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Aspirin Prevention of Cholesterol Gallstone Formation in Prairie Dogs

Abstract. *When prairie dogs (*Cynomys ludovicianus*) are fed a diet containing cholesterol, a marked increase in gallbladder mucin secretion parallels the evolution of cholesterol supersaturated bile. Gelation of mucin precedes the precipitation of cholesterol liquid and solid crystals and the development of gallstones. Aspirin given to prairie dogs inhibited mucin hypersecretion and gel accumulation and prevented gallstone formation without influencing the cholesterol content of supersaturated bile. This suggests that gallbladder mucin is a nucleation matrix for cholesterol gallstones.*

Cholesterol gallstones afflict 30 percent or more of the adult population in certain Western countries and ethnic groups (*1*). Their prevalence is increasing in the Orient where many Western dietary habits have been adopted (*2*). A critical step in the formation of cholesterol gallstones is the nucleation of cholesterol from supersaturated bile (*3*). Depending on the degree of supersaturation, the initial cholesterol microprecipitates may be either liquid-crystalline (*4*) or amorphous (*5*); both types contain substantial amounts of the biliary phospholipid, lecithin. These microprecipitates give rise to crystals of cholesterol monohydrate which in turn aggregate to form macroscopic gallstones (*6*). The importance of gallbladder mucin in the formation of gallstones has long been recognized. In 1856 Meckel von Hemsbach (*6a*), who had studied the formation of pearls, attributed gallstones to an accumulation of gallbladder mucin that served as a nucleus for the stones that formed. Womack (*7*) obtained evidence that hypersecretion of gallbladder mucin glycoprotein occurred in lithogenic hamsters and suggested that this high-molecular-weight polymer acted as an organic binder in the formation of cholesterol gallstones. Increased gallbladder mucin has been observed in other animal models (*8*) as well as in human gallbladders containing stones (*9*).

On the basis of morphologic studies, Hultén (*10*) suggested that cholesterol crystals grew predominantly in mucin-packed niches of the human gallbladder wall. More recent observations (*11*) suggest that a nucleation factor is present in supersaturated lithogenic human bile, and that this factor accelerates cholesterol crystal formation; the factor is not found in supersaturated nonlithogenic bile from control patients (*11*). Studies at our laboratory demonstrated (*12*) that marked gallbladder mucin hypersecretion occurred in prairie dogs fed a cholesterol diet for several days; this paralleled the enrichment of bile with cholesterol and occurred prior to nucleation and the formation of liquid crystals, crystals, or gallstones. Cholesterol crystals were particularly prominent in a visible mucin gel that accumulated in the gallbladder of the cholesterol-fed animals.

We designed the present study to test the hypothesis that gallbladder mucin glycoproteins are important in the nucleation of cholesterol microprecipitates from lithogenic bile. We inhibited gallbladder mucin glycoprotein secretion with aspirin, since this compound, as well as other nonsteroidal anti-inflammatory drugs, are known to inhibit mucin secretion in other organs (*13*) but are without effect on biliary lipid saturation (*14*). We found that aspirin administered to cholesterol-fed prairie dogs inhibited the nucleation of cholesterol-supersaturated bile without altering biliary lipid composition and prevented the appearance of cholesterol microprecipitates and gallstone formation. The prevention of crystals and stones in the aspirin-treated animals was correlated with the absence of a mucin gel and a marked diminution in gallbladder mucin concentration.

We first studied the effect of aspirin on gallbladder mucin secretion in vitro, using an organ culture technique (*15*). Gallbladder explants from cholesterol-fed and control prairie dogs were incubated with [^{3}H]glucosamine and various concentrations (1 to 25 m*M*) of aspirin (*16*). Aspirin at all doses inhibited gallbladder glycoprotein secretion from normal gallbladder explants as well as those from prairie dogs fed the cholesterol diet for 5 days. The addition of aspirin caused a dose-dependent inhibition of mucin secretion reaching 80 to 85 percent inhibition at 25 m*M*.

To confirm the inhibitory effect of aspirin on gallbladder mucin secretion, we compared mucin glycoprotein synthesis and secretion in gallbladder explants from animals fed cholesterol and animals fed cholesterol plus aspirin. Aspirin doses of 25, 50, 75, and 100 mg per kilogram per day were well tolerated. The largest dose of aspirin caused mild gastritis but did not cause significant hepatotoxicity, weight loss, or hemorrhage. Prairie dogs ($N = 6$) were then pair-fed with either the 1.2 percent cholesterol diet or the 1.2 percent cholesterol diet plus 100 mg of aspirin per kilogram of body weight per day for 6 days. As shown in Fig. 1, aspirin in vivo significantly ($P < .001$) inhibited the incorporation of [^{3}H]glucosamine into gallbladder tissue and secreted mucin glycoproteins after 6 and 24 hours of incubation.

We then tested the effects of aspirin on total and relative biliary lipid composition and the formation of cholesterol crystals and gallstones (*17*) in prairie dogs fed the cholesterol diet for 14 days. Three groups of prairie dogs were studied (Table 1). Control animals were given

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Table 1. Effects of aspirin on the physical state and lipid compositions of gallbladder bile in cholesterol-fed prairie dogs. For the analytical procedures see (*17*). Cholesterol crystals and stones were detected by both visual inspection and microscopy of aspirated bile. Mucin was detected as nonbirefringent viscoelastic aggregated threads by polarized light microscopy.

