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Why make gallstones, anyway?
Protein Kinase C and sodium transport in the gallbladder

In most people, bile is formed by the liver, held for a while in the gallbladder, and uneventfully delivered to the duodenum. In other people, the constituents of the bile do not remain in solution; sludge or stones result, and the biliary tract ceases to be a silent partner in digestive and excretory operations. Sometimes it’s pretty clear why this happens. A patient with chronic hemolysis, for example, may simply present a greater pigment load to the liver than can be excreted in clear bile; greenish-black pigment stones may then form. In other patients, abnormalities in lipid metabolism result in bile that has too much of one lipid or another, and a solution or smooth emulsion cannot reliably be maintained. In many patients, however, the reason for the propensity to make stones is not so readily discerned; interest continues to focus on the gallbladder, because there is ample opportunity for changes in the concentration and ionic composition of bile while it is being held there for release.

Many animals are ill-suited to the modeling of human gallbladder disease, because they differ importantly from people in biliary tract anatomy or physiology, or because there is no simple way to provoke gallstone formation. The black-tailed prairie dog, *Cynomys ludovicianus*, has a biliary tract that closely resembles that of people, and it tends to develop gallstones if fed a high-cholesterol diet (see *J Lab Clin Med*, 1992;120:976.). This model was used in a study presented beginning on page 227 of this issue by Dr. M. A. Abedin and colleagues from Drexel University and the University of Alabama, Birmingham.

It is already known that absorption of sodium by the gallbladder is increased during gallstone formation and that the concentration of Ca\(^{++}\) in gallbladder bile also rises; these changes may promote cholesterol nucleation and thus lead to sludge or stone formation. Protein Kinase C (PKC) is calcium-sensitive and inhibits basal sodium transport in the gallbladder; its state of activation could be a determinant of what happens to bile while held before release. The authors therefore sought to characterize Ca\(^{++}\)-dependent PKC isoforms in the gallbladder and to examine their roles on Na\(^{+}\) transport during gallstone formation.

Prairie dogs were fed either ordinary chow or chow that had been made more lithogenic by increasing its cholesterol content. At various intervals, the gallbladders were removed for study. As expected, PKC activation (by phorbol dibutyrate) decreased sodium flux and inhibited sodium...
uptake mediated by sodium/hydrogen exchange. The alpha isoform of PKC accounted for 42% of the total PKC activity at basal state, and inhibition of PKC-α blocked about 45% of the effect of the phorbol ester. During gallstone formation, PKC-α blockade became progressively less effective at blocking phorbol ester’s effect on sodium transport. The contribution of PKC-α (as opposed to other isoforms) to total PKC activity became progressively less during gallstone formation, even though levels of PKC-α mRNA and protein significantly increased. They conclude that PKC-α regulation of gallbladder sodium/hydrogen exchange becomes progressively dysfunctional during lithogenesis and may be part of the explanation for the increased sodium absorption during this process.

Dr. Karel J. van Erpecum, MD, of the University Hospital of Utrecht, the Netherlands, provides an editorial comment on page 202.

Tomatoes are red, and so is blood: Should I care?

Lycopene is a natural carotenoid antioxidant that accounts for much of the red color in tomatoes. It has been touted as an agent for the prevention of prostate cancer, heart disease and a number of other maladies. Many of the health claims are based on epidemiologic observations; these are hard to evaluate critically, because diets high in lycopene are almost always low in saturated fat and high in numerous other phytochemicals. In order to sort out the specific effects of lycopene, studying it in isolation is important, and one such study appears in the current issue of the Journal (see page 216).

Dr. George Hsiao and his associates from the Taipei Medical University asked whether lycopene might have an effect upon platelet function, and thus whether a beneficial effect on cardiovascular health might be credible on that basis. They started by simply exposing human platelets to lycopene in vitro, then measured the response of those platelets to traditional agonists, such as collagen, ADP or arachidonate. Micromolar concentrations of lycopene inhibited the aggregation response to each of these agents; the effect was seen in a washed-platelet system as well as in platelet-rich plasma. Lycopene also inhibited calcium mobilization, thromboxane generation and phosphoinositide breakdown—suggesting that its major effect was early in platelet activation. Supporting that idea, lycopene was also found to inhibit Protein Kinase C activation in platelets.

Lycopene was also found to have an effect on platelet responsiveness in vivo. UV-rich irradiation was used to provoke local tissue injury and platelet plug formation in exteriorized mesenteric vessels of mice that had received injections of fluorescein. The pre-administration of lycopene to these animals delayed the production of platelet plugs.

The authors conclude that there is an anti-platelet activity of lycopene, that it can occur in vivo and that the effect is worked early—perhaps through inhibition of activation of phospholipase C.

Making sense out of clotting tests in the face of bad liver function: The special case of portal vein thrombosis

Liver disease often confounds the evaluation of the coagulation system, while at the same time creating risks both of hemorrhage and thrombosis. The presence of impaired portal blood flow is a particularly common and particularly difficult example: sluggish flow may lead to thrombosis, but the formation of varices and the underproduction of coagulation factors may set the stage for catastrophic hemorrhage. From studies of lower-extremity venous thrombosis and pulmonary embolus, we know that a low D-dimer level makes clinically important thrombosis much less likely—can we say the same for portal thrombosis in patients with cirrhosis? Dr. F.L. Fimognari et amici from University La Sapienza, Rome, Italy (and its affiliated hospitals) asked that question and present their findings this month.

As described beginning on page 238, they examined 136 consecutive outpatients with stable cirrhosis. Each underwent color/power ultrasonography to test for the presence of portal thrombosis, which was found in 33 patients (24.2%). They found that the level of Factor VIII and the level of
D-dimer varied according to the severity of the underlying cirrhosis. For example, in patients without portal thrombosis, factor VIII was significantly higher in Child-Pugh class C patients than it was in patients who fit into class A or B. When they looked at class C patients with portal thrombosis, they found lower factor VIII levels than they had found in comparable patients without thrombosis.

The story for D-dimer was similar. That is, D-dimer level was significantly increased in class C patients compared to those in class A or B. Among class C patients, those with portal thrombosis had higher D-dimer values than did patients without thrombosis. The pattern that emerged was that, in patients with severe cirrhosis, a high D-dimer level and a low Factor VIII level identified a group that was quite likely to have portal thrombosis; a low D-dimer level and a high Factor VIII level identified a group that was quite unlikely to have thrombosis. The predictive value was less strong in patients with milder cirrhosis.

Race, ethnicity and distributive justice: there’s still no easy answer.

The concept of race in medical research is a thorny one (which we have addressed editorially in the past; see J Lab Clin Med 1999;133:10-12). The very concept is not very well or consistently defined, and specific racial groups are even less well defined. As a result, much research on race and health is based on ambiguous categorical variables—a serious structural problem. In studies that have explained their racial assignments, many have used assignments of convenience (such as the answer on an anti-discrimination form, or the racial descriptor in a house-officer’s admission note) rather than criteria appropriate to the research question at hand. It’s rare to see a study that critically assesses the actual racial assignments made, or one that describes (and defends) what has been done about study participants of mixed racial background. Moreover, racial assignments often turn out to be color assignments (in which recent Somali immigrants will be lumped together with 6th-generation slave descendants) or turn out to be surrogates for non-racial factors such as socioeconomic status. It’s tempting to decide that the whole issue of race is too imprecise for science.

But there are reasons for not doing so. Of course, there are diseases that are geographically distributed and thus correlate with race—enough to be clinically useful despite all the imprecision. I know, for example, that microcytosis is much more likely to be due to hemoglobin E or thalassemia if the patient before me is Lao or Thai rather than Norwegian. If a child presents with unexplained limb or belly pain, sickle-cell disease will be much higher on the list of likely diagnoses if she is known to be of equatorial African descent.

Beyond these obvious examples, though, are ones that are more difficult. As a profession, we physicians may be committed to equitable treatment of all who seek our assistance, but do we always live up to that ideal? When we fail, how much of that failure is attributable to our cultural or racial biases, how much is attributable to our patients’ cultural and racial biases, and how much is attributable to inequities in our medical care “system” or in the fabric of society more generally? (Precise definition of categorical variables may be necessary for the practice of good science, but it’s not needed for the practice of discrimination!) This sort of question is worthy of study, vexing though the study design issues may be.

It is also important for us to learn about health discrepancies among groups in our society, whether or not we can figure out immediately whether they are based in race or culture or socioeconomic status or something else altogether. In order to do that, we may need to know about barriers—some of them racial or cultural—that keep people from participating in research.

An article reviewing some of these issues is presented this month by Dr. Jada Bussey-Jones and her collaborators, from Emory University, and may be found on page 205.

For the Editors
Dale E. Hammerschmidt, MD
Editor-in-Chief
The gallbladder: Innocent bystander or major factor in cholesterol-gallstone formation?

**Abbreviations:** ABC = ATP-binding cassette; ERα = estrogen receptor-α; FXR = farnesoid X receptor; LXRα = liver X receptor-α; mRNA = messenger RNA; Muc1 = mucin gene 1; NHE = Na+/H+ -exchange protein; PKC = protein kinase C

Cholesterol gallstones represent a significant health and economic burden in all developed countries, and 10% of the combined population of these countries is affected. Approximately 1 million new cases are diagnosed each year in the United States, most involving cholesterol gallstones. Although cholesterol is poorly soluble in a pure aqueous environment, in gallbladder bile a relatively large amount (~20×10⁻³ mol/L) of the sterol can be kept in solution through the incorporation of cholesterol in mixed micelles together with solubilizing bile salts and phosphatidylcholine. Cholesterol supersaturation occurs when either too much cholesterol or not enough solubilizing bile salt and phosphatidylcholine molecules are secreted to allow complete micellar solubilization of all cholesterol. Excess cholesterol may be kept in vesicles (ie, spherical bilayers of cholesterol and phospholipids, without bile salts) or nucleate as cholesterol crystals, which is the beginning of gallstone formation. In recent years, gallstone research has been focused on the hepatocytic apical (canalicular) membrane, where an elaborate network of ABC transporters regulates biliary secretion and relative concentrations of bile salts, phosphatidylcholine, and cholesterol, thus determining whether cholesterol supersaturation and cholesterol crystallization can occur (Fig 1). Intrahepatocytic nuclear receptors have been found to regulate these canalicular membrane–transport proteins. First, the nuclear receptor FXR stimulates canalicular membrane–transport proteins for cholesterol-solubilizing bile salt and phosphatidylcholine molecules. In a pivotal recent publication, Moschetta et al described a high likelihood of cholesterol-gallstone formation in FXR-knockout mice. In addition, in wild-type mice consuming a lithogenic diet, biliary cholesterol supersaturation and gallstone formation were prevented by means of treatment with a synthetic FXR agonist: FXR-dependent increases in biliary secretion of cholesterol-solubilizing bile salt and phosphatidylcholine molecules restored cholesterol solubility and thereby prevented gallstone formation. Second, the nuclear receptor LXRα stimulates canalicular membrane–transport proteins for cholesterol. Indeed, overexpression of the hepatic LXRα gene after stimulation by a synthetic LXR ligand was found to promote cholesterol-gallstone formation in gallstone-resistant AKR/J mice. Third, ERα plays a crucial role in the formation of cholesterol gallstones in mice in response to estrogen, and the lithogenic actions of estrogen can be completely blocked by the antiestrogenic ICI 182,780. FXR, LXRα and ERα should therefore be considered promising therapeutic targets for the treatment or prevention of cholesterol-gallstone disease.

These exciting developments at the hepatic level notwithstanding, the timely and interesting article by Narins et al in the current issue of the JOURNAL bring the gallbladder back into focus. The gallbladder is where gallstones usually form, and several processes within the gallbladder may inhibit or enhance lithogenesis. First, gel-forming mucin secreted by the gallbladder wall (especially mucin encoded by mucin gene 1, MUC1) is considered an important in vivo promoter of cholesterol crystallization and gallstone formation. In addition, wild-type mice consuming a lithogenic diet, biliary cholesterol supersaturation and impaired gallbladder emptying (resulting from incorporation of absorbed cholesterol molecules into the sarcolemmal plasma membrane of the smooth-muscle cell) may promote cholesterol-crystal nucleation by providing time for the nucleation of cholesterol crystals and their aggregation into macroscopic stones.

Fourth (and of particular relevance with regard to the article by Narins et al), gallbladder bile is normally
concentrated fourfold to fivefold in the gallbladder, with a resulting enhanced risk of cholesterol crystallization.\textsuperscript{13} As illustrated schematically in Fig 1, after cholesterol and phosphatidylcholine molecules have been made available at the outer leaflet of the hepatocyte canalicular membrane by their specific transport proteins (ABC-G5/G8 resp. ABC-B4), detergent bile-salt monomers (secreted into the canalicular lumen by the bile salt–export pump ABC-B11) induce formation of nascent cholesterol-phosphatidylcholine vesicles. These nascent vesicles are stable because they are relatively cholesterol-poor (cholesterol/phosphatidylcholine ratio far below 1), and cholesterol crystallization does not occur. Bile salts are mainly present as monomers under these circumstances because the bile-salt concentration is initially quite low (in all probability, in fact, below the critical micellar concentration).

During bile concentration in the gallbladder, mixed cholesterol–phosphatidylcholine–bile salt micelles are increasingly formed because bile salt concentrations now exceed the critical micellar concentrations required for micelle formation. As a result, cholesterol and phosphatidylcholine transfer from vesicles to these mixed micelles takes place. However, because the solubilizing capacity of micelles for phosphatidylcholine is much higher than that for cholesterol, phosphatidylcholine transfer is prefered compared with cholesterol transfer. Therefore, although fewer vesicles remain, they are now cholesterol-super-saturated (ie, cholesterol-phosphatidylcholine ratio > 1) and may nucleate cholesterol crystals. This sequence of events explains why gallstones are normally formed in the gallbladder (the site of the highest bile concentrations) rather than in the bile ducts.

