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Cytosolic Phospholipase A_2-Mediated ICAM-1 Expression Is Calcium Dependent

Carlton C. Barnett, Jr., M.D.,*,†,[1] Ernest E. Moore, M.D.,† Christopher C. Silliman, M.D., Ph.D.,† Eddie K. Abdalla, M.D.,* David A. Partrick, M.D.,† and Steven A. Curley, M.D.*

*From the *Department of Surgery, The University of Texas, M.D. Anderson Cancer Center 1, Houston, Texas; and †Department of Surgery, The University of Colorado Health Sciences Center, Denver, Colorado*

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Background. **Some human malignancies such as virus-related hepatocellular cancer arise in a setting of chronic inflammation. Upregulation of ICAM-1 is a seminal late event in malignant transformation following chronic inflammation. Cytosolic phospholipase A_2 ($cPLA_2$) is a lipid-mediator activated by inflammatory stimuli, which has been shown to mediate ICAM-1 upregulation. As lipid mediators are known to work via calcium-dependent mechanisms in nearly all mammalian cells, we hypothesize that inflammatory-mediated ICAM-1 upregulation is dependent on both $cPLA_2$ and intracellular calcium.**

Materials and methods. **HUVEC were chosen as a representative cell line as they emulate hepatic sinusoids and are a well-established cell model. These were grown to confluence in T-25 flasks and stimulated with TNF-α or LPS for 6 h. Additional groups were preincubated with AACOCF3 (a specific $cPLA_2$ inhibitor) or BAPTA A.M. (a specific inhibitor of intracellular Ca^{2+}) prior to being exposed to inflammatory stimuli. ICAM-1 expression was determined by mean fluorescent intensity (MFI) as measured by FITC-labeled moAb to ICAM-1 via FACS. The role of intracellular Ca^{2+} on $cPLA_2$ activity was determined by thin-layer chromatography. Groups were compared using ANOVA with Scheffe's post hoc analysis; $^*P < 0.05$ vs control, $^\dagger P < 0.05$ vs LPS and TNF-α was considered significant; $N \geq 4$ all experimental groups.**

Results. **Both $cPLA_2$ and Ca^{2+} inhibition significantly inhibited inflammatory upregulation of ICAM-1. Pretreatment with BAPTA A.M. attenuated HUVEC $cPLA_2$ activity in response to LPS. These findings suggest that appropriate molecular target suppression may prevent malignant degeneration in the presence of chronic inflammation.** © 2001 Academic Press

Key Words: **phospholipase A_2; calcium; endothelial cells; cellular adhesion molecules; intercellular adhesion molecule-1.**

INTRODUCTION

Membrane phospholipids generate bioactive mediators, which are responsible for transmitting diverse cellular signals [1]. Mammalian cells contain structurally diverse forms of phospholipase A_2 (PLA_2), including secretory PLA_2 with a molecular weight of approximately 14 kDa, cytosolic PLA_2 ($cPLA_2$) with a molecular weight of approximately 85 kDa, and calcium-independent PLA_2 [2]. Cytosolic PLA_2, which preferentially cleaves arachidonic acid in the sn-2 position of lysophospholipids, is found at almost constant levels within most mammalian cells [3]. Arachidonic acid is an important regulator of specific cellular processes, including regulation of protein kinase C (PKC), phospholipase Cγ, and Ca^{2+} metabolism [4]. Increase of intracellular calcium levels causes translocation of $cPLA_2$ to cellular membranes, where the enzyme interacts with proteins containing C2 domains, playing a role in signal transduction as well as membrane trafficking [5]. Previously we and others have shown via the use of nonspecific PLA_2 inhibitors (quinacrine and bromophenacetin bromide), inhibition of cellular adhesion molecule upregulation, including ICAM-1, in response to inflammatory stimuli [6, 7]. Moreover, inflammatory mediators such as TNF-α and LPS have been shown to upregulate $cPLA_2$ activity [1, 8].

ICAM-1 is expressed in multiple types of cancer, in-

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[1] To whom correspondence should be addressed at Department of Surgical Oncology, Box 444, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Fax: (713)-792-0722. E-mail: ccbarnet@mdanderson.org.

