Acid- and bile-induced PGE$_2$ release and hyperproliferation in Barrett’s esophagus are COX-2 and PKC-ε dependent

Kaur, Baljeet S., and George Triadafilopoulos.


Barrett’s esophagus (BE) results from acid and bile reflux and predisposes to cancer. To further understand the mechanisms of acid- and bile-induced hyperproliferation in BE, we investigated the release of PGE$_2$ in response to acid or bile salt exposure. Biopsies of esophagus, BE, and duodenum were exposed to a bile salt mixture as a 1-h pulse and compared with exposure to pH 7.4 for up to 24 h, and PGE$_2$ release, cyclooxygenase-2 (COX-2), and protein kinase C (PKC) expression were compared. Similar experiments were also performed with acidified media (pH 3.5) alone, in the presence or absence of bisindolylmaleimide (BIM), a selective PKC inhibitor, and NS-398, a COX-2 inhibitor. One-hour pulses of bile salts or acid significantly enhanced proliferation, COX-2 expression, and PGE$_2$ release in BE. In contrast, the combination pulse of acid and bile salts had no such effect. Treatment with either BIM or NS-398 led to a dramatic decrease in PGE$_2$ release in BE explants and a suppression of proliferation. The acid- or bile salt-mediated hyperproliferation is related to PGE$_2$ release. Acid- and bile salt-induced induction of COX-2 and PKC may explain, at least in part, the tumor-promoting effects of acid and bile in BE.

Acid; bile salts; gastroesophageal reflux disease; protein kinase C; cyclooxygenase-2; prostaglandins; duodenogastric reflux
the possible roles of PKC and COX-2 in our ex vivo model of BE. Our experiments demonstrate an acid- and bile salt-mediated enhancement of PGE2 release that correlates well with PCNA expression as a marker of proliferation and may be suppressed by PKC or COX-2 inhibitors.

MATERIALS AND METHODS

Patients and tissue collection. Endoscopic mucosal samples from normal esophagus (squamous controls), BE, and duodenum (columnar epithelial controls) were obtained from individuals with BE undergoing endoscopic surveillance at the Veterans Affairs Palo Alto Health Care System and Stanford University Hospital as part of an endoscopic surveillance program. Biopsies were collected under a protocol that was approved by the Administrative Panel on Human Subjects in Medical Research, Stanford University.

Endoscopy and biopsy. Endoscopy was performed by using a video image endoscope, with patients receiving intravenous sedation and continuous cardiac-respiratory monitoring. BE was endoscopically defined as the presence of red columnar islands within squamous esophageal mucosa or by the presence of lighter squamous islands within a circumferential columnar mucosa present above the endoscopically identified gastroesophageal junction, as previously described (24). For the diagnosis of BE, both endoscopic and histological evidence for such an epithelium were required. Endoscopic biopsies were from each quadrant (4 biopsies) every 1 cm of the length of the BE (29). In addition, mucosal biopsies were obtained from the duodenum and from the proximal (squamous) esophagus and were used as controls. Endoscopic mucosal samples were immediately divided into two parts. One part was formalin-fixed for histopathological assessment; the other part was maintained in tissue culture medium (see Organ culture) for subsequent experimental use.

Histopathology. All specimens were analyzed independently by a staff pathologist to categorize normal esophageal mucosa, esophageal inflammation, metaplasia, and/or dysplasia. To confirm the presence of specialized intestinal metaplasia, Alcian blue staining was performed on all BE samples (10). Only BE biopsy samples with incomplete intestinal metaplasia, as defined by a specialized (intestinalized) surface and pit epithelium with goblet cells on hematoxylin and eosin (H&E) and Alcian blue stains, were included in the organ culture studies. Since all patients had established BE and were undergoing surveillance while receiving proton pump inhibitor therapy, there was no evidence of inflammation in the tissues other than the usual degree of scattered chronic inflammatory cells in the lamina propria. Furthermore, since dysplasia and COX-2 expression have been linearly related, we did not include any dysplastic samples in our ex vivo experiments to minimize variability of results. Morphological assessment by H&E stain was performed to ensure histological integrity of organ culture tissues for each time point up to 24 h.