In the prairie-dog model that was used in the study by Narins et al,\textsuperscript{7} increased fasting gallbladder bile-lipid concentrations were previously reported to occur during the earliest stages of gallstone formation (before stones had formed), and a reduced concentrating ability on the part of the gallbladder mucosa, induced with the drug amiloride, could prevent gallstone formation.\textsuperscript{14,15} Narins et al now explore in vitro in some detail potential underlying mechanisms.

In the fasting state, gallbladder bile is progressively concentrated: the apical plasma membrane protein NHE facilitates the exchange of Na\textsuperscript{+} in the gallbladder lumen for intracellular H\textsuperscript{+}, with secondary and osmosis-driven water transport from bile through the gallbladder mucosal layer. As a result, bile is acidified and becomes more concentrated.

The authors now demonstrate the presence of various PKC isoforms in the gallbladder mucosa. Under basal circumstances, PKC-\(\alpha\) was shown to impair the gallbladder bile concentration through NHE inhibition. The authors hypothesize that this phenomenon protects against cholesterol crystallization. During gallstone formation, PKC-\(\alpha\)-mediated regulation of gallbladder NHE became progressively dysfunctional, possibly contributing to increased Na\textsuperscript{+} absorption, a progressively increasing bile concentration, and cholesterol crystallization. Several criticisms are possible: The authors studied only unidirectional, not bidirectional Na\textsuperscript{+} fluxes, and their suggestion of different PKC-\(\alpha\)-mediated regulation of NHE2 versus NHE3 isoforms is not entirely convincing (results were not significant). Also, PKC-\(\alpha\)-mediated NHE inhibition would not only decrease bile concentration (a potentially beneficial effect) but also decrease bile acidification, with decreased calcium solubility in bile (a potentially detrimental effect that could promote cholesterol crystallization). Furthermore, other factors, such as periodic interdigestive gallbladder emptying, may prevent progressively increasing gallbladder bile concentration during the fasting period.\textsuperscript{16} Last, PKC activation is a key regulator of mucin gene expression, and its activity is required...
for phorbol ester–induced mRNA expression of some mucin genes.\(^{17}\) Therefore dysfunctional PKC could also up-regulate expression of mucin genes with enhanced secretion of this pronucleating glycoprotein, thereby promoting cholesterol crystallization and gallstone formation.

We recently explored in some detail the role of the gallbladder in the inbred-mouse model of gallstone formation.\(^{18}\) We noted progressive alterations in gallbladder histologic appearance while the animals consumed a lithogenic diet, with progressively increased gallbladder-wall thickness, stromal granulocyte infiltration, progressive fibrosis, edema, and epithelial-cell indentation. As a result, gallbladder emptying was increasingly impaired.

In contrast to the findings elicited with the prairie-dog model used by Narins et al.,\(^{7}\) we found that the gallbladder-bile concentration did not increase during the earliest stages of gallstone formation.\(^{19}\) We also detected aquaporin-1 and 8 in the murine gallbladder (at mRNA and protein level). These water channels may be involved in gallbladder water transport.

In conclusion, although Narins et al.\(^{7}\) have lit a small candle in the dark gallbladder, it remains unclear whether this organ is an innocent bystander or a major factor in gallstone formation. Much work remains to be done!

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According to the most recent census, the United States has become more racially diverse. If current demographic and health-care trends continue, the growing population of minorities will continue to be at risk for poor health outcomes. Although the U.S. health-care system as a whole struggles with the disparate needs of a racially diverse population, the potential role of health-care providers’ cultural bias in health disparities is perhaps even more compelling. Numerous studies have demonstrated that even when insurance status, socioeconomic status, and health status are similar, differences in treatment persist when comparing nonminority with minority populations. Disparities in receipt of different services had been, in part, attributed to differences in patient preference. However, in studies where patient preference was controlled, disparities remained, which suggests a role for other factors such as bias in clinical decision making.

Close examination of potential provider bias, patient preference, and doctor–patient communications can lead to an understanding of the extent to which these issues compromise health outcomes. Central to the concept of disparities of treatment and bias is a provider’s perception and determination of racial differences and racial categories. As much of the former discussions on the use of racial variables centers on research, this commentary will briefly discuss racial variables in research and the historical use of race as a backdrop. We will then focus our discussion on clinicians’ perception of racial characteristics, the selection and accuracy of racial variables in the clinical setting, as well as the possible clinical implications of these perceptions.

The use of racial variables by researchers has been controversial and widely debated. Important shifts in science policy have guided the use of race by epidemiologic and public health researchers. For example, the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry and the National Institutes of Health have issued inclusion policies for racial and ethnic minorities in externally funded research requiring grantees to collect race/ethnicity data. Other programs, such as the Food and Drug Administration, require drug sponsors to present data on efficacy and safety by race, age, and sex. In addition, biomedical journals increasingly mandate a statement regarding the purpose and the methods of determination of racial categories. Despite these guidelines, researchers have yet to reach consensus on the meaning or importance of racial categories in research.

This debate is, perhaps, heightened in the clinical setting. Although there is much in the literature regarding the use of race/racial variables by researchers, as well as the aforementioned guidelines for the use of these variables by researchers, audit, and publication, there is a relative dearth of information about the selection and use of racial categories by clinicians. This article seeks to add to this discourse by considering several questions regarding clinicians’ use of race: (1) Who/what determines a patient’s race? (2) Are racial categories attributed correctly? and (3) Should racial classification be used by clinicians to assess the role and consequences of race?

**WHO/WHAT DETERMINES A PATIENT’S RACE?**

The term “race” has a variety of definitions generally meant to describe a group of genetically related people who share certain characteristics. The *Merriam-Webster English Dictionary* defines race as “a division of mankind possessing traits that are transmissible by descent and sufficient to characterize it as a distinct human type.” Most authors have acknowledged that race is an unscientific term that can have biological significance only when it represents a uniform, closely inbred group—as in pure breeds of domesticated animals. These conditions are never met in humans. Although distinct, the term “ethnicity” is often interchangeable with race. It goes beyond physical and genetic features and incorporates shared language, country of origin, and sociocultural beliefs.

Ethnicity is arguably a more appropriate term given individual patient complexity and differing backgrounds, diets, beliefs, and practices, particularly for clinical settings. We use race in our discourse, however, as it seems to be more common in clinical discussions, with only occasional references to patients’ cultural background in both verbal and published clinical presentations.

Traditionally, concepts of race and skin color have served to categorize or group the population. The first U.S. Census of 1790 classified the population in terms of free white men, free white women, and other persons.
(such as American Indians) and slaves. Specific instructions required that the color of all slaves be documented. “Under heading 5, entitled ‘Color’ insert in all cases when the slave is black, the letter B; when he or she is mulatto, insert M. The 1890 Census discussed race in terms of white, black, mulatto, quadroon, octo-

Racial classifications, historically, have not only served to attribute physical characteristics but also psychological, social, and moral ones to members of given categories, justifying a discriminatory system with scientific claims of inferiority or superiority. The practice of apartheid in South Africa, genocide in Germany, and slavery and segregation in the United States were all based on the notion of social, cultural, and intellectual inferiority of certain “races.”

Concern about the use of race-perpetuating discrimination by clinicians is one of several arguments against this type of categorization. Clear definitions of race are questioned given that human variation is continuous. Allele frequencies vary gradually, leaving no clear demarcation of where 1 race begins and another ends. Most human variation is explained by geographic distance, with persons separated by the most distance having less similarities. Some traits vary independently of other traits. Race classifications then vary depending on the traits examined. A classification based on sickle cell trait, for example, could include Africans, Greeks, and Turks. Also within-group genetic variation is much greater than variation among “races.” One author evaluated blood group polymorphisms in different races and found that blood group variation among races explained only about 6% of total variation. Finally, racial classifications are inconsistently applied across time and place, often varying with changing political and social structure. These socially determined color lines are made even more dynamic by increasing minority populations, immigration, and intermarriage.

Race is, therefore, often thought of as a social construct. It has been used by researchers in the following ways: (1) to emphasize shared heritage that also highlights the power differentials that exist in society; (2) to identify social and economic factors, unequal treatment, public health policy, and health and coping behaviors as major determinants of unequal health outcomes; and (3) to identify shifting definitions and categories that correspond to social and political forces.

ARE RACIAL CATEGORIES ATTRIBUTED CORRECTLY?

One of the most common classifications to collect data on race is those of the U.S. Office of Management and Budget (OMB). Directive No. 15 of the OMB was developed in the 1970s with the intent to provide standard, unduplicated, exchangeable data by racial and ethnic categories. This directive was developed during a time when only 12.5% of the population was minority. The 2000 Census sought to expand these racial categories by allowing for 5 categories of race (American Indian or Alaska Native, White, Black or African American, Native Hawaiian or other Pacific Islander, and Asian) plus an Other category. For the first time, respondents were allowed to choose more than 1 category to indicate a multiracial background. Additionally, respondents were also asked whether they were Latino or Hispanic, giving 126 possible combinations of races and ethnicity.

For the purposes of clinical care, who places persons in these categories? Are the chosen categories representative and accurate? There is much in the literature about racial classification to suggest that attribution is approximate at best and often contradictory when querying different sources. Contradictory information, for example, has been found when comparing hospital discharge data, Medicare with Medicaid data, and birth and death certificates. One author found that when both parents reported the race of their child, nearly half (39 of 71 parent pairs) disagreed.

Many have suggested that information about race, when used by researchers, should be self-assigned. However this strategy may also be problematic. In the 1990 Census, 10 million people did not report their race. It has been suggested that Hispanics and patients with multiethnic backgrounds may have a difficult time with this classification. There seems to be more clarity, even in mixed-race children, when children were identified as African American. It may be a result of strong racial identification among this group or a reflection of the fact that society tends to treat any child with any African-American ancestors as African American (“one drop rule”).

RACIAL CLASSIFICATION IN THE CLINICAL SETTING

Motivated either by federal requirements or a desire to better understand, characterize, and serve their patients, many health-care organizations routinely record information about race in the clinical setting. The process of recording this information, which is maintained as a component of the clinical record, is often performed during initial registration by clerical staff. A small study found that 33% of 81 respondents in 1 setting and 22% of 59 respondents in another setting identified themselves differently than the way they were categorized in a clinical database. Furthermore, the database failed to capture the range of ways in which people self-identified. Twenty-five percent of the respondents indicated a preference for being allowed to identify multiple races. Additionally, the authors noted
that although the clerks were encouraged to ask the patient about their racial information, this occurred infrequently. Concern over patients’ expression of anger or lack of understanding of these questions led them to often assign race based on the patient’s last name or appearance.

Some have also questioned the current use of race by clinicians. Many students and trainees are trained in a culture that identifies patient race in clinical practice—often beginning their patient presentation by describing the age, gender, and race of the patient. The racial categories are often “black,” “white,” Hispanic, or Asian and may be rarely based on anything other than visual or language cues. One author suggests that the diagnostic and therapeutic value of these terms is at best imperfect and, in fact, may “obscure an accurate appraisal of a patient’s genetic and cultural backgrounds.”

These authors also surveyed clerkship directors of 48 medical schools in different regions of the United States and found that most students were taught, whether via oral tradition or formally, to use racial identification in introductory sentences in case presentations. Only 1 school out of the 48 reported that students were specifically taught not to use these terms unless relevant. Although tradition compels most training physicians to categorize patients by race, it is unclear whether their category selection is appropriate and valid. Issues with accuracy may be even more salient in the clinical encounter. In research, patients may be allowed to complete forms or to self-identify race via questionnaire; however, physicians that are face-to-face encounter with their patients may have similar reservations about using racial queries as described by clinical staff.

Also interesting are observations of how this information is used by clinicians. Often students and trainees present racial information for completeness or as a part of the learned ritual described above with little thought given to clinical relevance. One study examined chief resident case presentations over 2 months. They found that race was more often specified during the presentations of African-American patients. Additionally, for only a few of these patients were any “justifying” diagnoses considered for the discussion of race. Importantly, among patients to whom unflattering characteristics were attributed, race was more likely to be specified for blacks than for whites.

**SHOULD RACIAL CATEGORIES BE USED?**

Given varied definitions of race, misclassifications, and inaccuracies, some have suggested that the use of race by clinicians and researchers should be minimized, if not eliminated completely in clinical settings, public health reports, and research. There are, however, several reasons to consider race in the clinical encounter: (1) as an evaluation of progress on racial disparities in clinical care, (2) to discuss and address ways race may affect the clinical encounter, and (3) to identify potential risk and allow focused screening and treatment programs. We will discuss each of these reasons.

Many that argue against the use of race cite the undeniable ambiguity discussed above. Some even suggest that using racial categories perpetuates bias. Yet prejudice and social inequality are the reasons that others suggest that we should continue to include and examine this information. It is increasingly clear that “social perceptions of what a person is or is not influence the availability, delivery, and outcome of medical care.”

Focusing on the political, economic, social, and cultural factors that may lead to differences in health rather than small or potential biologic differences may help us recognize racism and its contributors. Many argue that using racial variables will allow monitoring of progress or setbacks in addressing racial inequalities in health.

Yet another reason to discuss race in the clinical encounter is the growing body of evidence demonstrating that patient race influences provider beliefs and expectations of patients and actions toward patients. In example, cardiac patients’ race and socioeconomic status were found to negatively influence physicians’ ratings of patients’ personality, education, intelligence, career demands, and adherence even after controlling for several physicians and patient characteristics. In another example, independent observers coded 150 physician–patient encounters and found that patient characteristics (race, sex, age, appearance) significantly influenced physician interpersonal behaviors. There are also examples of differing therapeutic plans based on patient race using standardized patient vignettes and videotapes. These studies support the premise that physicians’ perceptions of patient race may influence the content of clinical encounters as well as treatment recommendations.