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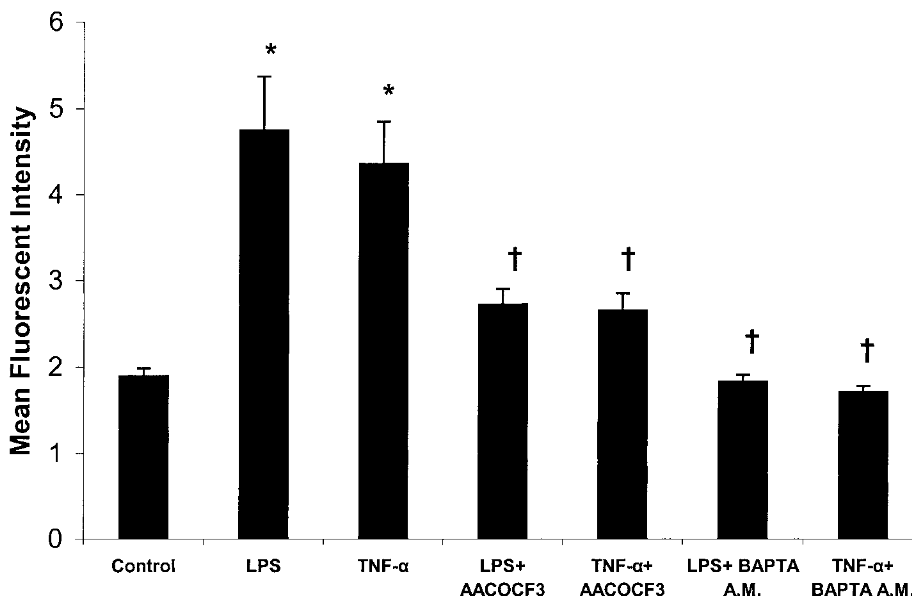

FIG. 1. ICAM-1 expression displayed as mean fluorescent intensity (MFI) is significantly upregulated by a 6-h incubation of HUVEC with either LPS (1 μg/ml) or TNF-α (5000 pg/ml). ICAM-1 upregulation in response to these inflammatory stimuli is attenuated by a 4-h pretreatment with a specific $cPLA_2$ inhibitor AACOCF3 (10 μM) or 4 h pretreatment with an intracellular Ca^{2+} chelator. BAPTA A.M. (100 nM). *difference from quiescent HUVEC; †difference from HUVEC incubated with LPS or TNF-α, $P < 0.05$; $N \geq 4$.

cluding hepatocellular carcinoma and adenocarcinoma of the breast [9–12]. More recently, upregulated levels of ICAM-1 have been reported to signal terminal differentiation into a malignant phenotype [9, 10]. Further, ICAM-1, via its interaction with macrophages and leukocytes, may play an important role in tumor cell invasion and metastasis [11, 12]. With the recent development of AACOCF3 (a specific inhibitor of cytosolic PLA_2) [13] as well as data indicating $cPLA_2$ activity is calcium dependent, we hypothesize that inflammatory-mediated ICAM-1 upregulation is dependent on both $cPLA_2$ and intracellular calcium [4, 5, 14].

METHODS

Reagents, hardware, cell lines, and moAb. HUVEC at pass one were purchased from American Type Culture Collection (Rockville, MD); Dulbecco's PBS, RPMI 1640, L-glutamine, penicillin, streptomycin, and trypsin were purchased from Mediatech (Herndon, VA); heat-inactivated FCS was obtained from Gemini Bio-Products (Calabasas, CA); carbon-14 (^{14}C)-labeled oleic acid substrate and silica-coated TLC plates were purchased from Amersham (Arlington Heights, IL); scintillation cocktail was procured from National Diagnostics (Atlanta, GA); BAPTA A.M. was purchased from Molecular Probes (Eugene, OR). Unless otherwise noted, all other reagents were purchased from Sigma (St. Louis, MO). T-25 flasks, 24-well plates, and 96-well plates were obtained from Falcon BD Labware (Lincoln Park, NJ). FITC-labeled 84H10 was purchased from Immunotech (Westbrook, ME).

