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The production of oxysterols in bile by activated human leukocytes[☆]

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Abstract

Oxysterols are naturally occurring intermediates in the conversion of cholesterol to bile acids, the major route for elimination of cholesterol. Additionally, they are important signaling agents, particularly in control of cholesterol synthesis; however, some species also are cytotoxic and carcinogenic. Oxysterols in plasma, contained in oxidized low-density lipoprotein, are strongly correlated with atherosclerosis. Oxysterols are found in infected human bile and the oxysterol content in gallstones correlates with bacterial DNA in the stones. Here we demonstrate that human leukocytes, activated by the presence of bacterial lipopolysaccharide, are able to oxidize cholesterol to a variety of oxysterols, including species known to be carcinogenic.

Keywords

Oxysterols; Cholesterol oxides; Leukocytes; Bile; Lipopolysaccharide; Infection; Cholangiocarcinoma; Gallbladder cancer

[☆]Abbreviations:

DMEM

Dulbecco's minimal essential medium

BSS

basic salts solution

BHT

butylated hydroxytoluene

LPS

bacterial lipopolysaccharide

3K

3-ketocholest-4-ene

3KD

3-ketocholesta-4,6-diene

7-beta

7 β -hydroxycholesterol

7K

7-ketocholesterol

7KD

7-ketocholesta-3,5-diene

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The occurrence of oxidized forms of cholesterol (oxysterols) in living tissues has now been known for more than six decades [1] and these compounds have been associated with atherogenicity for forty years [2]. It is now well established that oxysterols are closely associated with the genesis of atherosclerosis [3–5] and most probably with various forms of cancer [6–8]. Cancer of the biliary system has recently shown an alarming upsurge in incidence and mortality in both the UK and US [9,10] in addition to continuing increases in countries with already high incidence, such as Japan [11]. Little is known of the etiology of cholangiocarcinoma and gallbladder cancer. However, we have shown that bacterial infection is involved in the pathogenesis of mixed cholesterol stones and brown pigment stones [12] and that the content of oxysterols is negatively correlated with the cholesterol content of gallstones [13]. The presence of gallstones, particularly pigment stones, is strongly associated with gallbladder cancer [14] and intrahepatic (ductal) stones with cholangiocarcinoma [15]. A pathogenetic role of chronic infection or colonization by bacteria such as *Salmonella typhi* [16] and *Helicobacter* spp. [17] in biliary cancer also has been demonstrated.

We have demonstrated the presence of bacteria in gallstones [12] and of oxysterols in human gallstones and bile [13]. We also have shown that hepatic bile from patients with biliary infection contains significantly higher levels of oxysterols than does such bile from patients lacking infection of the biliary system [18]. We hypothesize that the oxidation of cholesterol in bile occurs as a result of the action of activated leukocytes on invading bacteria. Here we demonstrate that the incubation of activated human peripheral leukocytes with normal human bile, or with model bile, can result in the production of a wide range of oxysterols.

Materials and methods

Model bile

Model bile was made by dissolving 108 mg of sodium taurocholate (Calbiochem, La Jolla, CA) in 10 ml of methanol and adding 38 mg of phosphatidylcholine (Type XVII, Sigma, St. Louis, MO). Ten milligrams of three-times recrystallized cholesterol was added with sufficient chloroform to dissolve all components with gentle warming. The solvent was removed on a rotary evaporator at 40 °C, the flask filled with argon, sealed, and stored at -70 °C until used. At that time, 10 ml of DMEM (Gibco, Grand Island, NY) was added with warming to make a clear solution.

Leukocytes

One hundred milliliters of blood, drawn from volunteers, was mixed with 100 ml Hanks' BSS (Mediatech, Fisher Scientific, Los Angeles, CA) containing 1.5 mg/ml of disodium EDTA. Sixteen milliliters was carefully layered on top of 12 ml of Ficoll—Hypaque Plus (Amersham, Piscataway, NJ) in each of twelve 50 ml sterile centrifuge tubes. The tubes were centrifuged at 600g for 40 min, then the leukocyte layer was removed and diluted with a minimum of three volumes of Hanks' BSS. Leukocytes were precipitated by spinning at 200g for 20 min and then were resuspended in 1 ml of DMEM.

Incubation

To two 22-ml culture tubes were added 5 ml of model bile and 0.5 ml of leukocyte suspension. The LPS tube received 50 µg/ml of bacterial lipopolysaccharide from *Escherichia coli* (Sigma) and then it and the control tube were loosely stoppered with cotton and placed in a shaking water bath at 37 °C for 5 h. In addition to the mechanical shaking, the tubes were thoroughly shaken manually at 30-min intervals. At the end of the incubation time, each tube received 12 ml of chloroform/methanol (2:1 by volume) containing BHT as antioxidant and was capped and shaken well.