Identification of risks and focused screening programs is yet another practical reason for the use of racial information by clinicians. Although the Human Genome Project has provided evidence that the genetic code does not seem to have major differences that delineate 1 race from another. I author notes that small genetic differences have developed in populations around the world corresponding geographically to the major continents. This author challenges the thought that race has no biological relevance. These genetic differences may be associated with risks that vary among populations. Few question that the prevalence of certain alleles and mutations varies among populations. For example,
Pima Indians have a greater susceptibility to type-2 diabetes mellitus, and Ashkenazi women have increased susceptibility to breast cancer because of the BRCA-1 mutations. In central and west Africa, several mutations in the β-globin gene gave rise to different sickle hemoglobins with distinct geographic phenotypes. Other examples of genetic mutations that appear with increased frequency in certain groups include Tay-sachs, hemocromatosis, and varied responses to medications. Although these manifestations of allelic variation do not define race, these variations and mutations among people who share cultural or geographic origins may have clinical implications, for example, focused screening programs and targeted medication trials.

CONCLUSION

Many clinicians would agree that many factors may contribute to a patient’s risk for disease and therapeutic response, including both genetic and cultural influences. As work continues on the human genome, there is hope that this knowledge will end clinical work that involves imprecise variables such as race. When racial variables are used by clinicians and researchers, however, we suggest the following guidelines: (1) Health professionals should understand the rational for the use of racial variables and method of determination. These professionals (with the help of cultural competence training) should also recognize the potential influence of their personal as well societal values in their categorization of patients. It should be recognized that broad racial labels in clinical discussions are likely to be inaccurate and represent a crude shorthand that merely scratches the surface of patients’ cultural/ethnic background. (2) Accurate and detailed histories including patients’ socioeconomic and ethnic background, beliefs, diet, and health-care practices should be obtained rather than simple racial classifications, as this information is likely more relevant for clinical care. (3) Finally, it is best to empower patients to define their racial identity when this information is recorded. Although these personal descriptions of “race” and ethnic/cultural background may not provide information that is biologically or anthropologically relevant, they may instead provide important clues to the patient’s self-identity and allow a deeper understanding of the implications of race and racism in treatment and health outcomes.

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Increased glomerular and extracellular malondialdehyde levels in patients and rats with diabetic nephropathy

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Results from animal experiments have suggested that reactive oxygen species (ROS) play an important role in tissue damage associated with diabetes. To determine whether ROS are involved in patients with diabetic nephropathy, we measured the plasma and urinary levels of malondialdehyde (MDA), an important marker of lipid peroxidation, and assessed the immunoreactivity of MDA and superoxide dismutase (SOD) in glomeruli of patients and experimental rats with diabetic nephropathy. Both plasma and urinary MDA levels were significantly higher in patients with diabetic glomerulosclerosis (DGS) than those of diabetic patients without proteinuria, proteinuric patients without diabetes, and normal controls. In DGS patients, the plasma MDA was significantly correlated with urinary MDA ($p < 0.05$). The urinary MDA, but not plasma MDA, was significantly correlated with the degree of glomerulosclerosis and the index of mesangial expansion (both $p < 0.01$) in DGS patients. The immunostaining score of glomerular MDA and SOD were also significantly higher in DGS patients than in control kidneys. In rats with diabetes for more than one month, the glomerular immunostaining for both MDA and SOD were also significantly higher than in controls rats, and both were increased with the progression of diabetes. Our results suggest that oxidative stress is involved in the pathogenesis and the progression of DGS. (J Lab Clin Med 2005;146:210–215)

**Abbreviations:**

- CCr = creatinine clearance rate
- DGS = diabetic glomerulosclerosis
- DM = diabetes mellitus
- DM1M = diabetes for 1 month
- DM3M = diabetes for 3 months
- DM1W = diabetes for 1 week
- ESRD = end-stage renal disease
- IME = index of mesangial expansion
- LDL = low-density lipoprotein
- MDA = malondialdehyde
- NC = normal controls
- NSA = normal serum albumin
- PBS = phosphate buffered saline
- PRO = proteinuric patients without DM
- ROS = reactive oxygen species
- SOD = superoxide dismutase

The number of diabetic patients with ESRD is increasing dramatically worldwide, and in many countries, diabetes has become the most frequent cause of ESRD. Its progressive nature with the poorer response to renal replacement therapy has led nephrologists to seek a better understanding of its pathogenesis. Our recent study indicated that the glomerular production of superoxide, one of the most important, was enhanced in diabetic rats both in vitro and in vivo. Important ROS induce lipid peroxidation, mediate tissue injury induced by cytokines and growth factors, induce oxidation of LDL, and inactivate vasodilating nitric oxide. Although animal studies...
have suggested a role of ROS in the pathogenesis of diabetic nephropathy, studies on the ROS generation in human diabetics, however, are still rare.

One major process involved in ROS damage is lipid peroxidation. Degradation of lipid peroxides generates a wide variety of compounds that may be transported or that simply leak from the organ or tissue of origin into the blood stream and are excreted in urine. Among the various products, MDA has been suggested as an easily accessible biomarker of ROS damage that can be analyzed by relatively simple methods. Therefore, in this study, we measured the plasma and urinary MDA and assessed the glomerular immunostaining of MDA and SOD in patients with DGS, and compared our findings with those of DM patients and NC. Rats with diabetes induced by streptozotocin injection were also studied.

**MATERIALS AND METHODS**

**Materials.** Our study included plasma and urine from 15 type 2 diabetic patients with biopsy-proved DGS, 15 type 2 DM patients, 15 PRO patients, and 15 NC. The 15 DGS patients included 9 men and 6 women, with a serum creatinine concentration of 99 ± 11 μmol/L, and the CCR was 98 ± 9 mL/min. Their mean age was 47 ± 9 years old, mean duration of diabetes was 12.4 ± 3.6 years, and daily protein loss was 1.7 ± 0.8 g/day. The 15 DM patients included 7 men and 8 women, with a serum creatinine concentration of 82 ± 19 μmol/L, and CCR was 102 ± 11 mL/min. Their mean age was 51 ± 8 years old, mean duration of diabetes was 10.9 ± 4.4 years, and daily protein loss was below 150 mg/day. The 15 PRO patients included 8 men and 7 women, with a serum creatinine concentration of 88 ± 12 μmol/L, and the CCR was 99 ± 10 mL/min. Their mean age was 42 ± 11 years old, and daily protein loss was 1.9 ± 0.7 g/day. All diabetic patients were on diets and therapy involving hypoglycemic agents, and none of the selected subjects of this study was on statin therapy. None of the patients were receiving antihypertensive medications including angiotensin II converting enzyme inhibitors or angiotensin II receptor blockers. All subjects provided informed consent to participate in this study that was approved by the Human Ethics Committees of Kaohsiung Medical University.

Venous blood and urine from patients and controls was collected in standard tubes containing 5-mM EDTA. After centrifugation (600 g × 10 min, 4°C), samples were stored at −80°C until assay. All biochemical analysis was performed immediately after collection. Plasma glucose was evaluated by glucose-oxidase method, HbA1C was measured by affinity chromatography, and cholesterol and triglyceride concentration was assayed enzymatically on a clinical chemistry analyzer. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Glomerular immunoreactivity of MDA and SOD was also measured in diabetic rats. Male Wistar rats with body weights between 200 and 250 g were used for the study. These rats were injected with 55 mg/kg of streptozotocin intraperitoneally and used for experiment after confirming the diagnosis of DM. Rats were classified into 3 groups, each containing 10 rats. Group I was sacrificed at 1 week after inducing diabetes (DM1W), group II was sacrificed at 1 month (DM1M), and group III was sacrificed at 3 months (DM3M). All 3 groups had the same number of rats sacrificed at the same time to serve as NC. Groups II and III were administered insulin (heat-treated bovine ultralente insulin, Novo-Nordisk, Copenhagen, Denmark) every day to maintain a poorly controlled diabetic state. Plasma glucose levels were regularly checked and maintained above 450 mg/dL. All rats were allowed food and water ad lib. The study was approved by the Animal Care and Treatment Committee of our institution.

**Tissue processing, scoring of glomerulosclerosis, and IME.** Both kidney tissues obtained from humans and rats were immediately fixed in 10% neutral buffer formaldehyde overnight, dehydrated by alcohol, and then embedded in paraffin for light microscopy. The tissues were also immediately fixed in 2.5% glutaldehyde and 1% OsO4, dehydrated in graded alcohol, and embedded in Spur Resin for electron microscopy. The glomeruli demonstrating sclerosis were counted and each scored from 0 to 4 according to the percentage of glomeruli involved: 0% = 0, 0–25% = 1, 25–50% = 2, >50% = 3, and global sclerosis = 4. We counted 25 glomeruli to get the final score, with a range from 1 to 100 for each patient. The IME was determined by a semiquantitative estimate of the width of mesangial zones in each glomerulus with some modification. Briefly, 0 was used as normal thickness, 1.0 as twice the normal thickness, 2.0 as 3 times the normal thickness, and so on. Half grades were assigned where appropriate. The total grades of 25 glomeruli in each patient were determined and represent the IME score for each patient. This light microscopic parameter of IME has been reported to correlate highly with percentage total mesangium, percentage mesangial matrix, and percentage cellular mesangium.

**Measurement of plasma and urinary MDA.** Both plasma and urinary MDA were measured with a lipid peroxidation assay kit (Calbiochem Co., Darmstadt, Germany). Briefly, plasma or urine was added to 10.3 mM N-methyl-2-phenylindole in acetonitrile, vortexed, and then incubated at 45°C for 60 minutes after adding 12-N HCl. The samples were then cooled on ice, centrifuged at 15,000 x g for 10 minutes, and measured for absorbance at 586 nm.

**Immunohistochemical examination of glomerular MDA and SOD.** The avidin-biotin-peroxidase complex staining method was employed for the immunohistochemical study. The tissue sections were first deparaffinized in xylene and then subsequently washed with 100%, 95%, and 70% ethanol, which was followed by distilled water. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol,
followed by washing with standard PBS. The tissues were then blocked with 5% NSA using the Vectastatin ABC/DAB Elite Kit (Mouse IgG type, Burlingame, CA). Excess serum was blotted. A panel of primary antibodies specific for human MDA or SOD (Calbiochem Co., Darmstadt, Germany) was employed. The tissues were washed in PBS and incubated with biotinyl-conjugated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA) in an immunostaining kit for primary rabbit/mouse antibody. Sections were then studied with an optical photomicroscope (Olympus, Japan). Glomerular staining was graded in each glomerulus semiquantitatively, and each was scored from “0 to 4” according to the staining intensity (0 = 0, “1+” = 1, “2+” = 2, “3+” = 3, and “4+” = 4). We counted 25 glomeruli to get the final score, with the final score range from 1 to 100 for each patient.

Statistics. All data below were expressed as mean ± SEM, and unpaired t-test served to compare the difference between each group. Pearson correlation measured the relationship between groups. Analysis of variance compared the difference between different groups of diabetic rats.

RESULTS

Score of glomerulosclerosis. The average score in DGS patients was 24.9 ± 5.4, which was significantly higher than that of NC (1.7 ± 0.4, P < 0.05). The average score in diabetic rats was 10.4 ± 2.3, 4.3 ± 0.5, and 1.2 ± 0.2 in DM3M, DM1M, and DM1W, respectively. The sclerosing score was significantly higher in DM3M than that of DM1W and DM1M (P < 0.05 and P < 0.01, respectively), and the scores in DM1M and DM3M were significantly higher than that of their control rats (both P < 0.01).

Plasma MDA levels in DGS patients (Figure 1). Plasma MDA level was 1.14 ± 0.21 µmol/L in DGS patients, which was significantly higher than that of DM patients (P < 0.05) and NC (P < 0.01). In DGS patients, the plasma MDA level was not significantly correlated with either the score of glomerulosclerosis or the IME and was not significantly correlated with CCr or the amount of urinary protein.

Urinary MDA levels in DGS patients (Figure 2). Urinary MDA level in DGS patients was 1.28 ± 0.32 µmol/mmol Cr, which was significantly higher than that of DM patients (P < 0.05) and NC (P < 0.01). In DGS patients, urinary MDA was significantly correlated with either the score of glomerulosclerosis (P < 0.01) or the IME (P < 0.01) and was not significantly correlated with CCr and amount of urinary protein. Urinary MDA level was significantly correlated with plasma MDA in DGS patients (P < 0.05), but not in DM patients and NC.

Immunostaining for glomerular MDA in DGS patients. The immunostaining score for glomerular MDA (Figure 3) was significantly higher in DGS patients than that of NC (P < 0.01, Table I). The score was also significantly correlated with either score for glomerular sclerosis or IME (both P < 0.01, Table II) in DGS patients. The score was not significantly correlated with either CCr or the amount of daily protein loss. The score was significantly correlated with urinary level of MDA (P < 0.01) but not with plasma levels in DGS patients.

Immunostaining for glomerular SOD in DGS patients. The immunostaining score for glomerular SOD was significantly higher in DGS patients than that of NC (P < 0.01). The score was not significantly correlated with either glomerular sclerosing score or glomerular MDA score in DGS patients. However, the score was significantly correlated with that of the glomerular MDA score (P < 0.05).

Immunostaining for glomerular MDA in diabetic rats. The immunostaining score for glomerular MDA was significantly higher in DM3M rats than that of DM1W rats (P < 0.01, Table I), and the score was significantly higher in both DM1M and DM3M rats than their control rats (P < 0.05 and P < 0.01, respectively). The score was also significantly correlated with the score for glomerular sclerosis (r = 0.672, P < 0.01) and IME (r = 0.537, P < 0.05) in DM3M rats.
Immunostaining for glomerular SOD in diabetic rats.
The immunostaining score for glomerular SOD was significantly higher in DM3M rats than that of DM1W rats ($P < 0.01$, Table I), and the score was significantly higher in both DM1M and DM3M rats than their control rats (both $P < 0.01$). The score was significantly correlated with the score for glomerular sclerosis ($r = 0.771$, $P < 0.01$), IME ($r = 0.609$, $P < 0.05$), and glomerular MDA immunostaining score ($r = 0.752$, $P < 0.01$) in DM3M rats.

