Noninvasive Detection of Putative Biomarkers for Colon Cancer Using Fecal Messenger RNA[1]

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Abstract

Deaths from colon cancer number over 60,000 each year in the United States. Because early detection results in a high cure rate, development of noninvasive techniques for detection of colon cancer has received much interest. The ability to detect early changes in colonocyte genes and gene expression would provide valuable information. We have shown previously that alterations in protein kinase C (PKC) isoform expression are associated with changes in colonic cell proliferation, a key intermediate marker for the prediction of tumorigenesis. Here, we describe a method for the quantitative detection of mRNAs for select PKC isoforms isolated from rat feces containing exfoliated colonocytes. After total RNA extraction from fresh fecal material, polyadenylated RNA was selectively purified and quantitated with slot blotting and hybridization to oligodeoxythymidylic acid. Fecal polyadenylated RNA was used for semiquantitative (mimic) RT-PCR to quantitate PKC isoform mRNA expression. We detected mRNA for PKC-α, PKC-δ, PKC-ϵ, and PKC-ζ, but not for PKC-β or PKC-γ, which is consistent with the profile of isoforms detected previously in scraped colonic mucosa using immunoblot analysis. This noninvasive method, utilizing feces containing exfoliated colonocytes, is a sensitive noninvasive technique for quantitating luminal mRNAs. This provides a means to monitor gene expression of colonic epithelial cells, which may have predictive value in monitoring the neoplastic process.

Introduction

Colorectal cancer is the second most common cause of cancer-related mortality, with over 60,000 deaths in the United States each year (1, 2). With early detection, colorectal cancer has a high cure rate (3, 4). However, in general, detection involves invasive procedures, such as sigmoidoscopy and biopsy, with their inherent drawbacks and risks. The development of non-invasive techniques that provide early prognostic information would greatly aid in the management of this disease, resulting in early detection and treatment. It is known that several oncogenes, tumor suppressor genes, and intracellular signal transduction anomalies are involved in the multistep process of colon carcinogenesis (5, 6). The ability to detect gene mutations or to quantify levels of aberrant messages would provide valuable information as to the presence and/or progression of cancer.

Alteration in the expression and activation of specific PKC[3] isoforms plays a role in the malignant transformation process of the colon (7–11). We have demonstrated previously that levels and activity of several PKC isoforms in colonic mucosa, which are associated with changes in cell proliferation and differentiation, can be altered by diet (12, 13). In addition, it has been shown that overexpression of PKC-β_1 in HT29 cells, a colon cancer cell line, causes growth inhibition and tumor suppression (14). PKC isoforms can be detected in viable, exfoliated colonic cells isolated from human feces by using immunoblotting techniques (15). This technique provides an avenue for examining a host of factors with potential effects on PKC signal transduction. Approximately one-sixth to one-third of normal adult colonic epithelial cells are shed every day (16). This corresponds to the daily exfoliation of approximately 10^{10} cells. However, because the number of intact cells isolated from fecal material is relatively low, an enhanced detection system is required to amplify potential intermediate biomarkers of colon cancer. Therefore, we have optimized the use of semiquantitative "mimic" RT-PCR to detect the expression of genes with potential diagnostic value in the colon. Specifically, the isolation and quantitation of mRNA for several PKC isoforms, as well as cytokeratin from rat colonic epithelial cells sloughed into the feces is described. This experimental approach provides a sensitive method for detection of mRNA isolated from feces containing exfoliated colonocytes and a noninvasive means for monitoring changes in this population of cells.

Materials and Methods

Animals. All experimental procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Texas A&M University. As part of a larger study, male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX) were fed diets differing in type of fat (corn or fish oil) and type of fiber (cellulose or pectin). After 5 months on the experimental diets, feces were collected from two animals in each of the four dietary groups.