Reagents. All bile acid/salts (sodium glycocholate and taurocholate, glycocholic acid, and taurochenodeoxycholate) and anti-PCNA monoclonal antibodies (MAbs) were obtained from Sigma (St. Louis, MO). Anti-PKC MAbs were obtained from Oncogene Research Products (Cambridge, MA). The specific PKC inhibitor bisindolylmaleimide (BIM) was purchased from Boehringer-Mannheim (Indianapolis, IN). COX-2 antibodies were obtained from Cayman Laboratories (Ann Arbor, MI). Anti-PKC-ε MAbs were obtained from Oncogene.

Organ culture. Organ culture was performed essentially as previously described (8, 9). Briefly, multiple mucosal biopsies were cut into two fragments by using an aseptic technique and were randomly assigned to acid, bile salts, acid plus bile salts, or control groups to avoid sampling bias. Patient samples were not pooled together, and a single patient’s specimens were used for each individual experiment. Four to six biopsy fragments were placed on a sterilized stainless steel grid within 12-well plates so that culture medium (1 ml) covered the surface of the biopsy. Plates were placed on racks in a sterile sealed jar (Torsion Balance, Clifton, NJ), perfused with 95% O2-5% CO2, and then cultured at 37°C. For all experiments, we used medium 199 supplemented with 10% heat-inactivated fetal calf serum, 1 μg/ml insulin, 500 U/ml streptomycin, and 250 μg/ml penicillin. Tissues were exposed to a bile salt mixture (sodium glycocholate and taurocholate, glycocholic acid, and taurochenodeoxycholate; total final concentration 1 mM; pH 7.4) as a 1-h pulse, followed by incubation in normal medium (pH 7.4). This bile salt mixture concentration was selected because its effects have been previously described on patients with BE, and because it represents near-physiological constituents and concentrations (7). The concentration of 1 mM was chosen because it was not toxic to our experimental and control epithelia in dose-response experiments and because it exhibits uniformity of proliferative response in BE tissues in our ex vivo model. For acidic culture conditions, the medium was acidified for 1-h pulse with 0.1 M HCl (~20% vol/vol, pH 3.5). In all experiments, a volume of distilled water (~20% vol/vol) was added to the control nonacidified media to achieve an osmolality equal to that of the acid-treated media. Tissues were exposed to acid alone (in 1-h pulse, pH 3.5), bile salts alone (in 1-h pulse, pH 4.0, or control media (pH7.4). Experiments were conducted for 24 h, with collection time points of 1 h and 24 h. For the PKC experiments, a 1-h pulse of bile salts was administered either in the absence or presence of the PKC inhibitor BIM (17). Prior experiments (not shown) have demonstrated that the BIM dose chosen (10 μM) gives the best reproducibility of response in our ex vivo system. To confirm tissue viability after organ culture, lactate dehydrogenase (LDH) assays were performed by using an aliquot of tissue culture medium taken at the end of the culture period and an LDH assay kit (Sigma) (8). Explants that were homogenized in media with Triton X-100 and then sonicated (before measuring the LDH of the media) were used as positive controls. Control tissues were processed at the same pH as the experimental samples to adjust for pH sensitivity of the assay. The absorbance values (at 490 nm) were expressed as a percentage of the positive control and adjusted for protein content of the extracts.

Protein extraction and immunoblot analysis. This was performed to assess PCNA, PKC-ε, or COX-2 expression in BE epithelia treated with 1-h pulse of bile salts or acid (10, 17). Samples from 1 and 24 h of organ culture were assessed. For each time point, tissues were homogenized (4°C) in 1% deoxycholate, 1% Nonidet P-40, and 0.1% SDS in PBS (pH 7.4) containing 5 mM EDTA, 15 μg/ml aprotinin, 10 μM leupeptin, 10 μM pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride. The protease inhibitors were added just before solubilization, and homogenization was performed by using Kontes glass tissue grinders. Lysates were centrifuged at 16,000 g (20 min, 4°C), and protein concentrations were measured by the bicinchoninic acid protein assay as recommended by the manufacturer (Pierce, Rockford, IL). Proteins were separated by using 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH) in transfer buffer containing 12.5 mM...
Tris·HCl (pH 8.3), 100 mM glycine, and 20% methanol (40 V, 12 h, 4°C). After transfer, membranes were blocked for 3 h in MT buffer (5% dry nonfat milk in PBS containing 0.1% Tween 20), then incubated for 2 h (22°C) in 1:1,000 dilution of the anti-PCNA antibody, 1:1,000 dilution of the anti-COX-2 antibody, or 1:2,000 dilution of the anti-PKC antibody, respectively. Membranes were washed in PBS buffer (containing 0.1% Tween 20) and then incubated with peroxidase-conjugated secondary antibody (1:3,000 dilution) for 1 h at 22°C. After being washed once in PBS buffer (containing 0.1% Tween 20) for 1 h, bands were visualized by using an enhanced chemiluminescence system (NEN, Wilmington, DE).