**DISCUSSION**

Our study demonstrated that all plasma, urinary, and glomerular levels of MDA are increased in DGS patients, even though their renal function is still normal. MDA is a common product of lipid peroxidation that reflects the interaction between molecular oxygen and polyunsaturated fatty acids. Biologic membranes have a high polyunsaturated fatty acid content, so they are particularly susceptible to peroxidative attack by ROS. Patients with diabetic nephropathy are known to have a several-fold increase in risk of atherosclerosis over the risk for age-matched diabetics without nephropathy, and the oxidation of LDL has been proposed to explain the events initiating atherosclerosis. Patients with diabetic nephropathy usually present with increased serum LDL levels, and plasma lipid concentration may be the most important determinant of MDA level in diabetes.

Although urinary MDA has been shown to be elevated in diabetic patients and rats, and an increased MDA content had been found in kidney homogenate of STZ-induced diabetic rats, there have been only a few studies on urinary levels of MDA in patients with diabetes. Previous studies have revealed that urinary MDA level is a reliable index of renal damage. We have shown that urinary MDA is increased in DGS patients and that urinary MDA is significantly correlated with score of glomerulosclerosis and IME, which indicates that oxidative stress is closely related with the pathogenesis of DGS. Urinary MDA is not elevated in DM patients, which suggests that it is the glomerulosclerosis per se rather than the diabetes that is the main cause for the increased MDA.

Similar to a recent finding, we have also demonstrated that plasma MDA levels are elevated in DGS patients and that the plasma MDA is significantly correlated with urinary MDA. One previous study has demonstrated that plasma of type 2 diabetic patients is more prone to lipid peroxidation, and the possible source of the oxygen radicals may be the autooxidation...
of glucose and nonenzymatic glycation. Previous studies demonstrated that total radical-trapping antioxidant capacity of plasma is decreased in type 2 diabetic patients, and overexpression of SOD in diabetic rats attenuated the renal injury, which indicates a decrease in the defense against ROS injury in diabetes. Whether the increased plasma MDA come directly from the oversynthesis by kidneys or from the increased synthesis of ROS by blood cells in patients with diabetes is not known.

Although the products of lipid peroxidation in extracellular fluid may be useful markers of general oxidative stress, they are of limited use when attempting to assess the changes of oxidative stress in kidneys because they may originate from sources other than the kidney. We have demonstrated in earlier studies that the glomerular production of superoxide increased in diabetic rat glomeruli, and in this study, we have further found that glomerular MDA immunostaining was enhanced in DGS patients and that the score for glomerular staining correlated with the urinary MDA level and with the score of glomerular sclerosis and the IME. Therefore, increased glomerular MDA is responsible for the increased urinary MDA, and local lipid peroxidation may be one of the major causes for glomerulosclerosis in patients with DGS. Using antibodies to MDA-modified proteins, Haberland et al detected the MDA-protein epitopes in atherosclerotic plaque, which closely resembles glomerulosclerosis. One recent study also revealed that renal lipid peroxides were increased in lipid-induced glomerulosclerosis of rats, which further emphasizes the roles of oxidative stress in the DGS. Our results show that glomerular MDA staining was also significantly higher in STZ-induced diabetic rats and that the staining score increased with the progression of diabetes, which indicates that lipid peroxidation may be directly related to the development of DGS.

Although most studies have emphasized the enhanced generation of ROS, antioxidant defenses are likely to be equally important determinants of injury. Recent studies in diabetic kidneys revealed a decrease in the endogenous antioxidative enzymes including SOD, in contrast to our result, which was reversed by antioxidants supplementation. The reason why our diabetic rats showed an increase in SOD may because of the hyperglycemic status in our study, as the oxidative damage can be restored by insulin treatment. We have also shown that the glomerular SOD activities are increased in DGS patients and that the immunostaining score is significantly correlated with the score for MDA. Because SOD is inducible, our results indicate that SOD is induced for protecting the glomeruli against the enhanced lipid peroxidation status. It should be noted that because increased levels of SOD result in dismutation of superoxide to hydrogen peroxide, one could speculate that high glomerular SOD activity promotes oxidant stress by hydrogen peroxide.

In summary, our data demonstrate a significant elevation of plasma, urinary, and glomerular MDA levels in DGS patients, even though their renal function remains normal. The relevance of the observed results to the pathophysiological states remains to be determined, but a close relationship between the oxidative stress and DGS might be anticipated.

REFERENCES


| Table II. Correlation among plasma, urinary, and glomerular MDA and other parameters in patients with diabetic glomerulosclerosis |
|-----------------|-----------------|-----------------|
| Plasma MDA     | Urinary MDA     | Glomerular MDA  |
| Plasma MDA     | –               | –               |
| Urinary MDA    | $r = 0.604, P < 0.05$ | –               |
| Glomerular MDA | NS              | $r = 0.667, P < 0.01$ | –               |
| Glomerular SOD | NS              | NS              | $r = 0.541, P < 0.05$ |
| Glom. sclerosing score | NS | $r = 0.701, P < 0.01$ | –               |
| IME             | NS              | $r = 0.625, P < 0.01$ | $r = 0.714, P < 0.01$ |
| Serum glucose   | NS              | NS              | NS               |
| HbA1C           | NS              | NS              | NS               |
| Ccr             | NS              | NS              | NS               |
| Daily protein loss | NS          | NS              | NS               |
| Plasma cholesterol | NS          | NS              | NS               |
| Low-density lipoprotein | NS   | NS              | NS               |
| Serum triglyceride | NS           | NS              | NS               |
Inhibitory effects of lycopene on in vitro platelet activation and in vivo prevention of thrombus formation

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Lycopene is a natural carotenoid antioxidant that is present in tomatoes and tomato products. The pharmacologic function of lycopene in platelets is not yet understood. Therefore, in this study we sought to systematically examine the effects of lycopene in the prevention of platelet aggregation and thrombus formation. We found that lycopene concentration-dependently (2–12 μmol/L) inhibited platelet aggregation in human platelets stimulated by agonists. Lycopene (6 and 12 μmol/L) inhibited phosphoinositide breakdown in platelets labeled with tritiated inositol, intracellular Ca^{2+} mobilization in Fura-2 AM–loaded platelets, and thromboxane B_2 formation stimulated by collagen. In addition, lycopene (6 and 12 μmol/L) significantly increased the formations of cyclic GMP and nitrate but not cyclic AMP in human platelets. Rapid phosphorylation of a protein of 47,000 Da (P47), a marker of protein kinase C activation, was triggered by PDBu (60 nmol/L). This phosphorylation was markedly inhibited by lycopene (12 μmol/L) in phosphorus-32–labeled platelets. In an in vivo study, thrombus formation was induced by irradiation of mesenteric venules in mice pretreated with fluorescein sodium. Lycopene (5, 10, and 20 mg/kg) significantly prolonged the latency period for the induction of platelet-plug formation in mesenteric venules. These results indicate that the antiplatelet activity of lycopene may involve the following pathways: (1) Lycopene may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane B_2 formation, thereby leading to inhibition of intracellular Ca^{2+} mobilization. (2) Lycopene also activated the formations of cyclic GMP/nitrate in human platelets, resulting in the inhibition of platelet aggregation. The results may imply that tomato-based foods are especially beneficial in the prevention of platelet aggregation and thrombosis. (J Lab Clin Med 2005;146:216–226)

Abbreviations: DMSO = dimethylsulfoxide; EDTA = ethylenediaminetetraacetate; FITC = fluorescein isothiocyanate; Fura-2-AM = 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2’-amino-5’-methyl-phenoxy)ethane-N,N,N’,N’-tetraacetic acid; IP = inositol monophosphate; IP_2 = inositol-4,5-biphosphate; IP_3 = inositol-1,4,5-trisphosphate; GMP = guanosine monophosphate; NO = nitric oxide; PGE_1 = prostaglandin E_1; PRP = platelet-rich plasma; PSL = photostimulated luminescence

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Dietary intakes of tomatoes and tomato products containing lycopene have been shown to be associated in many studies with a decreased risk of illnesses such as cancer and cardiovascular disease. Lycopene is a potent antioxidant among various common carotenoids. The findings of several epidemiologic studies have suggested that fruits and vegetables are good sources of dietary carotenoids, including lycopene. Tomatoes are rich in carotenoids, especially lycopene. This product is a long-chain polyunsaturated aliphatic compound similar to carotene. The antioxidant properties of lycopene have been suggested to be responsible for the beneficial effects of these food products.

Epidemiologic evidence indicates that consumers of tomatoes have a lower risk of many types of chronic illnesses, including heart disease (eg, coronary heart disease) and key types of cancer (eg, lung, breast, ovary, intestinal tract, and prostate gland). The risks of myocardial infarction may be reduced in people with omega-3 fatty acids, phorbol-12, PDBu, apyrase, and heparin were status-matched controls.5

Furthermore, lycopene has been shown to be the only antioxidant that occurred at significantly lower levels in men in whom prostate cancer later developed than in status-matched controls.5

Intravascular thrombosis is a factor in the generation of a wide variety of cardiovascular diseases (eg, coronary heart disease). The initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Therefore, platelet aggregation may play a crucial role in atherothrombotic processes. Indeed, antiplatelet agents (eg, ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients.6

Although the epidemiologic studies conducted so far provide convincing evidence of the role of lycopene in the prevention of cardiovascular disease, this evidence is at best only suggestive and not proof of a causal relationship between lycopene intake and the risk of cardiovascular disease. We therefore systematically examined the influence of lycopene in washed human platelets and utilized the findings to characterize the mechanisms involved in this influence. We have previously reported that platelet thrombi are induced by lycopene (2–12 μmol/L) and heparin (6.4 IU/mL), then incubated for 10 minutes at 30°C and centrifuged at 500 g for another 10 minutes. The platelet pellets were suspended in 5 mL of Tyrode’s solution (pH 7.3); then apyrase (1 U/mL) and heparin (6.4 IU/mL) were added and the mixture was incubated for 10 minutes at 30°C. After centrifugation of the suspension at 500 g for 10 min, the washing procedure was repeated. The washed platelets were finally suspended in Tyrode’s solution containing bovine serum albumin (3.5 mg/mL) and adjusted to a concentration of 4.5 × 10^8 platelets/mL. The final concentration of Ca^2+ in Tyrode’s solution was 1 mmol/L.

**Platelet aggregation.** We applied the turbidimetric method to measure platelet aggregation, using a Lumi-Aggregometer (Payton, Canada). Both platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) and PRP were prewarmed to 37°C for 2 minutes (with stirring at 1200 rpm) in a silicone-treated glass cuvette. Lycopene (2–12 μmol/L) was added 3 minutes before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 minutes, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, we added 20 μL of a luciferin/luciferase mixture 1 minute before adding the agonists and compared ATP release with that of the control.

**Flow-cytometric analysis of the glycoprotein IIb/IIIa complex.** Triflavin, a specific fibrinogen receptor (glycoprotein IIb/IIIa complex) antagonist, was prepared as previously described. Fluorescence-conjugated triflavin was also prepared as previously described. The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/mL. Human-platelet suspensions were prepared as described above. Aliquots of platelet suspensions (4.5×10^8/mL) were preincubated with lycopene (6 and 12 μmol/L) for 3 minutes, followed by the addition of 2 μL of FITC-triflavin. The suspensions were then incubated for another 5 minutes, and the volume was adjusted to 1 mL/tube with Tyrode’s solution. The suspensions were then assayed for fluorescein-labeled

**METHODS**

**Materials.** Collagen (type I, bovine Achilles tendon), lycopene, sodium citrate, luciferin-luciferase, indomethacin, fluorescein sodium, Dowex-1 (100–200 μm mesh; X₈; chloride form), ADP, myo-inositol, prostaglandin E₁ (PGE₁), arachidonic acid, phorbol-12, PDBu, apyrase, and heparin were purchased from Sigma-Aldrich (St Louis, Mo). Fura 2-AM and FITC were purchased from Molecular Probes, Inc (Eugene, Ore). *Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[3H] inositol was purchased from Amersham (Buckinghamshire, UK). Thromboxane B₂, cyclic AMP, and cyclic GMP enzyme immunoassay kits were purchased from Cayman (Ann Arbor, Mich). Lycopene was dissolved in 0.5% DMSO for in vitro platelet-aggregation study and dissolved in 20% Tween-80 with normal saline solution for in vivo study. In this study, a vehicle solvent control was always included.

**Preparation of human PRP and washed platelet suspensions.** Human platelet suspensions were prepared as previously described. In this study, human volunteers gave informed consent. In brief, blood was collected from healthy volunteers, who had taken no medicine during the preceding 2 weeks, and was mixed with either 3.8% sodium citrate (9:1, vol/vol) for the preparation of PRP or acid/citrate/glucose (9:1, vol/vol) for the preparation of washed platelets. After centrifugation at 120 g for 10 minutes, the supernatant (PRP) was supplemented with PGE₁ (0.5 μmol/L) and heparin (6.4 IU/mL), then incubated for 10 minutes at 30°C and centrifuged at 500 g for another 10 minutes. The platelet pellets were suspended in 5 mL of Tyrode’s solution (pH 7.3); then apyrase (1 U/mL) and heparin (6.4 IU/mL) were added and the mixture was incubated for 10 minutes at 30°C. After centrifugation of the suspension at 500 g for 10 min, the washing procedure was repeated. The washed platelets were finally suspended in Tyrode’s solution containing bovine serum albumin (3.5 mg/mL) and adjusted to a concentration of 4.5 × 10^8 platelets/mL. The final concentration of Ca^2+ in Tyrode’s solution was 1 mmol/L.

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platelets with the use of a flow cytometer (FACScan System, Becton Dickinson, San Jose, Calif). We collected data from 50,000 platelets per experimental group. All experiments were repeated at least 5 times as a means of ensuring reproducibility.