RNA Isolation from Feces. Total RNA was isolated from rat feces using Ambion Totally RNA kit (Austin, TX). Immediately after defecation, 1–2 fecal pellets (0.2–0.5 g) were collected from rats and placed into cold denaturation solution

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[3] The abbreviations used are: PKC, protein kinase C; poly(A)$^+$ RNA, polyadenylated RNA; oligo (dT), oligodeoxythymidylic acid; RT, reverse transcriptase; SSPE, saline-sodium phosphate EDTA (1× SSPE = 10 mM NaH_2PO_4, 150 mM NaCl, 1 mM EDTA, pH 7.4).

(Ambion) and processed as per the kit instructions. The RNA pellet was resuspended in 600 μl 0.1 mM EDTA. Analysis on an agarose-formaldehyde denaturing gel indicated the presence of a significant amount of bacterial RNA (Fig. 1). To enrich the level of mammalian mRNA, poly(A)$^+$ RNA isolation was performed by using oligo (dT) cellulose micro spin columns (Collaborative Biomedical Products, Bedford, MA) and mRNA isolation buffers from BioTecx (Houston, TX). The resultant poly(A)$^+$ pellet was resuspended in 60 μl 0.1 mM EDTA and stored at −80°C before quantitation.

Quantitation of poly(A)$^+$ RNA. To normalize starting material for RT-PCR, poly(A)$^+$ RNA was quantitated by slot blotting and detection with biotinylated oligo (dT) probe (Promega, Madison, WI) (17). Serial dilutions of fecal poly(A)$^+$ RNA or total rat colonic mucosal RNA of known concentration (as calculated from absorbance at 260 nm) as the standard were blotted onto a positively charged nylon membrane (Boehringer Mannheim, Indianopolis, IN) by using a Hoefer slot blot filtration manifold (Hoefer, San Francisco, CA). RNA was diluted and membrane was prewetted in 50 mM sodium phosphate buffer, pH 6.8. To determine whether populations of nucleic acids other than rat poly(A)$^+$ RNA were hybridizing to the oligo (dT) probe, bacterial rRNA (Boehringer Mannheim), total bacterial RNA (isolated from rat colonic mucosa) and rat DNA (isolated from rat colonic mucosa with Ambion Totally RNA kit) were also blotted onto the membrane. The membrane was UV cross-linked with a Stratagene Stratalinker (La Jolla, CA) and hybridized with biotinylated oligo (dT) [50 pmol/ml hybridization solution, containing 5× SSPE, 5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% acetylated BSA), 0.1% SDS, and 50% formamide] at 44°C for 1 h followed by washing in 2× SSPE plus 0.1% SDS and 0.1× SSPE plus 0.1% SDS. Detection was with Gene Images Nucleic Acid Detection kit (United States Biochemical, Cleveland, OH) by using streptavidin-alkaline phosphatase followed by chemiluminescent substrate, Lumi-Phos 530 (Boehringer Mannheim). After film development, (Kodak X-OMAT; New Haven, CT), blots were quantitated on a Molecular Dynamics Computing densitometer (Sunnyvale, CA) and compared to a standard curve generated from rat colon RNA. The linear range of the colon RNA standard curve was used for quantitation of sample poly(A)$^+$ RNA concentrations. For simplicity of presentation, it is estimated that total colon RNA contains 2% poly(A)$^+$ RNA, based on the fact that typical concentrations of poly(A)$^+$ RNA are 1–4% of total RNA (18). With this assumption, relative quantitative values can be assigned to the poly(A)$^+$ samples, ensuring that equal amounts of isolated poly(A)$^+$ RNA are used for RT-PCR.