ICAM-1 expression. HUVEC were grown to confluence in T-25 flasks at 37°C, 5% CO_2 and then treated with either LPS (Salmonella Enteridities, 1 μg/ml) or TNF-α (5000 pg/ml) for 6 h. Doses for LPS and TNF-α were chosen based on previously performed dose-response curves [6, 8]. These concentrations fall within previously reported concentrations [8, 15, 16] and are intended to produce a desired *in vitro* effect rather than to reproduce actual *in vivo* conditions. Selected groups of HUVEC were pretreated for 4 h with either 100 nM BAPTA A.M. (a specific inhibitor of intracellular Ca^{2+}) [14] or 10 μM AACOCF3 [13] prior to being exposed to LPS or TNF-α. Dose-response curves as well as time courses for BAPTA A.M. and AACOCF3 showed these concentrations and times to be maximally effective in decreasing ICAM-1 expression, without causing HUVEC toxicity as determined by MTT assay. ICAM-1 expression was determined by flow cytometry using 10 μg/ml of anti-CD54 FITC-labeled antibody (84H10) or FITC-labeled isotypic antibody as a negative control. Mean fluorescent intensity (MFI) was determined as the experimental MFI divided by the negative control.

Cytosolic PLA_2 assay. HUVEC were grown to confluence in 24-well plates at 37°C, 5% CO_2. Cells are stimulated with LPS or TNF-α and preincubated with BAPTA A.M. (100 nM) for 4 h. Cells were then scraped after being washed two times with PBS+D and having 400 μl of Harvest buffer (5 mL 1 M Hepes, 30 mg EDTA (1 μM), 3.5 mg PMSF (20 μM), 0.1 mg leupeptin (25 μM), 150 mg benzamidine (mM), dH_2O to equal 100 ml). Cells were then sonicated on ice and centrifuged at 1200 RPM for 7 min to remove large debris. One hundred microliters of cell lysate, 40 μl 1 M Tris, 20 μL 1 M NaCl, 10 μl FA-free BSA, 2 μl DTT, 100 mM $CaCl_2$, 16 μl dH_2O were added to 2 μl of labeled substrate, 60 ml ^{14}C-labeled PCstaa, 19.8 ml cold PCstaa (407.5 mM), and 320.2 ml chloroform:methanol (1:1) The lysate was vortexed and incubated for 60 min at 37°C. Six hundred microliters of stop reagent (30 ml chloroform, 60 ml methanol, 30 mg PCstaa, 200 ml of 67 mg/ml arachidonic acid stock in choloroform) was then added with 40 μl dH2O in 200 ml 4 M KCl. The solution was vortexed and centrifuged at 4000 rpm for 2 min. A TLC plate was spotted (Si250-PA Silica Gel). The plate was developed with petroleum ether:diethyl ether:acetic acid (105:45:1.5). The plate was run over 30 min. PCstaa and AA standards were detected by iodine vapor. Standards were marked, scraped, and then added to 1 ml of dH_2O and 10 ml of scintillation fluid. Counts are measured for 2 min on a scintillation counter, (Beckman, Irvine, CA). A Lowry protein assay (Sigma, St. Louis, MO) is run concomitantly on 100 μl of each lysate. Final counts were divided by protein concentration μg/ml to give units (U) $cPLA_2$ activity.