**Measurement of the production of tritiated-inositol-labeled phosphates.** To measure the production of phosphates labeled with tritiated inositol, we used a method described previously. In brief, citrated human PRP was centrifuged and the pellets suspended in Tyrode’s solution containing tritiated inositol (75 μCi/mL). Platelets were incubated for 2 hours, then centrifuged, and finally resuspended in Ca²⁺-free Tyrode’s solution (5 × 10⁹/mL). Lycopene (6 and 12 μmol/L) was preincubated with 1 mL of loaded platelets at room temperature for 3 minutes, after which collagen (1 μg/mL) was added to trigger aggregation for 6 minutes. The reaction was stopped with the addition of 1 mL trichloroacetic acid (10% wt/vol), and samples were centrifuged at 1000g for 4 minutes. The inositol phosphates of the supernatants were separated in a Dowex-1 anion-exchange column. Only [³H]IP was measured as an index of total inositol phosphate formation.

**Measurement of platelet (Ca²⁺) mobilization.** Citrated whole blood was centrifuged at 120g for 10 minutes. The supernatant was protected from light and incubated with Fura 2-AM (5 μmol/L) at 37°C for 1 hour. Human platelets were then prepared as described above. Finally, the external Ca²⁺ concentration of the platelet suspensions was adjusted to 1 mmol/L. The increase in [Ca²⁺] was measured with the use of a fluorescence spectrophotometer (CAF 110; Jasco, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. We calculated [Ca²⁺] from the fluorescence, using 224 nmol/L as the Ca²⁺/H11001 containing tritiated inositol (75 Ci/mL). Platelets were incubated for 2 hours, then centrifuged, and finally resuspended in Ca²⁺-free Tyrode’s solution (5 × 10⁹/mL). Lycopene (6 and 12 μmol/L) was preincubated with 1 mL of loaded platelets at room temperature for 3 minutes, after which collagen (1 μg/mL) was added to trigger aggregation for 6 minutes. The reaction was stopped with the addition of 1 mL trichloroacetic acid (10% wt/vol), and samples were centrifuged at 1000g for 4 minutes. The inositol phosphates of the supernatants were separated in a Dowex-1 anion-exchange column. Only [³H]IP was measured as an index of total inositol phosphate formation.

**Measurement of protein kinase C activity.** Washed human platelets (2 × 10⁹/mL) were incubated for 60 minutes at 37°C with phosphorus-32 (0.5 mCi/mL). Platelet suspensions were next washed twice with Tris-saline buffer. The phosphorus-32–labeled platelets were preincubated with lycopene (6 and 12 μmol/L) an aggregometer at 37°C for 3 minutes, then PDBu (60 nmol/L) was added for 1 minute to trigger protein kinase C activation. Activation was terminated with the addition of Laemmli sample buffer, and the results were analyzed with the use of electrophoresis (12.5%, wt/vol) as described previously. The gels were dried, and the relative intensities of the radioactive bands were analyzed with the use of a bioimaging analyzer system (FAL2000; Fuji, Tokyo, Japan), and expressed as PSL per mm².

**Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice.** As we previously described, mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). After a tracheotomy, an external jugular vein was cannulated with polyethylene tubing (PE-10) for the administration of the dye and drug (by an intravenous bolus); additional tubing was threaded through the femoral artery as a means of monitoring blood pressure. A segment of the small intestine with its mesentery attached was loosely brought outside the body through a midline incision in the abdominal wall and placed on a transparent culture dish for microscopic observation. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules ranging in diameter from 30 to 40 μm were selected for irradiation to produce a microthrombus. In the epillumination system, light from a 100-W mercury lamp was passed through a filter (B-2A; Nikon, Tokyo, Japan) with a dichromic mirror (DM 510; Nikon). Filtering the light eliminated wavelengths below 520 nm, and this light was used to irradiate a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens (20×). The dose of fluorescein sodium used was 5 mg/kg. Various doses of lycopene (5, 10, and 20 mg/kg), solvent control (20% Tween-80 normal saline solution), or isovolumetric normal saline solution was administered 1 minute after the addition of fluorescein sodium. The injected volume of the lycopene and solvent control was less than 50 μL. We measured the time it took for a thrombus to form and cause cessation of blood flow. The elapsed time for the induction of platelet-plug formation was measured every 5 minutes during the irradiation of the venules.

**Statistical analysis.** The experimental results are expressed as the mean ± SEM and are accompanied by the number of observations. Student’s paired t test was used to determine significant differences between the solvent control and lycopene-treated groups in the study of fluorescein sodium–induced platelet thrombi in mice. We used analysis
of variance to assess the findings of the other experiments. If this analysis indicated significant differences among the group means, each group was compared using the Newman-Keuls method. *P* values of less than .05 were considered statistically significant.

**RESULTS**

**Effect of lycopene on platelet aggregation.** Lycopene (2–12 μmol/L) concentration-dependently inhibited platelet aggregation and the ATP-release reaction stimulated by collagen (1 μg/mL), ADP (20 μmol/L), and arachidonic acid (60 μmol/L) in both washed human platelets (Fig 1, A and C) and PRP (Fig 1, B and C). The inhibitory effect of lycopene in ADP (20 μmol/L)–induced platelet aggregation was present in fibrinogen (300 μg/mL) in washed-platelet suspensions. Furthermore, lycopene inhibited the ATP-release reaction when stimulated by agonists (eg, collagen; Fig 1, A). The 50% inhibitory concentration values of lycopene with regard to platelet aggregation induced by collagen, ADP, and arachidonic acid were estimated to be approximately 5.8, 6.2, and 6.8 μmol/L, respectively, in washed-platelet suspensions and approximately 6.5, 6.7, and 7.7 μmol/L, respectively, in PRP. Lycopene is approximately 170 times more potent than α-tocopherol in inhibiting platelet aggregation compared with the 50% inhibitory concentrations on a molar basis. Furthermore, when platelets were preincubated with a higher concentration of lycopene (50 μmol/L) and normal saline solution for 10 minutes, respectively, followed by 2 washes with Tyrode’s solution, we found no significant differences between the 2 aggregation curves stimulated by collagen (1 μg/mL) in both platelet suspensions, indicating that the effect of lycopene on inhibition of platelet aggregation occurs in a reversible manner (data not shown). In contrast to lycopene, solvent control (0.5% DMSO) did not significantly inhibit collagen (1 μg/mL)– or arachidonic acid (60 μmol/L)–induced platelet aggregation (data not shown). In the following experiments, we used collagen as an agonist to explore the inhibitory mechanisms of lycopene in platelet aggregation.

**Effect of lycopene on the glycoprotein IIb/IIIa complex in human platelets.** Triflavin is an arginine-glycine-Asp–containing antiplatelet peptide purified from *T flavoviridis* snake venom.9,15 Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex (αmβ3 integrin).11 A wealth of evidence suggests that the binding of fibrinogen to the glycoprotein IIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. We therefore decided to further evaluate whether lycopene binds directly to the platelet glycoprotein IIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of fluorescence of FITC-triflavin (2 μg/mL) bound directly to collagen (1 μg/mL)–activated platelets was about 998.9 ± 19.8 (Fig 2, A), and it was markedly reduced in the presence of 5 mmol/L EDTA (negative control) (Fig 2, B). Lycopene (6 and 12 μmol/L) did not significantly inhibit FITC-triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (Fig 2, C and D), indicating that the mechanism of lycopene’s inhibitory effect on platelet aggregation does not involve binding to the glycoprotein IIb/IIIa complex.

**Effect of lycopene on phosphoinositide breakdown.** Phosphoinositide breakdown occurs in platelets activated by many different agonists.16 In this study, we found that collagen (1 μg/mL) induced the rapid formation of radioactive IP, inositol-4,5-biphosphate (IP$_2$), and inositol-1,4,5-trisphosphate (IP$_3$) in human platelets loaded with $[^{3}H]$-inositol. We only measured $[^{3}H]$-IP formation as an index of total inositol phosphate formation. As shown in Fig 3, A, the addition of collagen (1 μg/mL) resulted in a marked increase in IP formation compared with that in resting platelets (9.1 ± 0.1 vs 37.5 ± 1.9)×10$^{3}$ cpm). In the presence of lycopene (6 and 12 μmol/L), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. We also measured free cytoplasmic Ca$^{2+}$ concentrations in human platelets using the Fura 2-AM–loading method. As shown in Fig 3, B, collagen (1 μg/mL) evoked an increase in [Ca$^{2+}$]$i$ from 47.9 ± 6.8 to 576.2 ± 45.9 nmol/L. This collagen-evoked increase in [Ca$^{2+}$]$i$ was markedly inhibited in the presence of lycopene (6 μmol/L, 50.3% ± 8.5%; 12 μmol/L, 82.3% ± 3.3%) (Fig 3, B). This finding suggests that lycopene exerts an inhibitory effect on phosphoinositide breakdown and [Ca$^{2+}$]$i$ mobilization in human platelets stimulated by collagen.

**Effects of lycopene on thromboxane B$_2$, cyclic AMP, cyclic GMP, and nitrate formation.** As shown in Table I, resting platelets produced less thromboxane B$_2$ than did collagen-activated platelets. PGE$_1$ (10 μmol/L) inhibited thromboxane B$_2$ formation in collagen-activated platelets by 82% (data not shown). Furthermore, results obtained with the use of various concentrations of lycopene indicated that lycopene (6 and 12 μmol/L) concentration-dependently inhibited thromboxane B$_2$ formation in PRP stimulated by collagen (1 μg/mL). In addition, the level of cyclic AMP in unstimulated platelets was about 18.6 ± 2.5 pmol/mL. The addition of PGE$_1$ (10 μmol/L) markedly increased the level of cyclic AMP (Table I). Lycopene (6 and 12 μmol/L) did not significantly increase cyclic AMP levels in human PRP (Table I). We also performed a similar study.
Fig 1. (A, B) Aggregation curves and (C) concentration-inhibition curves of lycopene on collagen (1 μg/mL, circles)—induced, arachidonic acid (60 μmol/L, triangles)—induced, and ADP (20 μmol/L, squares)—induced platelet aggregation in both washed human-platelet suspensions (black symbols) and PRP (white symbols). Platelets were preincubated with lycopene (2–12 μmol/L) for 3 minutes; agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (A). Data are presented as a percentage of the control (means ± SEM, n = 6) (C).
...phosphorylation experiments to examine the role of lycopene in the activation of protein kinase C in human platelets. When PDBu (60 nmol/L) was added to human platelets prelabeled with $^{32}$P-labeled O$_4$ for 2 minutes, a protein with an apparent molecular weight of 47,000 Da (P47) was predominately phosphorylated compared with resting platelets (Fig 4, A and B). On the other hand, lycopene (12 &mu;mol/L) significantly inhibited the phosphorylation of P47 in human platelets stimulated with PDBu. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (PSL per mm$^2$) of the radioactive bands (Fig 4, B). Moreover, lycopene (6 and 12 &mu;mol/L) also significantly inhibited collagen (1 &mu;g/mL)–induced phosphorylation of P47 in human platelets (data not shown).

**Effect of lycopene on thrombus formation in microvessels of mice.** Lycopene inhibited platelet aggregation induced by agonists in vitro; therefore, we further examined its effect on the formation of platelet-rich thrombi in this in vivo model. Mice were treated with isovolumetric normal saline solution and solvent control (20% Tween-80 normal saline solution), respectively. We found that administration of solvent control did not significantly affect occlusion time compared with that of the group treated with normal saline solution (152.6 ± 12.8 s vs 157.1 ± 11.7 s, P > .05, n = 5). When lycopene was administered at concentrations of 5, 10, and 20 mg/kg, the occlusion time was significantly prolonged: 1.4-fold (120.4 ± 16.7 s vs 169.6 ± 21.6 s, n = 7, P < .001), 1.7-fold (136.3 ± 32.1 s vs 215.4 ± 33.3 s, n = 7, P < .001), and 1.9-fold (157.8 ± 12.8 s vs 289.0 ± 19.3 s, n = 7, P < .001) compared with the individual solvent control (Fig 5). Our data reveal that platelet aggregation usually occurred first in venules rather than in arterioles. This may be explained by the higher flow velocities found in arterioles, resulting in delayed adhesion of platelets to arteriolar endothelial cells.

**DISCUSSION**

In this study, platelet aggregation induced by these agonists (eg, collagen) appeared to be affected in the presence of lycopene, implying that lycopene affects Ca$^{2+}$ release from intracellular Ca$^{2+}$-storage sites (eg, dense tubular systems or dense bodies) (Fig 6), and this is in accord with the concept that intracellular Ca$^{2+}$ release is responsible for platelet aggregation. Although the action mechanisms of various platelet aggregation agonists such as collagen, ADP, and arachidonic acid differ, lycopene significantly inhibited platelet aggregation stimulated by all of them. This implies that lycopene blocks a common step shared by these inducers. These results also indicate that the site of action of lycopene is not at the receptor level of individual agonists. Triflavin acts by binding to the glycoprotein IIB/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor.3,10,15 In this study, we found that lycopene did not significantly affect FITC-triflavin binding to the glycoprotein IIB/IIIa complex.
Fig 3. Effect of lycopene on collagen-induced (A) IP formation and (B) intracellular Ca\(^{2+}\) mobilization in human-platelet suspensions. Platelets were preincubated with tritiated inositol or Fura 2-AM (5 \(\mu\)mol/L), then exposed to collagen (1 \(\mu\)g/mL) in the absence or presence of lycopene (6 and 12 \(\mu\)mol/L), which was added 3 minutes before the addition of collagen. (A) Data are presented as the mean ± SEM (n = 5). *\(P < .001\) vs the resting group; *\(P < .05\) and **\(P < .001\) vs the collagen group. (B) The profiles are representative examples of 4 similar experiments.
Table I. Effect of lycopene on thromboxane B₂, cyclic AMP, cyclic GMP, and nitrate formation in PRP

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration</th>
<th>Thromboxane B₂ (ng/mL) (n = 4)</th>
<th>Cyclic AMP (pmol/mL) (n = 4)</th>
<th>Cyclic GMP (pmol/mL) (n = 4)</th>
<th>Nitrate (μmol/L) (n = 4)</th>
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<tbody>
<tr>
<td>Resting</td>
<td></td>
<td>25.7 ± 4.8</td>
<td>18.6 ± 2.5</td>
<td>4.7 ± 0.2</td>
<td>1.4 ± 0.2</td>
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<td>Collagen (μg/mL)</td>
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<td>278.6 ± 23.5*</td>
<td>—</td>
<td>—</td>
<td>6.3 ± 0.5*</td>
</tr>
<tr>
<td>+ lycopene (μmol/L)</td>
<td>6</td>
<td>108.2 ± 15.3†</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>68.6 ± 7.3†</td>
<td>—</td>
<td>—</td>
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<tr>
<td>PGE₁ (μmol/L)</td>
<td>10</td>
<td>—</td>
<td>156.4 ± 18.9*</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Nitroglycerin (μmol/L)</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>39.5 ± 4.1*</td>
<td>—</td>
</tr>
<tr>
<td>Lycopene (μmol/L)</td>
<td>12</td>
<td>—</td>
<td>23.7 ± 4.2</td>
<td>8.1 ± 0.6*</td>
<td>4.5 ± 0.4*</td>
</tr>
</tbody>
</table>

PRP was preincubated with lycopene (6 and 12 μmol/L) for 3 minutes at 37°C, and then collagen (1 μg/mL) was added to trigger thromboxane B₂ formation. Addition of PGE₁, nitroglycerin, and collagen to the PRP served as positive controls of cyclic AMP, cyclic GMP, and nitrate formation, respectively. Data are presented as the mean ± SEM.