RT-PCR. Approximately 70–140 pg of poly(A)$^+$ RNA was reverse transcribed into cDNA by using Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). A negative control contained no RT. A 50-μl PCR reaction contained the following: 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 1× Taq DNA polymerase buffer, 2% DMSO, 0.5 ng/μl concentrations of each forward and reverse primer, 1.25 units of Taq DNA polymerase (GIBCO-BRL), and 10 μl of the RT reaction. The product sizes and primers used were as follows: PKC-α (325 bp), 5′-TGAACCCTC AGTGGAATGAGT-3′ (forward), 5′-GGCTGCTTCCTGTCTTCTGAA-3′ (reverse); PKC-β (639 bp), 5′-TGTGATGGAGTATGTGAACGGGGG-3′ (forward), 5′-TCGAAG TTGGAGGTGTCTCGCTTG-3′ (reverse); PKC-γ (347 bp), 5′-TTGATGGGGAAG ATGAGGAGG-3′ (forward), 5′-GAAATCAGCTTGGTCGATGCTG-3′ (reverse); PKC-δ (352 bp), 5′-CACCATCTTCCAGAAAGAACG-3′ (forward), 5′-CTTGCCATA GGTCCCGTTGTTG-3′ (reverse); PKC-ε (389 bp), 5′-CGAGGACGACTTGTTTGA ATCC-3′ (forward), 5′-CAGTTTCTCAGGGCATCAGGTC-3′ (reverse); PKC-ζ (681 bp), 5′-CGATGGGGTGGATGGGATCAAAA-3′ (forward), 5′-GTATTCATGTCAGGG TTGTCTG-3′ (reverse); and cytokeratin 8 (644 bp), 5′-TTGCTGAAGTTCGTGCCCAG-3′ (forward), 5′-ACACCAGTTTCCCATCTCGG-3′ (reverse). PCR was performed on a Perkin-Elmer-Cetus Gene Amp 9600 thermal cycler with 93°C, 30 s denaturation; 59°C, 45 s annealing; and 74°C, 45 s extension for 35 cycles. PCR products were separated on precast 4% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, ME) in 0.5× Tris-borate EDTA (1× Tris-borate EDTA = 89 mM tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) and stained with ethidium bromide for visualization. Subcloned and sequenced PCR products from a preliminary experiment confirmed the fidelity of amplification.

Semiquantitative Competitive RT-PCR (Mimic RT-PCR). For the purpose of generating competitive internal standards, cDNA was synthesized as described above by using ~200 pg poly(A)$^+$ RNA as template. PKC-δ and PKC-ζ primers were as described above, yielding products of 352 and 681 bp, respectively. Selected tubes were spiked with varying amounts of the appropriate internal standard (mimic) which was synthesized by using the following primers: PKC-δ, 5′-CACCATCTTC-CAGAAAGAACGACATGAGCC CCACC-3′ (forward), 5′-CTTGCCATAGGTCCCGTTGTTG-3′ (reverse); PKC-ζ,5′-CGATGGGGTGGATGGGATCAAAA-3′ (forward), 5′-GTATTCATGTGAGGG TTGTGTGGATTTCGGGGGCG-3′ (reverse), generating mimic products of 299 bp (PKC-δ) and 561 bp (PKC-ζ). These internal standards have similar length and GC content as the target sequence and are amplified with the same primers as the target, resulting in equivalent amplification efficiency. Amplification of mimic *versus* endogenous target was titrated to determine molar equivalence (19). PCR products were analyzed by electrophoresis on 4% agarose gels and DNA visualized by ethidium bromide staining.

Results

Total RNA isolated from rat feces contained a large proportion of bacterial RNA. As seen in Fig. 1, 18S and 28S rRNA are present in colonic mucosal total RNA (Lane 2), whereas fecal RNA contains 16S and 23S RNA (Lane 3), indicative of bacterial RNA. After poly(A)$^+$ isolation with oligo (dT) cellulose spin columns, rRNA was not evident on ethidium bromide stained gels (data not shown), indicating poly(A)$^+$ RNA had been preferentially concentrated.