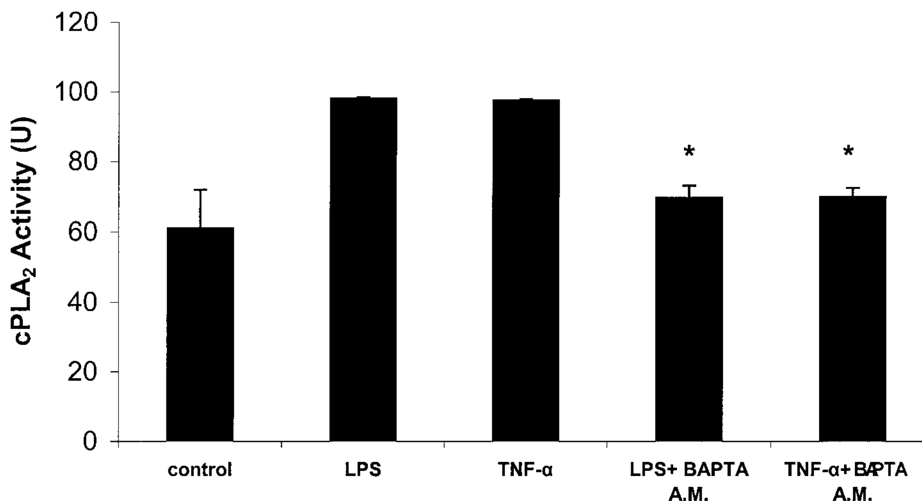

FIG. 2. Preincubation of HUVEC with BAPTA A.M. (100 nM) for 4 h prior to exposure to LPS or TNF-α significantly decreases cPLA$_2$ activity; $^*P < 0.05$; $N \geq 4$.

MTT assay to determine cell viability was determine as previously described [17].

Statistical analysis. Data are presented as means ± SEM, with each study group repeated 4 or 5 times. One way analysis of variance (ANOVA) testing was performed to determine the significance of observed differences. Scheffe's F procedure was used for post hoc comparisons. $P < 0.05$ represents significance at the 95% confidence interval.

RESULTS

Treatment of HUVEC with the specific cPLA$_2$ inhibitor (AACOCF3) as well as a specific inhibitor of intracellular calcium (BAPTA A.M.) significantly reduced ICAM-1 upregulation in response to the inflammatory mediators LPS and TNF-α (Fig. 1). Additionally, pretreatment with BAPTA A.M. significantly attenuated HUVEC cPLA$_2$ activity in response to LPS (Fig. 2).

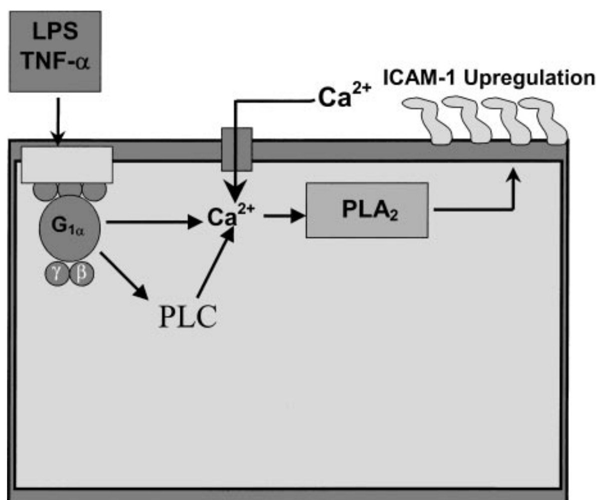

FIG. 3. Inflammatory stimuli LPS or TNF-α promotes ICAM-1 upregulation via intracellular calcium and cytosolic PLA$_2$ activity.

DISCUSSION

Multiple cytokines and mitogens increase the activity of cPLA$_2$ while concomitantly causing upregulation of ICAM-1 expression [2, 6–8]. This inflammatory-mediated increase in cPLA$_2$ activity allows the liberation of arachidonic acid, which itself is an important regulator of specific intracellular processes, including regulation of protein kinase C, phospholipase Cγ, and intracellular Ca^{2+} transients [18–20]. Arachidonic acid may be further converted into potent inflammatory mediators by lipoxygenase and cyclooxygenase pathways. Various models have shown an intimate relationship between cPLA$_2$ and COX 2 activity [21]. In a convergence of signal transduction pathways, both COX 2 and cPLA$_2$ have to been found to play important roles in the induction of the protooncogene c-Fos [22–24]. Moreover, overexpression of c-Fos has also been found to induce ICAM-1 expression on endothelial cells [25]. Finally the dedifferentiation of human hepatocellular carcinoma has been shown to be mediated by cPLA$_2$ activity as well as C-fos activation [22–24], and ICAM-1 upregulation has been shown to be a seminal late event in malignant dedifferentiation of hepatocellular cancer [9].