Extraction

After centrifugation, the lower (chloroform) layer was transferred to a fresh tube and evaporated to dryness. One milliliter of 5% methanolic KOH was added and allowed to act for 30 min, in order to hydrolyze the lecithin. The non-saponified lipids were extracted with ether, washed, and the ether evaporated to dryness. The sterols were derivatized with BSTFA (Supelco, Bellefonte, PA) in pyridine and finally taken up in 100 µl of hexane for analysis by gas chromatography/mass spectrometry (GC/MS).

Analysis

Oxysterols were analyzed by GC/MS as described previously [13], with identification by comparison with standard mass spectra from the NIST 98 and Wiley Registry of Mass Spectra (7th edition).

Results and discussion

Table 1 shows the results of incubating peripheral leukocytes (control or activated) with human bile or model bile. Similar results were obtained using live *E. coli* instead of LPS (data not shown). There is remarkable variation in both the amount and species of oxysterol produced by leukocytes between subjects and also great variation between times with leukocytes from the same subject. The observation that control incubations sometimes contained oxysterols may best be explained by some degree of autoxidation, which would be impossible to avoid during the cell incubation period. This is particularly likely for 7-hydroxycholesterol and 7-ketocholesterol. It is also quite likely that some degree of activation of the leukocytes may have pre-existed, due to sub-clinical infections in our volunteer donors. The use of various preparations of multivitamins and antioxidants, consumed as food supplements by our donors, also may have exaggerated the wide biological scatter of the species and quantities of oxysterols produced during incubation of their leukocytes.

One concern which always arises with oxysterols is that they may be artifacts, either of the extraction procedure or generated in the GC. For extraction, we always used solvents containing the antioxidant BHT and the procedures were carried out under nitrogen. Pure standards processed through the same steps showed no autoxidation. In the GC, we have noted that production of 7-ketocholestadiene (7KD) from 7-ketocholesterol (7K) can occur by thermal dehydration in the injection port, particularly if the injection port ***is contaminated by non-volatile material deposited from samples injected by other users; thus, we always run an injection of 7K standard before the samples are analyzed. Interestingly, we have observed that 7K taken up from model bile by dog gallbladder epithelial cells (data not shown) is converted by the cells to 7KD to a significant extent, confirming that this is a biological process.

In addition to the oxysterols reported, we have observed the presence of both 4- and 6-hydroxycholesterols, together with other compounds whose mass spectra strongly suggest that they are cholesterol diols, but do not correspond with standards in the libraries of mass spectra.

Recently, we described the discovery of previously unknown oxysterol species in gallstones and in bile samples of patients with gallstones [13] and noted that the quantity of oxysterols in the gallstones correlated with the abundance of bacterial DNA. These new findings generated intriguing questions as to the origin of oxysterols and their pathological significance, leading us [8] and others [19] to hypothesize that they mediate important biological effects, including cytotoxicity [8] and carcinogenesis. The hypothesis that chronic, low grade or asymptomatic bacterial infection, such as present in biliary stones and primary sclerosing cholangitis, can be pathogenetically linked to cancer formation is reinforced by observations in carriers of *Salmonella typhi*. These totally asymptomatic individuals harbor a biofilm of *S. typhi*

colonizing the biliary epithelium. The risk of biliary cancer in this group is at least 40 times that in age- and sex-matched controls from the same geographic region [16].

These experiments were designed not to study the kinetics of oxysterol production, or to evaluate all the oxysterol species correlating with leukocyte biological/metabolic function, but simply as a proof of principle that endogenous biliary cholesterol can be modified during a bacterial infection. Here, we have shown that human leukocytes, activated by a bacterial product (LPS), can convert biliary cholesterol into oxysterols. Thus, the present data provide a strong logistical link between bacterial infection of the biliary system and the development of gallbladder cancer and cholangiocarcinoma.