Poly(A)$^+$ RNA was quantitated by hybridization of slot-blotted samples with biotinylated oligo (dT) probe. To determine the specificity of this procedure, total bacterial RNA, rRNA and DNA at levels 25 times that of the highest colon RNA standard were also blotted and probed. As seen in Fig. 2, only rat fecal poly(A)$^+$, containing RNA from sloughed colonic epithelial cells, generated a positive signal with the oligo (dT) probe (rRNA not shown). This indicates that poly(A)$^+$ regions in bacterial RNA and other potential contaminants of the rat poly(A)$^+$ samples, such as DNA, were insignificant or non-existant. Therefore, reverse transcription of poly(A)$^+$ samples with oligo (dT) primers should yield products originating only from rat RNA. The amount of poly(A)$^+$ RNA isolated varied between animals due to a variety of factors, including number of colonocytes sloughed into the feces, interanimal variation and efficiency of purification. The range of values obtained was 0.84–12.7 ng poly(A)$^+$ RNA/g feces (n = 18; mean, 4.43 ± 0.88).

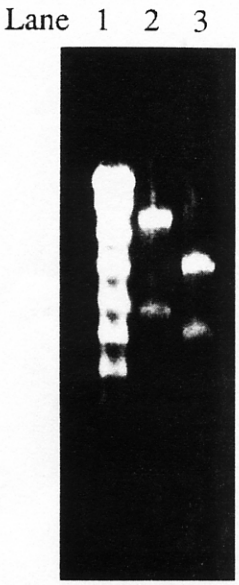

Fig. 1. Agarose-formaldehyde gel electrophoresis analysis of rat colonic mucosal total RNA (*Lane 2*) and rat fecal RNA (*Lane 3*). *Lane 1*, RNA molecular size markers.

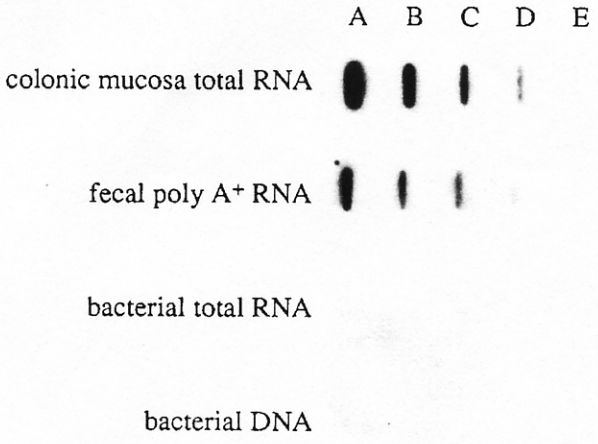

Fig. 2. Detection of poly(A)$^+$ RNA. Two-fold serial dilutions of samples were slot blotted onto nylon membranes and detected by using biotinylated oligo (dT) followed by streptavidin-alkaline phosphatase, Lumi-Phos 530 substrate, and exposure to film. Concentrations, as assessed by absorbance at 260 nm, were as follows: colonic mucosal RNA: 10 ng (*A*), 5 ng (*B*), 2.5 ng (*C*), 1.25 ng (*D*), 0.63 ng (*E*); bacterial RNA and DNA: 250 ng (*A*), 125 ng (*B*), 62.5 ng (*C*), 31.3 ng (*D*), 15.6 ng (*E*). Fecal poly(A)$^+$ RNA concentration was determined after densitometric scanning of the colonic mucosal total RNA standard curve, assuming colonic RNA contains 2% poly(A)$^+$ RNA.

Cytokeratin, a cytoskeletal protein expressed by epithelial cells (20), was used as a positive control in RT-PCR. Fecal poly(A)$^+$ samples from all 8 rats yielded an amplified cytokeratin product after RT-PCR. PCR products of fecal poly(A)$^+$ and colonic mucosal RNA from a representative animal using primers for six PKC isoforms, as well as cytokeratin, are shown in Fig. 3. PCR products for cytokeratin and PKC-α, PKC-δ,

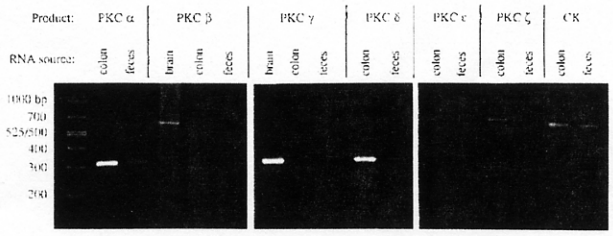

Fig. 3. RT-PCR analysis of rat colonic mucosal total RNA, rat fecal poly(A)$^+$ RNA, and rat brain total RNA by using primers for six PKC isoforms and cytokeratin 8 (*CK*). PCR products were separated on 4% agarose gels and visualized with ethidium bromide staining. *Far left lane*, DNA molecular size markers.