**PGE2 immunoassays.** PGE2 release was measured by using the Biotrak EIA assay (Amersham Pharmacia Biotech). PGE2 was extracted from the organ culture medium by using the Biotrak assay kit protocol and evaporated to dryness under nitrogen. Samples were reconstituted in the assay buffer provided in the kit. Twenty-five microliters of each sample were assayed in duplicates and triplicates for accuracy. A standard curve was run in duplicates and under similar conditions as the samples. Assay data were calculated from the standard curve by using Excel software. PGE2 release was measured in separate experiments with biopsy tissues from four different patients. Since pilot experiments (not shown) revealed an excellent correlation among tissue weight, PGE2 release, and protein content, we report all of the PGE2 data for milligrams of tissue placed in each dish.

To examine the synthesis of PGE2 in more detail, we also measured the effects of acid and bile salts on the production of PGE2 when an excess of arachidonic acid (500 µM) was added to the system. These experiments were done because PGE2 production can be affected by changes in the activity of phospholipase A2, which provides the substrate for COX-catalyzed reactions. Adding excess arachidonate minimizes any contribution of phospholipase A2 activity to the rate of production of PGE2. In these experiments, arachidonic acid was added to the media along with other treatments or alone, in the case of controls. The treatment lasted 1 h in each case.

**COX-2 and PKC-ε inhibition.** NS-398, a specific COX-2 inhibitor, was used at 0.1-, 0.5-, 1.0-, 5.0-, 10.0-, and 20.0-µM concentrations to determine the ideal concentration for our experiments with biopsies in the ex vivo organ culture setting (17). Final concentration of 10.0 µM NS-398 was then used in all of our experiments. To inhibit PKC-ε, BIM at 10 µM was used in all experiments. However, BIM is not a specific inhibitor of PKC-ε; rather, it inhibits all isoforms of PKC.

**PKC-ε activity.** Acid- and bile salt-treated BE samples were homogenized, and the soluble fraction of total protein was collected. Protein (50–100 µg) was used to immunoprecipitate PKC-ε with specific PKC-ε antibody. Immunoprecipitates (equal amounts) were used for PKC activity assay. Immunoprecipitates were incubated for 10 min at 30°C with 2 µg myelin basic protein (MBP)/whole protein in the presence of 5 µCi of [γ-32P]ATP and phospholipids. Kinase reaction mixture was subjected to SDS-PAGE to resolve the phosphorylated MBP and then to autoradiography.

**RESULTS**

**PGE2 release in BE.** We first measured PGE2 release at 1 and 24 h in the media of mucosal biopsy explants from normal esophagus, BE, and duodenum using a specific immunoassay. Figure 1 shows the amount of PGE2 (pg/ml) released in the medium of such explants at 1 and 24 h of organ culture in standard media (see MATERIALS AND METHODS). The amount of PGE2 released was significantly higher in BE tissues compared with either normal esophageal or duodenal control tissues at both 1 and 24 h (P < 0.001). These changes could not be attributed to inflammation, since the number of chronic inflammatory cells in the lamina propria of BE epithelia was similar to that of normal duodenum. At 24 h, there was no significant LDH release compared with the Triton X-extracted, positive controls; light microscopy of H&E-stained sections revealed that tissue architecture was well preserved (data not shown).

**PGE2 release in BE is enhanced in response to either acid or bile salt pulses.** We then determined the pattern of PGE2 release from BE explants in response to either acid or bile salt 1-h pulses in the presence or absence of excess arachidonic acid (500 µM). All experiments were performed in the presence and absence of arachidonic acid (not all shown). Figure 2 depicts the PGE2 release from mucosal biopsy BE explants after 1 h of acid or bile salt exposure, followed by culture in regular media for 24 h. Organ culture media were retrieved after 1 and 24 h of organ culture and then analyzed by immunoassay as described in MATERIALS AND METHODS. An increase in PGE2 was noted in response to 1-h pulse of acid (Fig. 2A) or bile salts (Fig. 2B) compared with arachidonic acid-containing control media. This effect was noted as early as 1 h after exposure and was more pronounced at 24 h and in the presence of arachidonic acid (P < 0.005).

