3. Amplification of H-Ras specific Hind III sites. Two distinct bands are noted in Lane 1 and 2 in control DNA samples. These bands are present in the low dose of equol (Lane 3) and coumestrol (Lane 5 and 7). However, at the high doses of equol and coumestrol (Lane 4 and 6) there were no bands detected.

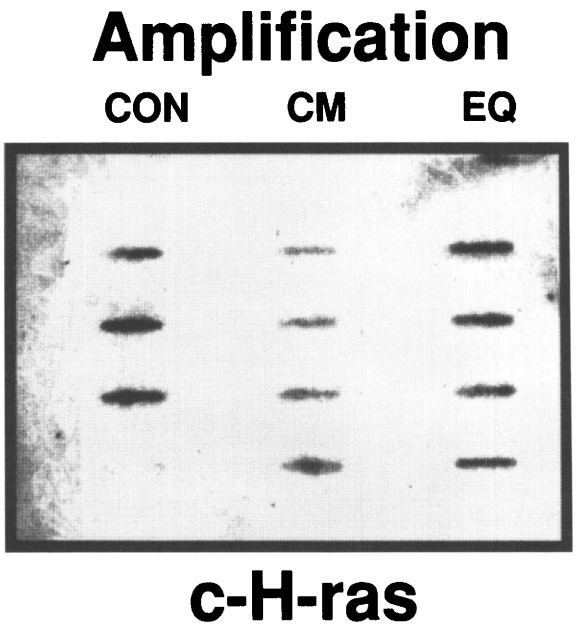

Figure 4. Slot-blot analysis of H-ras gene amplification. Note the decrease in gene amplification in the coumestrol sample (CM) compared with the control, (CON). Also note the decrease in amplification in the equol sample, (EQ) when compared with the control.

that contain no methyl groups. These studies show that equol and coumestrol treatment resulted in hypermethylated DNA profiles in the H-ras gene. This suggests that they may play a role in "turning off" genes which may be involved in the development of pancreatic cancers. Activation of both H- and K-ras protooncogenes has been shown to enhance the development of pancreatic cancer (12). Hypermethylation (inactivation) of the H-ras gene by the phytoestrogens, equol and coumestrol, suggests that phytoestrogens may exert anticarcinogenic effects on a specific protooncogene in the exocrine pancreas through this epigenetic mechanism, thus turning off genes that may play a role in carcinogenesis. Activation of the H- and K-ras genes, by epigenetic or point mutations, is known to play a role in carcinogenesis of a number of tumors, including pancreatic cancer (13).

The phytoestrogens may also affect the methylation status of the estrogen receptor (ER) gene. Estrogens are known to increase the incidence of hormone-dependent cancers through their receptor (14). Recently, abnormal DNA methylation in transcriptional inactivation of the ER gene has been demonstrated (15). These studies showed that estrogen-negative human breast cancer cells growing in culture lack ER RNA; however they have a higher capacity to methylate DNA, and display extensive methylation of the CpG island in the 5′ promoter region of the ER gene, which would correlate with silencing of expression. Methylation in CpG islands *in vitro* can block transcription of downstream sequences. The investigators suggested that increased methylation of the ER gene would block it's transcription without a structural alteration in the gene's CpG island in the 5′ regulatory region. Further molecular studies are needed to examine the role of phytoestrogens on the methylation of specific CpG sites in the ras and the ER gene in rat exocrine pancreas. The present study was carried out on neonatal animals, and studies are currently ongoing to examine the effects of phytoestrogens on adult pancreatic acinar cells.

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