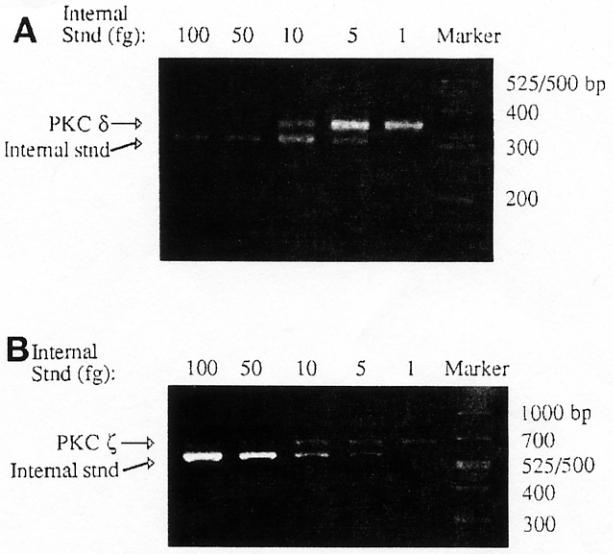

Fig. 4. *A*, semiquantitative (mimic) PCR of PKC-δ. Before PCR, decreasing amounts of PKC-δ internal standard were added to sequential tubes containing the same amount of RT reaction. After PCR, products were separated on 4% agarose gels. *Far right lane*, DNA molecular size markers. *B*, semiquantitative PCR of PKC-ζ.

PKC-ϵ, and PKC-ζ using fecal poly(A)$^+$ RNA and colonic mucosal total RNA were detected in all samples. In contrast, PKC-β and PKC-γ were not detectable after 35 PCR cycles in either colonic mucosal total RNA or fecal poly(A)$^+$ RNA, but bands were seen for these isoforms amplified from brain (positive control; Fig. 3). Negative controls processed without RT yielded no detectable amplified products (data not shown). RT-PCR performed before poly(A)$^+$ selection resulted in no amplified cytokeratin or PKC products, possibly due to the interference of large amounts of bacterial RNA.

Using semiquantitative mimic PCR for PKC-δ and PKC-ζ, the level of expression of these isoforms in a representative fecal poly(A)$^+$ sample could be quantitated (Fig. 4, *A* and *B*). This sample contained approximately 10 fg of PKC-δ and 5 fg of PKC-ζ/15 pg poly(A)$^+$ RNA. By using this methodology, a 2-fold difference in message could be detected.

Discussion

Changes in cell proliferation have been considered the "gold standard" of intermediate markers for the prediction of tumor-

igenesis (21, 22). Previous work in our laboratory has shown that alterations in fat and fiber composition of the rat diet influence the colonic expression of several PKC isoforms and that these changes are associated with altered cell proliferation (12, 13). Indeed, of all cancers, colon cancer appears to be the most affected by diet (23, 24). Relative amounts of PKC isoforms vary between normal and colon tumor cells, with PKC-β virtually undetectable in normal cells but highly expressed in carcinomas (15, 25). In addition, select PKC isoforms have been found to influence cell and tumor growth (14). In an attempt to develop a noninvasive method for examining changes in colonocyte expression of PKC isoforms, we examined the feasability of using feces, which contains exfoliated colonocytes (20).