**Modulation of acid- or bile salt-induced PGE2 release in BE.** We then studied the effects of acid (pH 3.5, Fig. 3A) and bile salt (1 mM, Fig. 3B) pulses (1 and 24 h) in the presence of the COX-2 inhibitor NS-398, the PKC inhibitor BIM, and their combination on PGE2 release under similar experimental conditions. For both acid and bile salt-treated tissues, there was a significant
The effect of combined acid (pH 3.5) and bile salt (1 mM) pulses (1 h) on PGE2 release in media from BE explants in the presence or absence of NS-398, BIM, and the combination of the two inhibitors at 1 and 24 h

![Graph A](image1.png)

**A**: effect of acid pulses (1 h, pH 3.5) on PGE2 release in media from BE explants with and without the cyclooxygenase-2 (COX-2) inhibitor NS-398 (NS), the nonselective protein kinase C (PKC) inhibitor bisindolylmalemide (BIM), and the combination of the two inhibitors (NS/BIM) at 1 and 24 h of organ culture. For both acid- and bile salt-treated tissues, there is a significant degree ($>80\%$) of inhibition of PGE2 release by BIM and NS-398 at both time points. A near-complete inhibition of PGE2 release is also accomplished by the combination of inhibitors ($P < 0.005, n = 4$).

![Graph B](image2.png)

**B**: effect of bile salt pulses (1 mM) on PGE2 release in media from BE explants with and without the COX-2 inhibitor NS-398 (NS), the nonselective protein kinase C (PKC) inhibitor bisindolylmalemide (BIM), and the combination of the two inhibitors (NS+BIM) at 1 and 24 h of organ culture. For both acid- and bile salt-treated tissues, there is a significant degree ($>80\%$) of inhibition of PGE2 release by BIM and NS-398 at both time points. A near-complete inhibition of PGE2 release is also accomplished by the combination of inhibitors ($P < 0.005, n = 4$). Experimental conditions are the same as in Fig. 2.

**Fig. 2.** A: effect of excess arachidonic acid (AA) on acid-induced (pH 3.5) PGE2 release from BE organ culture explants using a specific immunoassay. AA1 and AA24, non-acid-treated AA controls; A1 and A14, acid-treated BE tissues. A+AA1 and A+AA24, acid + AA-treated tissues. B: effect of excess AA on bile salt (1 mM) mixture-induced PGE2 release from BE, AA1 and AA24, non-bile salt-treated controls; BS1 and BS24, bile salt-treated tissues (1 mM); BS+AA1 and BS+AA24, bile salt + AA-treated tissues. In the presence of excess AA, both acid and bile salts further enhance PGE2 release in BE compared with control conditions ($P < 0.005, n = 4$).

**Fig. 3.** A: effect of acid pulses (1 h, pH 3.5) on PGE2 release in media from BE explants with and without the cyclooxygenase-2 (COX-2) inhibitor NS-398 (NS), the nonselective protein kinase C (PKC) inhibitor bisindolylmalemide (BIM), and the combination of the two inhibitors (NS+BIM) at 1 and 24 h of organ culture. B: effect of bile salt pulses (1 mM) on PGE2 release in media from BE explants with and without the COX-2 inhibitor NS-398, BIM, and the combination of both at 1 and 24 h of organ culture. For both acid- and bile salt-treated tissues, there is a significant degree ($>80\%$) of inhibition of PGE2 release by BIM and NS-398 at both time points. A near-complete inhibition of PGE2 release is also accomplished by the combination of inhibitors ($P < 0.005, n = 4$).
of organ culture is shown in Fig. 4. The combined acid and bile salt pulses induce PGE2 release in media from BE explants that is similar to controls (see Fig. 3). There was a significant degree of inhibition of PGE2 release by BIM, NS-398, and the combination at both time points $(P < 0.005, n = 4)$. These studies further characterize the degree of inhibition of PGE2 release in response to these inhibitors.