*P < .001 vs resting groups.
†P < .001 vs the collagen group.

Fig 4. Effect of lycopene on phosphorylation of a protein of 47,000 Da (P47) in human platelets challenged with PDBu. Platelets were preincubated with lycopene (6 and 12 μmol/L) before challenge with PDBu (60 nmol/L). Lane 1, platelets with Tyrode’s solution only (resting group); lane 2, platelets with PDBu (60 nmol/L); lane 3, platelets with lycopene (6 μmol/L); lane 4, platelets with lycopene (12 μmol/L) for 3 minutes followed by the addition of dibutyrate (60 nmol/L). (A) The profiles are representative examples of 4 similar experiments. The arrow indicates a protein of 47,000 Da (P47). (B) The relative detection densities of the radioactive bands are expressed as PSL per mm².
indicating that the antiplatelet activity of lycopene may not be directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

Stimulation of platelets by agonists (eg, collagen) results in phospholipase C–catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of IP₃ and diacylglycerol (Fig 6).¹⁸ There is strong evidence that IP₃ induces the release of Ca²⁺ from intracellular stores (Fig 6).¹⁹ Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by lycopene, suggesting that inhibition of platelet aggregation by lycopene is related to inhibition of phospholipase C activation. Moreover, thromboxane A₂ is an important mediator of the release reaction and aggregation of platelets (Fig 6).²⁰ The collagen-induced formation of thromboxane B₂, a stable metabolite of thromboxane A₂, was markedly inhibited by lycopene (6 and 12 μmol/L) (Table I). It has been demonstrated that phosphoinositide breakdown can induce thromboxane B₂ formation by way of free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A₂ release from membrane phospholipids (Fig 6).²¹ It therefore seems likely that thromboxane B₂ formation plays a role in mediating the inhibitory effect of lycopene on human platelets.

Lycopene significantly inhibited PDBu–induced activation of protein kinase C. PDBu is known to intercalate with membrane phospholipids and form a complex with protein kinase C translocated to the membrane.²² Moreover, increased cyclic GMP formation can negatively affect agonist-induced protein kinase C activation (Fig 6).²³ Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca²⁺-mobilizing second messengers.²⁴ Lycopene increased the level of cyclic GMP in human platelets; therefore the inhibitory effect of lycopene in PDBu–induced activation of protein kinase C may be due at least in part to mediation of the increase in the formation of cyclic GMP. In this study, we found that lycopene induced NO formation in human platelets; this result is in accord with the result of a cyclic GMP study; most cellular actions of NO occur by way of stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP.²⁴ The platelet-derived NO/cyclic GMP not only modulates platelet activation but also, and more importantly, markedly inhibits atherothrombotic diseases.²⁵

Platelet aggregation plays a pathophysiologic role in a variety of thromboembolic disorders. Inhibition of platelet aggregation by drugs may represent an in-

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**Fig 5.** Effect of lycopene (5, 10 and 20 mg/kg, hatched bars) on occlusion times in the induction of thrombus formation during light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (5 mg/kg). Data are presented as the mean ± SEM of occlusion time (seconds) of platelet-plug formation (n = 7). *P < .001 vs the individual solvent control (open bars).
creased therapeutic possibility for such diseases. In this study, we evaluated the inhibition of thrombus formation by lycopene in vivo and found that lycopene significantly prevented platelet-plug formation. Electron microscopy has shown that the thrombus formed in this experiment was mainly composed of the activated platelets adhesion to the underlying damaged endothelium. It shortened the occlusion time of thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. Because the light beam covered the entire microscopic field, we were able to observe arterioles and venules simultaneously. In this system, the occlusion time was related to the blood flow rate, the diameter of the microvessel, and the dose of fluorescein dye. In this study, lycopene caused occlusion times to be significantly prolonged in mice pretreated with fluorescein sodium, mainly because of its inhibition of platelet aggregation. In the thrombotic study, the mesenteric venules were continuously irradiated with fluorescein sodium throughout the entire experimental period, leading to serious damage to endothelial cells, as described previously. The dosage of lycopene employed in this model was relatively higher than that in platelet aggregation.

In conclusion, the most important observations of this study suggest that lycopene inhibits agonist-induced human platelet aggregation. This inhibitory effect may involve the following mechanisms: (1) Lycopene may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A2 formation, thereby leading to inhibition of intracellular Ca2+ mobilization. (2) On the other hand, lycopene increases cyclic GMP/nitrate formations and subsequently inhibits phosphoinositide breakdown and protein kinase C activation, ultimately resulting in inhibition of platelet aggregation.

**Fig 6.** Signal transduction of platelet aggregation. Agonists can activate several phospholipases, including phospholipase C (PLC) and phospholipase A2 (PLA2). (1) The products of phospholipase C’s action on phosphatidylinositol 4,5-bisphosphate (PIP2) include 1,2-diacylglycerol (DAG) and IP3. DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47,000-Da protein. IP3 induces the release of Ca2+ from the dense tubular system (DTS). (2) PLA2 cleaves membrane phospholipids (eg, PC, phosphatidylcholine) and liberates arachidonic acid (AA), which is converted into a cyclic endoperoxide by platelet cyclooxygenase. Thromboxane synthase subsequently converts the cyclic endoperoxide into thromboxane A2 (TXA2). (3) NO activates guanylate cyclase to increase the production of cyclic GMP. The cyclic GMP stimulates cyclic GMP-dependent protein kinase (CGK), which then inhibits platelet aggregation. Lycopene may inhibit (1) the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A2 formation, thereby leading to inhibition of intracellular Ca2+ mobilization. Lycopene may also increase (3) cyclic GMP/nitrate formations and subsequently inhibits phosphoinositide breakdown and protein kinase C activation, ultimately resulting in inhibition of platelet aggregation.
eficial in the prevention of platelet aggregation and thrombosis.

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Protein kinase C-α regulation of gallbladder Na⁺ transport becomes progressively more dysfunctional during gallstone formation

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Gallbladder Na⁺ absorption and biliary Ca²⁺ are both increased during gallstone formation and may promote cholesterol nucleation. Na⁺/H⁺ exchange (NHE) is a major pathway for gallbladder Na⁺ transport. Ca²⁺-dependent second messengers, including protein kinase C (PKC), inhibit basal gallbladder Na⁺ transport. Multiple PKC isoforms with species- and tissue-specific expression have been reported. In this study we sought to characterize Ca²⁺-dependent PKC isoforms in gallbladder and to examine their roles in Na⁺ transport during gallstone formation. Gallbladders were harvested from prairie dogs fed either nonlithogenic chow or 1.2% cholesterol enriched diet for varying periods to induce various stages of gallstone formation. PKC was activated with the use of phorbol dibutyrate, and we assessed gallbladder NHE regulation by measuring unidirectional Na⁺ flux and dimethylamiloride inhibitable 22Na⁺ uptake. We measured gallbladder PKC activity with the use of histone III-S phosphorylation and used Gö 6976 to determine PKC-α contributions. Gallbladder PKC isoform messenger RNA and protein expression were examined with the use of Northern- and Western-blot analysis, respectively. Prairie dog and human gallbladder expresses PKC-α, βII, and δ isoforms. The PKC activation significantly decreased gallbladder JNa.ms and reduced baseline 22Na⁺ uptake by inhibiting NHE. PKC-α mediated roughly 42% of total PKC activity under basal conditions. PKC-α regulates basal gallbladder Na⁺ transport by way of stimulation of NHE isoform NHE-2 and inhibition of isoform NHE-3. PKC-α blockade reversed PKC-induced inhibition of JNa.ms and 22Na⁺ uptake by about 45% in controls but was progressively less effective during gallstone formation. PKC-α contribution to total PKC activity is progressively reduced, whereas expression of PKC-α mRNA, and protein increases significantly during gallstone formation. We conclude that PKC-α regulation of gallbladder NHE becomes progressively more dysfunctional and may in part account for the increased Na⁺ absorption observed during gallstone formation. (J Lab Clin Med 2005;146:227–237)

Abbreviations: bp = base pair; cDNA = complementary DNA; DMA = dimethylamiloride; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; GBEC = gallbladder epithelial cell; Gö 6976 = 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a) pyrrolo (3,4-c)-carbazole; HOE-694 = 3-methylsulfonyl-4-piperidinobenzoyl guanidine methanesulfonate; JNa.ms = mucosa-to-serosa Na⁺ fluxes; kb = kilobase; mRNA = messenger RNA; M₃S = molecular size; NHE = Na⁺/H⁺ exchange; PDB = 4-β-phorbol-12,13-dibutyrate; PCR = polymerase chain reaction; PKC = protein kinase C; RT = reverse transcription

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Gallbladder epithelium has both absorptive and secretory functions, and alterations in these functions are critical in gallstone formation. Gallbladder absorption of Na⁺ and H₂O is increased during the early stage of gallstone formation and may promote cholesterol nucleation by concentrating biliary composition. The secretory function of gallbladder epithelium contributes to gallstone formation by increasing the luminal levels of pronucleating factors such as mucin and calcium. Gallbladder Na⁺ absorption is mediated mostly through apically restricted Na⁺/H⁺ exchange and Cl⁻ secretion probably involves Na⁺/H⁺/Cl⁻/HCO₃⁻ exchangers and chloride channels.

Na⁺/H⁺ exchange–mediated gallbladder Na⁺ absorption is influenced by state of feeding and Cl⁻ transport. During the fasting state, when gallbladder absorption generally dominates, Na⁺ is absorbed and gallbladder bile is acidified. In contrast to the fasting state, after a meal such gastrointestinal hormones as secretin and vasoactive intestinal polypeptide can activate a cyclic AMP–dependent chloride channel, cystic fibrosis conductance regulator, resulting in Cl⁻ secretion. Increases in intracellular cyclic AMP subsequently inhibit Cl⁻/HCO₃⁻ and Na⁺/H⁺ exchangers at the apical membrane, with inhibition of net NaCl entry as a result.

NHE is a plasma-membrane protein that facilitates the electroneutral exchange of extracellular Na⁺ for intracellular H⁺ and regulates intracellular pH and cell volume. Several distinct isoforms of the NHE family, each with a tissue-specific pattern of expression, have been reported. The NHE-1 isoform is ubiquitous to all cell types and is located on the basolateral membrane in polarized cells where it maintains intracellular pH and cell volume. The NHE-2 and NHE-3 isoforms reside on the luminal or apical surfaces of polarized cells, where they may function in transcellular Na⁺ absorption. Gallbladder expression of NHE-1 and NHE-3 has been reported in rabbits, human beings, and calves. We recently characterized the gallbladder NHE isoforms NHE-1 through NHE-3 and have shown that NHE-2 and NHE-3 are involved in vectorial Na⁺ transport in the prairie dog. Our subsequent studies have shown that gallbladder NHE activity is up-regulated before crystal formation and may account for the increase in Na⁺ absorption observed during gallstone formation. The study reveals that the dramatic increases in NHE activity cannot be fully accounted for by modest increase in NHE protein expression, suggesting alterations in gallbladder NHE regulation similar to those described in other epithelial tissues.

The NHE activity in various cells is modulated by diverse stimuli, including intracellular Ca²⁺. The effects of intracellular Ca²⁺ are mediated mainly through Ca²⁺-dependent second messengers, including PKC. We have previously shown that PKC inhibits basal gallbladder Na⁺ transport in prairie dogs by way of NHE. Modulation of NHE activity by PKC appears to differ according to the specific NHE isoform. Recent studies in other tissues suggest that PKC activation stimulates NHE-1 and NHE-2 activity and inhibits NHE-3. PKC is a family of structurally related serine kinases; molecular cloning has identified 12 PKC isoforms to date, divided into 3 groups on the basis of their relative requirements for intracellular Ca²⁺, diacylglycerol, and phospholipid for activation. The classical group (PKC-α, βI, βIII, and γ) requires all 3 factors, whereas the novel group (PKC-δ, ε, η, θ) is Ca²⁺-independent and the atypical group (PKC-ξ, λ, μ) requires phospholipid only. PKC isoform expression is species- and tissue-specific. Recent studies have shown that differential regulation of NHE in other tissues appears to be mediated mainly by the classical-group isoform PKC-α. The expression of specific PKC isoforms in gallbladder epithelium and their role in the regulation of gallbladder NHE during gallstone formation is not known at this time. Alterations in gallbladder Na⁺ transport observed during gallstone formation may reflect alterations in PKC expression or activity resulting in subsequent changes in the regulation of gallbladder NHE.