Normal adult colonic epithelial cells turn over every 3–4 days (16), resulting in the daily exfoliation of approximately 10^{10} cells. Recently, human stool samples have been used to detect the presence of colorectal tumors (26) and colonic inflammation (27). In addition, the detection of K-*ras* mutations in the stool of patients with pancreatic adenocarcinoma and pancreatic ductal hyperplasia has been reported (28). Although we did not use human feces in this study, previous work has successfully examined DNA (26, 29) and proteins (15, 27) in colonocytes obtained from feces. Therefore, it is likely that the methodology described here for the rat could be utilized for the analysis of human samples.

Although the prognostic value of detecting accumulated DNA damage leading to the activation of oncogenes in stool samples has been well documented (29, 30), the amplification/suppression of gene expression as a biomarker in the colon has not been determined. In this study, we examined the feasability of quantitating PKC isozyme gene expression in rat fecal samples. It is apparent that the major source of RNA in the feces is from the colonic bacterial population (Fig. 1). Sloughed colonocyte RNA was present to a much lower extent than was bacterial RNA because the 18S/28S rRNA bands in total fecal RNA were not visible in ethidium bromide stained gels. However, after poly(A)$^+$ selection, bacterial rRNA content was significantly reduced. Although procaryotes do contain poly(A)$^+$ sequences, they are rapidly degraded and are present in low amounts compared with eukaryotic poly(A)$^+$ RNA (31).

To examine whether any potential bacterial poly(A)$^+$ RNA could complicate the RT-PCR analysis, we quantitated poly(A)$^+$ populations in bacterial total RNA, rRNA, and DNA by using a biotinylated oligo (dT) probe. At RNA levels 25 times above the highest colon total RNA amount and 400 times greater than the lowest detectable total colon RNA amount, we could not detect poly(A)$^+$ populations in any colonic bacterial preparation. This indicates that bacterial poly(A)$^+$ components are negligible and do not interfere with the RT-PCR amplification of fecal poly(A)$^+$ RNA.

Using scraped colonic mucosa, we have examined previously the expression of rat and human PKC isoforms by immunoblot analysis (13, 15). PKC-α, PKC-δ, PKC-ϵ, and PKC-ζ are expressed in significant amounts, whereas only very low levels of PKC-β are detected in some samples. PKC-γ is not expressed in colon (15). However, due to the limiting sample size, an enhanced detection system is required to routinely amplify potential cancer risk biomarkers. By incorporating the exquisite sensitivity of RT-PCR, we detected mRNAs for PKC-α, PKC-δ, PKC-ϵ, and PKC-ζ isoforms in feces containing sloughed colonocytes. These data are consistent with previous results obtained on expression of PKC protein in scraped colonic mucosa. Additional studies are needed to determine the relevance of alterations in fecal PKC mRNA expression.

Several semiquantitative techniques are available to measure changes in gene expression. These include the Northern blot and the more sensitive RNase protection assay. However, for the purpose of detecting fecal mRNAs, neither method is sufficient to detect quantitative differences between samples.[4] Competitive RT-PCR is ideally suited to determine the relative levels of extremely rare mRNAs or mRNAs in small numbers of cells or in small amounts of tissue (19, 32). This procedure relies on the use of an external standard that mimics or closely imitates target mRNA species because the standard and target sequences actually compete for the same primers and, therefore, for amplification. Because the target:internal standard ratio remains constant during the PCR reaction, it is possible to obtain useful data after the reaction has reached the plateau phase. Our findings suggest that this technique is well suited for the quantitation of fecal mRNAs isolated from control and treatment groups.

Although we have developed noninvasive mimic RT-PCR methodology to quantitate fecal PKC isoform gene expression, many messages could be amplified with this powerful technique. After fecal RNA isolation and poly(A)$^+$ selection, an apparently clean preparation (Fig. 3) is available for RT-PCR, and many variations including enriched, multiplex or semiquantitative PCR can be pursued. Although there is no simple way to determine colonic disease activity, the ability to use fecal material containing exfoliated colonic epithelial cells as a means to monitor gene expression may have predictive value in terms of detecting the neoplastic process.

Acknowledgments

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