**Effect of acid or bile salts on PCNA expression.** We also examined the effect of acid or bile salts on cell proliferation by assessing PCNA expression. For this, we performed organ culture experiments on BE exposed to 1-h pulses of media alone (pH 7.4), acid (pH 3.5), and bile salts (pH 7.4), followed by continuous culture in regular media (pH 7.4). Figure 5 depicts PCNA immunoblot results of a representative of three experiments. Baseline PCNA expression (36 kDa, Fig. 5, top) was markedly increased after a pulse of acid or bile salts (Fig. 5, middle). Both baseline and acid or bile salt-stimulated PCNA expression were significantly decreased by either of the two inhibitors.

**Effects of COX-2 and PKC inhibitors.** Figure 6, top, depicts Western blots showing the effects of NS-398, BIM, and their combination on the expression of COX-2 in BE using a specific MAb. Both inhibitors caused a significant decrease in COX-2 expression that paralleled the decrease in PGE2 release (see Fig. 3). Figure 6, bottom, depicts Western blots showing the effects of NS-398, BIM, and their combination on the expression of the 93-kDa protein (PKC-ε) in BE by using a specific MAb. Both inhibitors cause a significant decrease in PKC-ε expression that parallels the decrease in COX-2 expression. We could not show any significant differences when the combination of inhibitors was used, but there was a trend suggesting that the combination was more inhibitory, raising the possibility that other factors may be involved. Similar findings were noted with acid exposure (not shown).

**PKC-ε activity in the presence and absence of PKC inhibition.** Figure 7 depicts acid- and bile salt-induced phosphorylation of MBP both at 1 and 24 h, whereas a complete inhibition is observed in the presence of BIM (see MATERIALS AND METHODS).

**DISCUSSION**

In contrast to normal esophagus or duodenum, the metaplastic, hyperproliferative, and premalignant epithelium of BE releases PGE2 that may be modulated ex vivo by pulse exposure of acid or bile salts. Such PGE2 release is COX-2- and PKC-ε-dependent and leads to increased PCNA expression.

The pathogenesis of BE and its propensity to dysplasia and esophageal adenocarcinoma remain poorly understood (28). A growing number of abnormalities in
cell proliferation, oncogene activation, tumor suppressor gene inactivation, and growth factor effects have been described that suggest a multifactorial and multistage disease. To understand the malignant progression of BE to adenocarcinoma, an understanding of the spectrum and the relationship of environmental factors involved is critical. Understanding the role of environmental factors coming in contact with the BE, such as acid and/or bile, will allow their modification by medical or surgical therapy and may in turn alter the natural history of this condition (9). For example, one could imagine a scenario wherein an environmental trigger (i.e., acid, bile salts) for an increase in cell proliferation predisposes cells to genetic mutations. As a result of these genetic alterations, cell proliferation is deregulated further and more genetic abnormalities accumulate. A vicious cycle is thus set up, and the accumulation of critical genetic errors (e.g., p53 mutations) may eventually lead to a clone of malignant cells capable of invasion (30).

In this study, we examined the relationship between BE and environmental factors to which this metaplastic tissue is exposed. Specifically, because BE usually develops in the context of chronic reflux of stomach acid and intestinal juices into the esophagus, we studied how acid or bile salts affect its proliferation. In the experiments described, we used tissue samples from patients with BE undergoing surveillance endoscopy and monitored their cellular behavior in the presence or absence of acid and bile in the laboratory setting of organ culture. We hypothesized that, if components of the duodenogastric refluxate increase PGE2 release and cell proliferation, effective inhibition of PGE2 release through COX-2 or PKC inhibition or prevention of acid or bile reflux with medical or surgical therapy would substantially decrease the risk for esophageal adenocarcinoma.

Work from our laboratory (33) and others (22, 23, 36) provides evidence that COX-2 is important for the genesis of cancer. Hence there is evidence to link the activity of PKC to carcinogenesis, in part via the induction of COX-2 and overproduction of prostaglandins. This study adds to this evidence by showing that both acid and bile salts upregulate PGE2 release, suggesting a plausible mechanism underlying esophageal adenocarcinoma formation: PKC and COX-2 induction.
The main emphasis of our work was to link COX-2 (expression and activity) to proliferation (PCNA) in the context of environmental stimulation by acid or bile salts. The nonspecific PKC inhibitor BIM was used to point out the involvement of PKC in these processes. Using specific antibodies to the PKC-ε isoform that is specific for BE, we demonstrated that PKC-ε is the dominant isoform involved. However, more work will be needed to address the specificity and regulation of PKC involvement in these processes.