As cytosolic phospholipase A$_2$ is known to be expressed at near constant levels in mammalian tissue, we have chosen HUVEC as an *in vitro* surrogate to investigate the relationship among intracellular calcium, cPLA$_2$, and ICAM-1. MTT assays of HUVEC viability showed-minimal cytotoxicity (all groups >90% viable), making it unlikely that the effects seen are secondary to nonspecific reagent effects.

Our findings show ICAM-1 cell surface expression is markedly upregulated in response to both LPS and TNF-α (Fig. 1). Further, these inflammatory mediators

were found to increase $cPLA_2$ activity (Fig. 2). Pretreatment with either a specific inhibitor of $cPLA_2$ or intracellular Ca^{2+} antagonist reduces ICAM-1 expression in response to these mediators, suggesting a pivotal role for intracellular Ca^{2+} and $cPLA_2$ in inflammatory mediated ICAM-1 upregulation (Fig. 3). Moreover, inhibition of $cPLA_2$ activity by BAPTA A.M. suggests an obligate, proximal role for intracellular Ca^{2+} in inflammatory-mediated ICAM-1. Our findings implicate specific molecular and pharamacologic targets in patients at risk of developing malignant lesions secondary to chronic inflammatory stimulation, e.g., HCC from hepatitis or squamous cell carcinoma of the esophagus. Targets may include inhibition of ICAM-1 leukocyte interaction [26], COX 2 inhibition [27], or chronic Ca^{2+} channel blockade.