Group	*N*	Diet	Number of animals with		Total lipid concentration (g/dl)*	Lipid analysis (moles/100 moles)*			Cholesterol saturation (%)
			Cholesterol crystals and stones	Mucin gel		Cholesterol	Bile salts	Phospholipids	
1	20	Control	0	0	9.3 ± 2.6	2.3 ± 1.1	86.1 ± 2.3	12.2 ± 2.8	48 ± 13†
2	15	Cholesterol (1.2 percent)	15	15	9.6 ± 3.5	7.6 ± 2.8	75.4 ± 5.3	16.9 ± 4.7	125 ± 24†
3	15	Cholesterol (1.2 percent) plus aspirin (100 mg/kg-day)	0	0	8.9 ± 4.1	6.9 ± 2.5	77.3 ± 6.1	15.8 ± 3.6	123 ± 21†

*Mean ± standard deviation. †Group 2 versus group 3, not significant by Student's *t*-test; groups 2 and 3 versus group 1, $P < .001$.

free access to a low cholesterol diet, and experimental animals were fed either a 1.2 percent cholesterol diet or 1.2 percent cholesterol plus 100 mg of aspirin per kilogram of body weight per day and were pair-fed in order to ensure equivalent intake of cholesterol. The gallbladder bile of prairie dogs fed the control diet was 48 percent saturated with cholesterol (Table 1) and, by polarizing light microscopy, contained no cholesterol crystals, stones, or mucin gel. Gallbladder bile of all of the 15 animals fed cholesterol contained mucin gel, cholesterol crystals, and gallstones and, as expected, the mean percentage of cholesterol saturation was increased (125 percent). The gallbladder biles of the animals fed cholesterol plus aspirin were comparably saturated with cholesterol (123 percent); however, none of these biles contained mucin gel, cholesterol crystals, or gallstones.

To further confirm our microscopic and gross findings, we quantified mucin concentration in gallbladder bile using the Alcian blue technique (*18*) after first partially purifying the mucin glycoproteins by gel filtration on Sepharose 4B (*19*). In the gallbladder bile from the aspirin-treated animals ($N = 3$) the concentration of mucin glycoproteins was one-third of that of the cholesterol-fed animals and was apparently below the concentration where gel formation occurs.

The ability of gallbladder mucin to nucleate cholesterol from supersaturated bile was directly tested by mixing gelled human gallbladder mucin (*12*) with supersaturated hepatic bile from cholesterol-fed prairie dogs (*20*). By polarizing microscopy these biles were initially clear. Birefringent liquid crystals first appeared in the mucin gel after 2 hours and by 6 to 8 hours numbered between 20 to 50 per high-power field. By 16 hours these liquid crystals had begun their transformation into solid crystals and 4 to 8 hours later, only cholesterol monohydrate crystals with the typical notched rhombohedral plate configuration (*21*) were visible. Control slides containing a portion of the same samples of hepatic bile alone or with added albumin (20 mg/ml) failed to form liquid or solid cholesterol crystals even after 24 hours of incubation.

These experiments document the importance of gallbladder mucin in the development of cholesterol gallstones and the ability of aspirin to inhibit mucin production and gallstone formation without altering biliary lipid composition. A gallbladder-derived "nucleating factor" has long been postulated to explain the preponderance of gallstones in the gallbladder compared with hepatic bile (*22*) and the prevention of cholesterol gallstones in the biliary tree after cholecystectomy even though hepatic bile may remain supersaturated (*23*). Furthermore, supersaturated bile per se does not necessarily result in cholesterol precipitation or gallstones (*3*). As postulated (*11*), lithogenic gallbladder bile from humans with gallstones appears to contain a nucleating agent not found in supersaturated gallbladder bile from patients without stones, but previous attempts to identify the putative agent have failed (*11*).

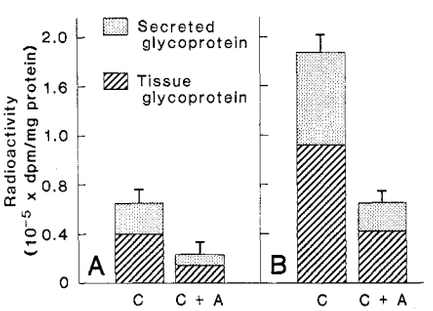

Fig. 1. Incorporation of [^{3}H]glucosamine into secreted and tissue mucin glycoproteins after (A) 6 and (B) 24 hours of organ culture of gallbladder explants. Prairie dogs ($N = 6$) were pair-fed either a 1.2 percent cholesterol diet (*C*) or a 1.2 percent cholesterol diet plus 100 mg of aspirin per kilogram of body weight per day ($C + A$) for 6 days. In each animal 85 percent of the incorporated radioactivity was present in a high-molecular-weight (> 1 million) mucin glycoprotein fraction as determined by Sepharose 4B gel chromatography (*12*). The reduction in incorporation of the radioactive label into both tissue and secreted glycoproteins in the aspirin-fed group is significant ($P < .001$) at both time points and reflects reduced glycoprotein synthesis. The rate of incorporation is linear with time (6 → 24 hours) and lower in the aspirin-fed group. For details of methods, see (*12, 15, 16*).