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References

- [1]. Haslewood GAD. Isolation of 7-hydroxycholesterol and of "hepatols" from ox liver. *Biochem. J* 1939;33:709–712. [PubMed: 16746963]
- [2]. Cook RP, MacDougall JDB. Experimental atherosclerosis in rabbits after feeding cholestanetriol. *Br. J. Exp. Pathol* 1968;49:265–271. [PubMed: 5665440]
- [3]. Hodis HN, Crawford W, Sevanian A. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel; further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis* 1991;89:117–126. [PubMed: 1793439]
- [4]. Chisolm GM, Ma G, Irwin KC, Martin LL, Gunderson MG, Linberg LF, Morel DW, DiCorleto PE. 7- β -hydroxycholest-5-ene-3 β -ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low-density lipoprotein. *Proc. Natl. Acad. Sci. USA* 1994;91:11452–11456. [PubMed: 7972083]
- [5]. Brown AJ, Leong SL, Dean RT, Jessup W. 7-Hydroperoxycholesterol and its products in oxidized low-density lipoprotein and human atherosclerotic plaque. *J. Lipid Res* 1997;38:1730–1745. [PubMed: 9323583]
- [6]. Morin, RJ.; Peng, SK. Cholesterol oxides in plasma and tissues. In: Peng, SK.; Morin, RJ., editors. *Biological Effects of Cholesterol Oxides*. CRC Press; Boca Raton, FL: 1992. p. 89-101.
- [7]. Yoon JY, Canbay AE, Werneburg NW, Lee SP, Gores GJ. Oxysterols induce cyclooxygenase-2 expression in cholangiocytes: implications for biliary tract carcinogenesis. *Hepatology* 2004;39:732–738. [PubMed: 14999691]
- [8]. Seo DW, Choi HS, Lee SP, Kuver R. Oxysterols from human bile induce apoptosis of canine gallbladder epithelial cells in monolayer culture. *Am. J. Physiol. Gastro. Liver Physiol* 2004;287:G1247–G1256.
- [9]. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001;33:1353–1357. [PubMed: 11391522]
- [10]. Taylor-Robinson SD, Toledano MB, Arora S, Keegan TJ, Hargreaves S, Beck A, Khan SA, Elliott P, Thomas HC. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968–1998. *Gut* 2001;48:816–820. [PubMed: 11358902]
- [11]. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002;2:10. [PubMed: 11991810]
- [12]. Lee DK, Tarr PI, Haigh WG, Lee SP. Bacterial DNA in mixed cholesterol gallstones. *Am. J. Gastroenterol* 1999;94:3502–3506. [PubMed: 10606311]
- [13]. Haigh WG, Lee SP. Identification of oxysterols in human bile and pigment gallstones. *Gastroenterology* 2001;121:118–123. [PubMed: 11438500]
- [14]. Fox JG, Dewhirst FE, Feng Y, Taylor NS, Pasta BJ, Ericson RL, Lau CN, Correa P, Araya JC, Roa I. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 1998;114:755–763. [PubMed: 9516396]

- [15]. Kim MH, Sekijima JH, Lee SP. Primary intrahepatic stones. Am. J. Gastroenterol 1995;90:540–548. [PubMed: 7717308]
- [16]. Ko, CW.; Lee, SP. Gallstones. In: Yamada, T.; Owyang, C.; Powell, DW.; Laine, L., editors. Textbook of Gastroenterology. Williams and Wilkins; Lippincott, Philadelphia: 1999. p. 225-280.
- [17]. Murata H, Tsuji S, Tsuji M, Fu HY, Tanimura H, Tsujimoto M, Matsuura N, Kawano S, Hori M. *Helicobacter bilis* infection in biliary tract cancer. Aliment. Pharmacol. Ther 2004;20S:90–94. [PubMed: 15298612]
- [18]. Yoshida T, Matsuzaki Y, Haigh WG, Fukushima S, Ikezawa K, Tanaka N, Lee SP. Origin of oxysterols in hepatic bile of patients with biliary infection. Am. J. Gastroenterol 2003;98:2275–2280. [PubMed: 14572579]
- [19]. Wang DQ, Afdhal NH. Good cholesterol, bad cholesterol: role of oxysterols in biliary tract diseases. Gastroenterology 2001;121:216–218. [PubMed: 11438511]

Table 1
Production of oxysterols by incubation of human leukocytes with human bile or model bile, in the absence or presence of bacterial lipopolysaccharide

Subject	3K		3KD		7-beta		7K		7KD	
	Control	LPS	Control	LPS	Control	LPS	Control	LPS	Control	LPS
<i>Model bile</i>										
T-1	0	0	0	0	0	0	47.3	36.4	10.4	53.6
T-2	0	0	0	0	0	0	23.4	0	0	31.1
G-1	0	1.5	0	0	0	0	0	0	0	1.2
G-2	35.9	8.7	0	0	135.4	21.8	5.3	0	0	1.6
G-3	0	0	58.4	34.6	0	67.4	41.6	13.9	5.9	0
G-4	0	0	0	0	0	1127.7	75.7	20.9	9.2	16.1
G-5	0	0	0	0	0	406.1	0	258.8	0	0
S-1	0	0	0	36.2	41.1	48.9	7.1	6.5	0.1	0.4
I-1	0	50.5	0	0	0	0	0	0	8.7	31.3
I-2	0	0	0	0	81.1	91.5	70.5	32.3	0	0
R-2	0	0	0	0	124.2	129.1	48.8	149.3	0	0
<i>Human bile</i>										
A	2.2	1.5	0	0	0.27	0.28	0.63	0.43	0.2	0.1
B	2.2	2.3	0.22	0.18	0	0	2.6	0.76	0.4	0.3
C	10.4	12.2	0	0	0.91	2.6	0.2	0	1	2.1

Concentrations are in ng/ml in the final 100 µl sample.