REFERENCES

1. Leslie, C. C. Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.* **272:** 16709, 1997.
2. Dennis, E. A. Diversity of group types, regulation, and function of phospholipase A_2 *J. Biol. Chem.* **269:** 13057, 1994.
3. Miyashita, A., Crystal, R. G., and Hay, J. G. Identification of a 27 bp 5′-flanking region element responsible for the low level constitutive expression of the human cytosolic phospholipase A_2 gene. *Nucleic Acids Res.* **25:** 293, 1995.
4. Graber, M. N., Alfonso, A., and Gill, D. L. Ca^{2+} pools and cell growth: Arachidonic acid induces recovery of cells growth-arrested by Ca^{2+} pool depletion. *J. Biol. Chem.* **271:** 883, 1996.
5. Nalefski, E. A., and Falke, J. J. Location of the membrane-docking face on the Ca^{2+}-activated C2 domain of cytosolic phospholipase A_2. *Biochemistry* **37:** 17642 1998.
6. Barnett, C. C., Jr., Moore, E. E., Moore, F. A., Carl, V. S., and Biffl, W. L. Endotoxin induced ICAM-1 upregulation on endothelium is mediated via PLA_2 activity. *Surg. Forum* **XLVI:** 144, 1995.
7. Yokote, K., Morisaki, N., Zenibayashi, M., Ueda, S., Kanzaki, T., Saito, Y., and Yoshida S. The phospholipase-A_2 reaction leads to increased monocyte adhesion of endothelial cells via the expression of adhesion molecules. *Eur. J. Biochem.* **217:** 723, 1993.
8. Lee, C. H., Reid, Y. A., Jong, J. S., and Kang, Y. H. Lipopolysacharide-induced differential cell surface expression of intercellular adhesion molecule-1 in cultured human umbilical cord vein endothelial cells. *Shock* **3:** 96, 1995.
9. Mei, M., Xu, J., Shi, Q., Chen, Q., and Qin, L. Measurement of serum circulating intercellular adhesion molecule-1 and its clincical significance in hepatocellular carcinoma: Preliminary report. *J. Hepatobiliary Pancreatic. Surg.* **6:** 181, 1999.
10. Sun, J. J., Zhou, X. D., Liu, Y. K., Tang Z. Y., Feng, J. X., Zhou, G., Xue, Q., and Chen, J. Invasion and metastasis of liver cancer: Expression of intercellular adhesion molecule 1. *J. Cancer Res. Clin. Oncol.* **125:** 28, 1999.
11. van Netten, J. P., Ashmead, B. J., Parker, R. L., Thorton, I. G., Fletcher, C., Cavers, D., Coy, P., and Brigden, M. L. Macrophage-tumor cell association: A factor in metastasis of breast cancer? *J. Leukocyte Biol.* **54:** 360–362, 1993.
12. Welch, D. R., Schissel, D. J., Howrey, R. P., and Aeed, P. A. Tumor-elicited polymorphonuclear cells, in contrast to "normal" circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. *Proc. Natl. Acad. Sci. USA* **86:** 5859–5863, 1989.
13. Wenche, S., Brekke, O., and Johansen, B. Secretory and cytosolic phospholipase A_2 regulate the long-term ctytokine-induced eicosanoid production in human keratinocytes. *Cytokine* **12:** 1189, doi:10.1006/cyto.199.0727.
14. Lei, S., and Carpenter, G. Epidermal growth factor activation of NF-κB is mediated through IκBα degradation and intracellular free calcium. *Oncogene* **16:** 2095, 1998.
15. Meng, X., Ao, L., Brown, J. M., Meldrum, D. R., Sheridan, B. M., Cain, B. S., Banerjee, A., and Harken, A. H. LPS induces late cardiac functional protection against ischemia independent of cardiac and circulating TNF-α. *Am. J. Physiol.* **273:** H1894, 1997.
16. Biffl, W. L., Moore, E. E., Moore, F. A., and Barnett, C. C., Jr. Nitric oxide reduces endothelial expression of intercellular adhesion molecule(ICAM)-1. *J. Surg. Res.* **63:** 328, 1996.
17. Jiao, H., Soejima, Y., Ohe, Y., Miura, K., Tomohide, T., and Nagahiro, S. Differential macrophage-mediated cytotoxicity to P388 leukemia cells and its drug-resistant cells examined by a new MTT assay. *Leukemia Res.* **16:** 1175, 1992.
18. Shuttleworth, T. J. Arachidonic acid activates the noncapacitative entry of Ca^{2+} during $[Ca^{2+}]$i oscillations. *J. Biol. Chem.* **271:** 21720, 1996.
19. McPhail, L. C., Clayton, C. C., and Snyderman, R. A potential second messenger role for unsaturated fatty acids: Activation of Ca^{2+}-dependent protein kinase. *Science* **224:** 622, 1984.
20. Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. Activation of phospholipase C-gamma by the concerted action of tau proteins and arachidonic acid *J. Biol. Chem.* **271:** 18342, 1996.
21. Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L. L. Cytosolic phospholipase A_2. *J. Lipid Mediat. Cell Signal.* **12:** 83, 1995.
22. Kim, B. C., Ha, K. S., Park, J. B., and Kim, J. H. Evidence for role of phospholipase A_2 in phosphatidic acid-induced signaling to c-fos serum response element activation. *Biochem. Biophys. Res. Commun.* **247:** 630, 1998.
23. Koga, H., Sakisaka, S., Ohishi, M., Kawaguchi, T., Tanguchi, E., Sasatomi, K., Harada, M., Kusaba, T., Tanaka, M., Kimura, R., Nakashima, Y., Nakashima, O., Kojiro, M., Kurohiji, T., and Sata, M. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: Relevance to tumor dedifferentiation. *Hepatology* **29:** 688, 1999.
24. Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., and Hori, M. Activation of mitogen-activatedprotein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. *Hepatology* **27:** 951, 1998.
25. Wang, N., Verma, L., Hardy, S, Forsayeth, J., Zhu, Y., and Stemerman, M. B. Adenovirus-mediated overexpression of c-Jun and c-Fos induces intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2078, 1999.
26. Mulligan, M. S., Vaporciyan, A. A., Warner, R. L., Jones, M. L., Foreman, K. E., Miyasaka, M., Todd, R. F., 3rd, and Ward, P. A. Compartmentalized roles for leukocytic adhesion molecules in lung inflammatory injury. *J. Immunol.* **154:** 1350, 1995.
27. Rao, C. V., Rivenson, A., Simi, B., Zang, E., Kellof, G, Steele. V., and Reddy, B. S. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Res.* **55:** 1464, 1995.