Mucin gel in the gallbladder of the cholesterol-fed prairie dog appears to act as a nucleating agent for cholesterol. Although salicylates in general cause an increase in bile salt-independent bile flow (*14, 24*), the salutary effect of aspirin in our studies was mediated through inhibition of gallbladder mucin synthesis, which thus decreased secretion and accumulation of mucin gel in gallbladder bile. Binding of lipids to mucin gel has been described during studies on cholesterol adsorption in the intestinal tract (*25*), and may also occur in gallbladder bile. One possibility is that gallbladder mucin, like bovine cervical mucin (*26*), contains repeating hydrophobic sequences of amino acids in the unglycosylated regions of the peptide core. Cholesterol-lecithin microprecipitates from supersatured bile might, because of their hydrophobicity preferentially bind to these regions of the polymer and thus initiate crystal growth. An analogous configuration is used in industry to grow pure crystals from supersaturated systems. Here a gel serves to lower the activation energy required for nucleation to occur (*27*). If confirmed in man, these results raise the possibility that gallstones might be prevented in high-risk patients (*28*) by nonsteroidal anti-inflammatory drugs or other pharmacological agents capable of inhibiting the secretion of mucin glycoproteins from the gallbladder wall.

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17. Gallbladder bile was analyzed immediately by visual inspection and by polarizing microscopy on a heated stage at 37°C. Biliary lipids were measured in bile as described by Carey and Small (*3*). The percentage of cholesterol saturation of bile was calculated from the total and relative biliary lipid compositions [M. C. Carey, *J. Lipid Res.* **19**, 945 (1978)].
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Repair of the Ultraviolet-Irradiated Male Genome in Fertilized Mouse Eggs

Abstract. *Unscheduled DNA synthesis occurred in both male and female pronuclei of the mouse zygote in response to irradiation with ultraviolet light, indicating a capacity for excision repair. Furthermore, damage to DNA of the male gamete before fertilization can be repaired after the sperm enters the egg cytoplasm.*

Environmental agents that damage DNA produce mutations and lead to malignancies in humans and laboratory animals (*1*). When genetic damage occurs in the germ cells, there is a risk of increased malignancy or genetic disease in the offspring (*2*). Although mammalian cells in general can repair damaged DNA, the human disease xeroderma pigmentosum is characterized by a deficiency in the repair of DNA (*3*). The high incidence of malignancies in this disease indicates that repair of DNA damage may play a significant role in controlling mutagenesis and carcinogenesis. Because the mature sperm of mice, rats, rabbits, and humans are also known to be deficient in excision repair (*4*), the period after the entry of sperm into the egg cytoplasm is the only stage at which damage to sperm DNA could be repaired before the zygote genome begins active DNA replication and cell division.

We studied the repair of mouse sperm DNA between entry into the egg cytoplasm and the beginning of the first S phase by means of autoradiographically detectable DNA synthesis in response to ultraviolet irradiation. Such non-S-phase, or unscheduled, DNA synthesis is thought to indicate the activity of a DNA repair system that removes damaged bases (*5*). In one approach, both male and female pronuclei were irradiated in one-cell embryos after fertilization in vivo; in another, mature sperm were irradiated before they were used for fertilization in vitro.

Embryos were obtained by superovulation (*6*) of ICR mice (Flow Labs, Dublin, Virginia) and cultured as described in (*6*). After removal of follicle cells, washed embryos were irradiated 15 to 16 hours after administration of human chorionic gonadotropin (hCG) in medium without bovine serum albumin (exposure, 1.25 J/m^2/sec), then incubated in medium containing [^{3}H]thymidine (20 μCi/ml; specific activity, 40 Ci/mmole, Amersham). After 1 hour, embryos were washed and incubated in medium containing nonradioactive thymidine, fixed, and air-dried on slides (*7*). Slides were exposed to Kodak NTB emulsion for 2 weeks, developed, and stained with Giemsa.

Unscheduled DNA synthesis occurred in both female and male pronuclei of one-cell mouse embryos that had been irradiated with ultraviolet light 16 hours after treatment with hCG (Fig. 1A). In unirradiated (control) embryos, nuclei showed up to 10 grains, a cellular background seen in mouse oocytes and embryos (*8*). About 10 percent of nuclei

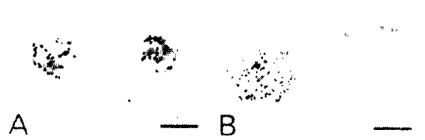

Fig. 1. (A) Unscheduled DNA synthesis in pronuclei of a one-cell mouse embryo fertilized in vivo and then exposed to ultraviolet light. (B) Unscheduled DNA synthesis in mouse embryo fertilized in vitro after sperm were exposed to ultraviolet light. Scale bars are 10 μm.

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