In this study we sought to examine the gallbladder expression of Ca²⁺-dependent and Ca²⁺-independent groups of PKC isoforms and their roles in the regulation of gallbladder NHE isoforms under basal conditions and during various stages of gallstone formation in prairie dogs. Relative expression of PKC isoforms may play an important role in regulating basal gallbladder Na⁺ transport, and alterations in their expression or regulation may alter gallbladder Na⁺ absorption, promoting gallstone formation.

**METHODS**

Materials. We purchased Na⁺ from New England Nuclear (Boston, Mass). HOE-694 was a gift of Dr H. J. Lang (Hoechst, Frankfurt, Germany). DMA and PDB were from Sigma-Aldrich (St Louis, Mo). Gö 6976 was obtained from Calbiochem (La Jolla, Calif). Electrophoresis reagents were purchased from Bio-Rad (Richmond, Calif), and all other reagents were selected from vendors as noted in the text.

Animals and dietary regimen. Adult male prairie dogs (Cynomys ludovicianus) weighing about 1 kg were obtained from Flyers Specialty Pets (Lubbock, Texas) and fed either nonlithogenic chow (Ralston-Purina, St Louis, Mo) or a 1.2% cholesterolenriched diet (Teklad, Madison, Wis) for different periods to induce various stages of gallstone formation: cholesterol-saturated bile in 4 to 6 days, crystal in 9 to 12 days,
and gallstones in 14 to 21 days, as established previously. At the end of each feeding period, animals were anesthetized after an overnight fast and their gallbladders harvested by way of cholecystectomy, after which gallbladder bile was examined under a microscope for the presence of crystals or gallstones.

Unidirectional Na⁺ flux in native gallbladders. Gallbladders were obtained from control prairie dogs and those given a cholesterol-enriched diet at various stages of gallstone formation, after which unidirectional mucosa-to-serosa Na⁺ fluxes (JNa⁺) were measured in Ussing chambers under short-circuit current conditions in accordance with a previously published method. In brief, about 2 μCi of 22Na⁺ was added to 10 mL of modified Ringer’s solution in the mucosal reservoir and the steady-state JNa⁺ was measured from 4 10-minute flux periods either in the absence of pharmacologic intervention (baseline) or in the presence of 10 μmol/L PDB alone or with 20 nmol/L Gö 6976 for 24 hours in culture as previously described. 30

To assess the relative contributions of PKC-α to JNa⁺, we determined the baseline of differential sensitivity to 20 nmol/L of Gö 6976 in the presence of PDB. The effects of PKC activation and PKC-α blockade on gallbladder NHE activity were assessed in parallel experiments in the presence of either 100 μmol/L dimethylamiloride or 50 μmol/L HOE-694.

PKC-activity assay. We measured PKC activity in gallbladder cytosol obtained from control and cholesterol diet–fed prairie dogs within 1 week of lysate preparation, in accordance with the method of Donowitz. In brief, gallbladder lysates were prepared as previously described and total PKC activity was assayed on the basis of the measurement of transfer of phosphorus-32 from ATP to histone III-S in the presence of phosphatidylycerine, PDB, and Ca²⁺, compared with that in the presence of Ca²⁺ alone. All assays were performed over the course of 10 minutes at 30°C and terminated with the addition of 1 mL 25% trichloroacetic acid/0.2% sodium pyrophosphate to each tube. Results were expressed as picomoles of phosphate per milligram of protein.

RNA isolation and RT-PCR analysis. Total RNA was extracted from gallbladders with the use of an RNA-isolation kit (Stratagene, La Jolla, Calif) and purified as described previously. Approximately 200 ng of total RNA was used for RT, in accordance with the manufacturer’s protocol (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, Mass). The RT-PCR reaction was performed as described previously with isoform-specific primers of PKC-α, βII, and δ, designed on the basis of maximum homology among rat, rabbit, and human nucleotide sequences (Table I). The 30-cycle amplification profile involved denaturation for 1 minute at 94°C, primer annealing for 1 minute at 58°C (PKC-α and PKC-βII), 60°C (PKC-δ), and extension for 1 minute at 72°C. The elongation phase was lengthened by 1 second per cycle. PCR products were analyzed by means of electrophoresis on 1% agarose gels and visualized with the use of 0.05% ethidium bromide staining.

Cloning of PKC isoforms. PCR products corresponding to the predicted size for PKC-α, βII, and δ were ligated into the PCR 2.1 vector with the use of the deoxynucleosine and deoxyadenosine (TA) one-shot cloning kit (Invitrogen, San Diego, Calif). The transformed Escherichia coli cells were identified with the use of blue/white plaque screening and grown in Luria Broth (LB)/ampicillin medium overnight, after which the plasmids were isolated with the use of the Qiagen miniprep kit (Chatsworth, Calif). The PKC-α, βII, and δ plasmids were subsequently sequenced with the use of the Sanger dideoxynucleotide method and the deduced amino-acid sequences of the amplified regions determined.

Northern-blot analysis. Purified total RNA was fractionated on 1% agarose-formaldehyde gels and transferred to nylon Zeta-probe (Bio-Rad) membranes as described earlier. In brief, after UV cross-linking, RNA was hybridized with radiolabeled cDNA probes specific for PKC isoforms.
PKC inhibits both unidirectional Na⁺ flux and apical ²²Na⁺ uptake in the gallbladder. The effects of PKC activation and PKC-α blockade on unidirectional mucosal-to-serosa Na⁺ flux (J\textsubscript{Na\textsuperscript{ms}}) were examined in native gallbladders obtained from prairie dogs fed a control diet, and the results are summarized in Fig 1A. Native gallbladders demonstrated significant Na⁺ transport under basal conditions (17.6±1.4 μEq cm⁻² hr⁻¹). Mucosal exposure to 10 μmol/L PDB significantly decreased gallbladder J\textsubscript{Na\textsuperscript{ms}} (−18%; *P<.05) compared with the baseline value. PDB-induced reduction in J\textsubscript{Na\textsuperscript{ms}} was partially recovered by mucosal application of 20 nmol/L Gö 6976 (−45%; P<.05) compared with baseline. The effects of PKC activation and PKC-α blockade on apical ²²Na⁺ uptake were examined in primary cultures of GBECs harvested from control animals and are summarized in Fig 1B. Baseline ²²Na⁺ uptake was 72.3±4.9 nmol-mg protein⁻¹ min⁻¹ and was reduced by about 41% (42.6±7.2 nmol-mg protein⁻¹ min⁻¹, *P<.05) by treatment of cultured GBECs with 10 μmol/L PDB. PDB-induced inhibition of apical ²²Na⁺ uptake was completely reversed by 20 nmol/L Gö 6976 (98.8±15.7 nmol-mg protein⁻¹ min⁻¹).

PKC regulates gallbladder Na⁺ transport through Na⁺/H⁺ exchange. To determine whether PKC-induced inhibition of gallbladder Na⁺ transport is mediated through NHE, the effects of PKC activation and PKC-α blockade on DMA and HOE-inhibitable ²²Na⁺ uptake were measured in primary cultures of GBECs harvested from animals fed a control diet (Fig 2). DMA treatment caused significant inhibition of ²²Na⁺ uptake compared with the baseline value (−48%, *P<.05). HOE treatment also significantly inhibited ²²Na⁺ uptake in these cells compared with baseline (−43%, *P<.05).
resulted in inhibition of basal \( { }^{22}\text{Na}^+ \) uptake greater than that seen with HOE, PDB, and Gö. Data expressed as mean±SEM; \( n = 4 \). *P<.05 vs baseline, \( ^{*}P<.05 \) vs DMA, \( ^{\#}P<.05 \) vs DMA and PDB, \( ^{\#}P<.05 \) vs DMA, PDB, and Gö, \( ^{\#}P<.05 \) vs HOE, \( ^{\#}P<.05 \) vs HOE with PDB, \( ^{\#}P<.05 \) vs HOE, PDB, and Gö

Fig 2. PKC mediates gallbladder apical \( { }^{22}\text{Na}^+ \) uptake by way of \( \text{Na}^+/\text{H}^+ \) exchange in prairie dog gallbladder epithelium. PKC activation does not alter the extent of \( { }^{22}\text{Na}^+ \) uptake inhibition by DMA or HOE. PKC-α blockade results in uptake recovery over DMA with PDB and HOE with PDB. Recovery in DMA with PDB and Gö was greater than that seen with HOE, PDB, and Gö. Data expressed as mean±SEM; \( n = 4 \). *P<.05 vs baseline, \( ^{*}P<.05 \) vs DMA, \( ^{\#}P<.05 \) vs DMA and PDB, \( ^{\#}P<.05 \) vs DMA, PDB, and Gö, \( ^{\#}P<.05 \) vs HOE, \( ^{\#}P<.05 \) vs HOE with PDB, \( ^{\#}P<.05 \) vs HOE, PDB, and Gö

\( P<0.05 \)). Simultaneous treatment of primary cultures of GBECs with 100 μmol/L DMA and 10 μmol/L PDB resulted in inhibition of basal \( { }^{22}\text{Na}^+ \) uptake in GBECs by about 54% (\( P<.05 \) vs baseline) that was not significantly different from the result elicited by DMA treatment alone. Simultaneous treatment of GBECs with 50 μmol/L HOE-694 and 10 μmol/L PDB inhibited basal \( { }^{22}\text{Na}^+ \) uptake by about 51% (\( P<.05 \) vs baseline) and was not statistically different from the result elicited with HOE treatment alone. The net effect of PKC activation in GBECs trended toward increased (\( \sim 20\% \)) NHE-2–mediated \( { }^{22}\text{Na}^+ \) uptake and inhibition (\( \sim 70\% \)) of NHE-3, as shown in Fig 3, A and B, respectively. Addition of 20 nmol/L Gö 6976 resulted in significant uptake recovery over DMA with PDB (\( \sim 53\%, P<.05 \)) and HOE with PDB (\( \sim 43\%, P<.05 \)). PKC-α inhibition in GBECs trended toward decreased NHE-2 activity (\( \sim 16\%, \text{not significant} \)) and increased NHE-3 activity (\( \sim 110\%, P<.05 \)) (Fig 3, A and B).

Gallbladder epithelium expresses PKC isoform mRNAs. The expression of PDB-sensitive classical group (α, βΙΙ, βΙΙΙ, γ) and novel group (δ) PKC isoform mRNAs were examined in human and prairie dog gallbladder epithelium by means of RT-PCR with the use of PKC isoform–specific primers listed in Table I. RT-PCR demonstrated the expression of Ca\(^{2+}\)-dependent PKC-α, βΙΙΙ, and Ca\(^{2+}\)–independent PKC-δ mRNA in both human and prairie dog gallbladder epithelia (Fig 4). Expression of PKC-βΙΙ and γ mRNAs could not be demonstrated in either human or prairie dog gallbladder epithelium (data not shown). The sizes of the RT-PCR products observed matched those predicted and listed in Table I. To eliminate the possibility of amplification of contaminating genomic DNA in gallbladder samples, we ran PCR reactions with RNA that had not been subjected to RT, and no PCR product was observed (data not shown). The PKC-α, βΙΙΙ, and δ RT-PCR products were sequenced to confirm their authenticity, and the corresponding amino acid sequences were deduced. The results are summarized in Table II. Nucleotide-sequence analysis of prairie dog PKC-α, βΙΙΙ, and δ showed roughly 90% identity to human, rabbit, and rat. Deduced amino acid sequences were also highly similar to those of human, rabbit and rat (Table II).

Fig 3. Differential regulation of apically restricted gallbladder NHE isoforms by PKC in prairie dogs. (A) A nonsignificant trend toward stimulation of NHE-2 was observed with PDB-induced PKC activation. PKC-α blockade results in a nonsignificant trend toward reduced NHE2 activity. (B) A nonsignificant trend toward inhibition of NHE-3 was observed with PDB-induced PKC activation. PKC-α blockade results in significant augmentation of NHE-3 activity. Data expressed as mean±SEM; \( n = 4 \). *P<.05 vs baseline, \( ^{*}P<.05 \) vs PDB.

Fig 4. Expression of PKC isoforms in gallbladder epithelium. Total RNA isolated from human and prairie dog gallbladder tissues was analyzed with the use of RT-PCR. Results demonstrate the expression of PKC-α, βΙΙΙ, and δ in gallbladder epithelium. Nucleotide-sequence analysis of prairie dog PKC-α, βΙΙΙ, and δ showed roughly 90% identity to human, rabbit, and rat. Deduced amino acid sequences were also highly similar to those of human, rabbit and rat. (A) Human: lane 1, 100-bp ladder; lane 2, PKC-α (443 bp); lane 3, PKC-βΙΙΙ (260 bp). (B) Prairie dog: lane 1, 100-bp ladder; lane 2: PKC-α (443 bp). (C) Prairie dog: lane 1, 100-bp ladder; lane 2: PKC-βΙΙ (309 bp); lane 3: PKC-δ (260 bp).
### Table II. Comparison of cDNA nucleotide and deduced amino-acid sequences of prairie dog PKC isoforms

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<td>Homology to human (%)</td>
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PKC isoforms from prairie dog gallbladder epithelium demonstrate significant homology to human, rabbit, and rat isoforms.

**Gallbladder epithelium expresses PKC-isoform proteins.** We examined the expression of PKC-isoform protein in human and prairie dog gallbladder epithelium by means of Western-blot analysis with the use of anti-PKC-α, anti-PKC-βII or anti-PKC-δ isoform-specific antibodies. The monoclonal anti-PKC-α antibody recognized a roughly 90-kD polypeptide in both prairie dog and human gallbladder epithelium (Fig 5, lanes 2 and 3, respectively). The polyclonal anti–PKC-βII antibody recognized a roughly 85-kD polypeptide in both prairie dog and human gallbladder epithelium (Fig 5, lanes 5 and 6) and anti–PKC-δ antibody recognized a roughly 80-kD polypeptide in both prairie dog and human gallbladder epithelium (Fig 5, lanes 8 and 9). The Mr's of these polypeptides are in good agreement with the reported Mr's for PKC-α, βII, and δ.\(^{37}\)

When nonimmune antibodies were substituted for the PKC isoform–specific antibodies, none of the polypeptides was recognized (Fig 5, lanes 1, 4 and 7).