The activator protein-1 and -2 recognition and nuclear factor-κB binding sites are several possible elements that mediate the effects of PKC (14, 19, 37). Nevertheless, irrespective of the precise mechanism by which acid and bile salts induce COX-2, our results suggest that PKC inhibitors could be useful for down-regulating COX-2 and thereby preventing and or treating BE and adenocarcinoma. Further studies will be needed to identify the responsible COX-2 promoter elements that lead to PGE2 release by acid or bile salts and to identify the precise role of the specific PKC-ε isoform that mediates such effects. In the present study, although it appears that PKC-ε is the predominant isoenzyme involved, the experiments with BIM are not isoform specific. Nevertheless, preliminary observations from our laboratory suggest that PKC-ε (Fig. 5, bottom) is specific to BE and contributes to enhanced COX-2 expression and proliferation (16).

The concentrations of acid and bile salts required to induce COX-2 and PGE2 release in our study were similar to those found in bile and gastric secretions (5) and similar to those used in other studies (40). There is no physiological level of bile salts in the esophagus. Bile acids may enter the mucosal cells in the nonionized form through the lipophilic cell membrane and then accumulate, because intracellular ionization results in entrapment (32). Furthermore, conjugated bile acids may be precipitated out of solution at higher pH (3). In our experiments, however, we did not observe any precipitation of bile salts, nor did we observe any morphological damage by either histology or LDH assays of the explants and surrounding media. Recently, observations with a rat model of esophageal carcinogenesis have shown bile-induced COX-2 overexpression in vivo and suppression of such expression with selective and nonselective COX-2 inhibitors (6). Furthermore, PKC inhibitors block the transcription of COX-2 in Barrett’s adenocarcinoma cell lines (40). In other cell systems, inhibition of PKC inhibits COX-2 induction and transcription (26, 35), whereas PKC activation stimulates PGE2 release (20). All of these efforts should help our understanding of the precise mechanisms by which components of the gastroduodenal refluxate induce proliferation and facilitate the development of chemopreventive strategies to diminish the risk of adenocarcinoma in BE. To what degree such strategies will regress the BE surface also remains unclear.

In conclusion, we have demonstrated by using a nondysplastic, ex vivo BE culture model that acid or bile salts, administered as 1-h pulses, induce PGE2 release and cell proliferation in BE through PKC-ε- and COX-2-dependent mechanisms. Indeed, our results suggest that the sequence of events is that of an early PKC-ε activation, followed by upregulation of COX-2 expression, enhanced PGE2 production, and, thus, enhanced cell proliferation (Fig. 8). However, such a sequence should be seen only as a working model, before further experiments clarify the precise events involved. Additional studies will be needed to address other COX-2-modulating factors and to assess whether such altered proliferation leads to the development of dysplasia and adenocarcinoma. One possible therapeutic implication of our proposed model is that the recently available COX-2 inhibitory therapy may suppress cell proliferation in BE, particularly if it is associated with effective inhibition of acid and bile reflux. Therefore, the current clinical practice of acid suppressive therapy using proton pump inhibitors and aiming at completely abolishing any acid pulses affecting the BE epithelia may be enhanced by COX-2 inhibitors to prevent dysplasia and adenocarcinoma. Indeed, all clinical studies to date have failed to show a reduction of dysplasia risk in patients with BE treated with acid-suppressive therapy alone (1). Because of the disturbing increase in the incidence of BE and adenocarcinoma in the western world, understanding of the mechanisms by which the various components of the gastroduodenal refluxate affect the phenotype and behavior of BE epithelia will, in turn, allow more effective disease prevention and treatment.
We thank Kris Morrow for medical illustration and the faculty, fellows, and staff of the Endoscopy Units of the Veterans Affairs Palo Alto Health Care System and Stanford University Hospital for their assistance in retrieval of mucosal specimens.

This research was funded in part by the Cancer Research Foundation of America (to B. S. Kaur).

REFERENCES