**PKC-α regulation of gallbladder unidirectional Na\(^+\) flux is lost during gallstone formation.** The effects of PKC activation and PKC-α blockade on unidirectional mucosa-to-serosa Na\(^+\) flux \(J_{Na}\) in prairie dog native gallbladders obtained during the various stages of gallstone formation are summarized in Fig 6. Under basal conditions, \(J_{Na}\) was increased by about 57% in animals with cholesterol-saturated bile compared with that in controls (27.6±3.5 vs 17.6±1.4 μEq·cm\(^{-2}\)·hr\(^{-1}\), \(P<.05\)) and appeared to be progressively decreased in animals with crystals (23.2±1.6 μEq·cm\(^{-2}\)·hr\(^{-1}\) and gallstones (19.8±1.7 μEq·cm\(^{-2}\)·hr\(^{-1}\)). These findings are consistent with observed increases in basal gallbladder apical \(^{22}\)Na\(^+\) uptake and gallbladder NHE activity during the earliest stage of gallstone formation.\(^{58}\) PKC activation resulted in similar reductions in \(J_{Na}\) (−18%, \(P<.05\) vs corresponding baseline) in both controls and in various stages of gallstone formation, suggesting that PKC is involved in gallstone formation. However, PKC-α response seemed to be different during various stages of gallstone formation. Although PDB-induced reduction in \(J_{Na}\) was almost recovered (−92%) with mucosal application of 20 nmol/L Gö 6976 in control animals, PKC-α blockade failed to recover the PKC-induced reduction in \(J_{Na}\) during the various stages of gallstone formation.

**PKC-α regulation of gallbladder apical \(^{22}\)Na\(^+\) uptake is progressively lost during gallstone formation.** We examined the effects of PKC activation and PKC-α blockade...
Fig 7. Effects of PKC activation and PKC-α blockade on 22Na⁺ uptake in cultured GBECs. (A) PKC inhibits 22Na⁺ uptake under basal conditions and during various stages of gallstone formation. PKC-α blockade results in recovery of PKC-mediated inhibition of 22Na⁺ uptake under physiologic and lithogenic conditions. (B) No significant difference in the extent of 22Na⁺ uptake inhibition in response to PKC activation during the various stages of gallstone formation was observed. (C) Recovery of 22Na⁺ uptake by PKC-α blockade was significantly decreased during the various stages of gallstone formation. Data expressed as mean±SEM; n = 4. *P<.05 vs control (CON) baseline, †P<.05 vs baseline for bile with crystals (CRYS), ‡P<.05 vs corresponding baseline, ‡P<.05 vs corresponding PDB. PreCRYS = cholesterol-saturated bile; GS = gallstones.

Fig 8. PKC activity in gallbladders of control and animals at various stages of gallstone formation. We measured PKC activity in crude gallbladder lysates obtained from control and cholesterol diet-fed prairie dogs, using histone III-S as a substrate, under conditions of maximal stimulation by Ca²⁺ (130 μmol/L), phosphatidylserine (40 μmol/mL), and PDB (10 μmol/L) (Fig 8). Total PKC activity in controls was 0.39±0.03 pmol phosphate/mg protein and did not change significantly during various stages of gallstone formation. However, contributions by PKC-α to total PKC activity varied during gallstone formation. Inhibition of PKC-α reduced total PKC activity in controls (CON) by about 32%. PKC-α blockade (Gö 6976) reduced total gallbladder PKC activity by about 25% in cholesterol-saturated bile (PreCRYS), 23% in bile with crystals (CRYS), and 23% in gallstones (GS); these values were not statistically significant.
PKC-α isoform protein expression in gallbladder epithelium increases during gallstone formation. We examined the expression of PKC-α isoform protein in prairie dog gallbladder epithelium during the various stages of gallstone formation by means of Western-blot analysis using the anti–PKC-α isoform–specific antibody. Densitometric analysis of protein blots revealed a mean ratio of PKC-α/β-actin protein expression of 0.25±0.08 (arbitrary densitometry units) in controls, which was set at 1. The mean ratio of PKC-α/β-actin protein expression increased by about 20% in animals with cholesterol-saturated bile, 48% in animals with bile with crystals (P<.05), and 64% in animals with gallstones (P<.05), respectively, compared with the findings in controls.

PKC-α isoform mRNA expression in gallbladder epithelium increases during gallstone formation. The expression of PKC-α mRNA was examined in gallbladder epithelium obtained from control and cholesterol-fed animals by means of Northern-blot analysis. The PKC-α cDNA specifically hybridized to 4.3- and 10-kb transcripts; the sizes of the PKC-α transcripts are in agreement with those previously reported for PKC-α mRNAs described in other tissues. Densitometric analysis of autoradiographs revealed a mean ratio of PKC-α/GAPDH mRNA expression of 0.78±0.02 for the 4.3-kb PKC-α mRNA transcript in controls; this value was increased by about 36% in animals with cholesterol-saturated bile, 51% in animals in bile with crystals (P<.05), and 62% in animals with gallstones (P<.05) over control values, respectively. The control value was set at 1. PKC-α mRNA transcript at 4.3 kb at various stages of gallstone formation. GAPDH mRNA transcript at 1.7 kb at various stages of gallstone formation.

Discussion

Alterations in gallbladder Na⁺ absorption have been linked to the pathogenesis of cholesterol gallstones. We have recently characterized gallbladder NHE isoforms and have shown that NHE-2 and NHE-3 are involved in vectorial Na⁺ transport in prairie dogs. Our subsequent studies have shown that gallbladder NHE activity is upregulated before crystal formation and partly accounts for the increase in Na⁺ absorption, suggesting alterations in NHE regulation during gallstone formation. Indeed, we have previously shown that Ca²⁺-dependent second messengers, including PKC, inhibit basal gallbladder Na⁺ transport. The results of the study reported here demonstrate that PKC regulation of gallbladder Na⁺ transport is mediated through apically restricted gallbladder NHE. We have also shown that PKC-α is a major contributor to PKC-mediated regulation of gallbladder NHE under basal condition and that PKC-α regulation of gallbladder NHE is altered during gallstone formation. Loss of PKC inhibition of gallbladder NHE may result in increased Na⁺ absorption observed prior to gallstone formation.

The finding that PKC-α blockade significantly reversed the effects of PKC activation on NHE-mediated Na⁺ uptake suggests that the effects of PKC-α on gallbladder Na⁺ transport are mediated through NHE.
The observed trend of PKC-α blockade towards decreased NHE2 and increased NHE3 activity shows that the functional role of PKC-α in prairie dog gallbladder epithelium is NHE isoform specific, with a stimulatory effect on NHE2 and an inhibitory effect on NHE3, consistent with the previous findings. Our study further assessed PKC phosphorylation activity in prairie dog gallbladder, demonstrating that diacylglycerol-responsive PKC isoforms are functional in gallbladder under basal conditions. Significant reductions in total PKC activity observed by PKC-α blockade with 20nM Gö 6976 confirmed that PKC-α is both functionally active and is a significant contributor to total gallbladder PKC activity under basal conditions.

Having demonstrated that PKC-α is a major contributor to PKC-mediated regulation of gallbladder Na⁺ transport under basal conditions and differentially regulates gallbladder NHE-2 and NHE-3, we examined alterations in PKC function and expression during various stages of cholesterol gallstone formation. Our previous data showing that Ca²⁺ homeostasis in GBECs is altered after exposure to lithogenic bile suggests that the activity of Ca²⁺-sensitive second-messenger systems, including the classical group of PKC isoforms, is altered during gallstone formation. The observation that PKC activation significantly inhibits gallbladder JNa⁰⁰ transport under physiologic conditions and during the various stages of gallstone formation suggests that PKC is still functionally active during gallstone formation. The failure of PKC-α blockade to result in recovery of gallbladder JNa⁰⁰ to the same extent in cholesterol diet-fed tissues, as observed in controls, suggests a dysfunction of PKC-α regulation during gallstone formation. The finding that PKC-α blockade demonstrated an increasingly reduced capacity to reverse PDB-induced inhibition of apical JNa uptake during various stages of gallstone formation further suggests that PKC-α regulation of gallbladder Na⁺ transport is progressively lost during gallstone formation. This progressive loss of regulation by PKC-α parallels an observed decline in its phosphorylation capacity.

Given our findings that the functional role of PKC-α in prairie dog gallbladder epithelium is NHE isoform-specific under basal conditions and that PKC-α becomes dysfunctional during the earliest stage of gallstone formation, progressive dysfunction of PKC-α-mediated regulation of gallbladder NHE may in part account for the alterations in gallbladder NHE activity observed during gallstone formation. Decreased PKC-α function during the earliest stage of gallstone formation may result in both decreased stimulation of NHE-2 and reduced inhibition of NHE-3, which agrees closely with our findings demonstrating alterations in the contributions of NHE-2 and NHE-3 to total NHE activity in GBECs during gallstone pathogenesis.

The findings that expression of the roughly 90-kD PKC-α polypeptide and the 4.3-kb PKC-α transcript progressively increased during the various stages of gallstone formation suggest that exposure of the gallbladder epithelium to a lithogenic stimulus results in an increase in PKC-α protein and mRNA expression that appears to be paradoxical to the observed reduction in functional activity. Such increases in PKC-α mRNA and protein expression may represent compensatory adaptations to the perceived loss of PKC-α function during gallstone formation and observed increases in NHE-3 activity.

The mechanisms by which PKC-α becomes dysfunctional during gallstone formation are not clear, but cholesterol may be a candidate, as suggested by our previous data showing that increased cholesterol content in model bile solutions stimulates Na⁺ uptake in GBECs. Increased biliary cholesterol has been shown to increase gallbladder-tissue cholesterol concentrations in prairie dogs and may involve the exchange of cholesterol between bile and the epithelial cell membrane. Increased membrane cholesterol content may alter the membrane-cytoskeleton interactions necessary for the translocation of second messengers or alter the formation of cholesterol-enriched microdomains of the plasma membrane, “lipid rafts,” that coordinate signal pathways through regulation of apical protein targeting and are recognized to have important roles in the membrane localization of NHE in ileal brush-border epithelial cells. Furthermore, increased membrane cholesterol content has been shown to increase bilayer curvature and prevent the insertion of proteins, including PKC, into the plasma membrane, an interaction that alters the protein conformation essential for their activation. Failure of PKC-α to translocate to the plasma membrane or undergo membrane-assisted conformational changes by cholesterol may contribute to the loss of PKC-α-mediated inhibition of gallbladder NHE observed during gallstone formation. Cholesterol may also affect biosynthetic pathways required for activation of PKC-α. Studies in other systems have demonstrated alterations in PKC signaling pathways mediated by membrane biosynthetic events in cells enriched with cholesterol. It is likely that increased cholesterol in bile during gallstone formation affects PKC-α activity by several mechanisms simultaneously and that cholesterol-dependent alteration of PKC-α activity contributes to the altered regulation of gallbladder NHE observed during gallstone formation. However, the roles of other components of lithogenic bile in the regulation of PKC of gallbladder transport remain unclear and will require additional investigation.
In summary, these results demonstrate that PKC activation regulates gallbladder Na\textsuperscript{+} transport by way of NHE. We have also shown that gallbladder epithelium expresses PKC-\(\alpha\), \(\beta\)II, and \(\delta\) isoforms and that PKC-\(\alpha\) plays a significant role in the regulation of gallbladder NHE. We have further demonstrated that regulation of gallbladder NHE by PKC-\(\alpha\) is progressively lost during gallstone formation. Loss of the inhibitory effect of PKC-\(\alpha\) on gallbladder NHE results in increased gallbladder Na\textsuperscript{+} absorption and may promote gallstone formation.

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Evaluation of D-dimer and factor VIII in cirrhotic patients with asymptomatic portal venous thrombosis

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ROME, ITALY

D-dimer and factor VIII levels raise in advanced cirrhosis. We investigated the behavior and the diagnostic usefulness of D-dimer and factor VIII in cirrhotic patients with asymptomatic portal venous thrombosis. Factor VIII coagulant and D-dimer values were measured in 136 consecutive outpatients with stable cirrhosis divided according to Child-Pugh (CP) classification, who underwent color/power ultrasonography to detect portal thrombosis. Portal thrombosis was found in 33 patients (24.2%). In patients without thrombosis, factor VIII was significantly higher in CP class C compared with class A and B. Conversely, class C patients with portal thrombosis had lower factor VIII levels than those without thrombosis. In both groups, D-dimer was significantly increased in class C compared with class A and B. In class C, thrombotic patients showed higher D-dimer values than did patients without thrombosis. In class C, a D-dimer value ≥ 0.55 μg/mL provided a sensitivity and a negative predictive value for portal thrombosis of 100%, and a factor VIII coagulant level ≤ 80% showed a specificity and a negative predictive value of 76% and 84%, respectively. In class B, a D-dimer value ≥ 0.225 μg/mL had a sensitivity of 89% and a negative predictive value of 82%. In conclusion, our study shows that factor VIII values increase in severe cirrhosis but significantly decrease in the presence of concomitant portal thrombosis, likely because of consumption during thrombosis; D-dimer is enhanced by both liver failure and portal thrombosis; in severe cirrhosis, normal D-dimer and factor VIII values may safely exclude the presence of asymptomatic portal thrombosis. (J Lab Clin Med 2005;146:238–243)

Abbreviations: AUC = area under the curve; CP = Child-Pugh; CPDU = color/power Doppler ultrasonography; FVIIIc = factor VIII coagulant; LC = liver cirrhosis; PVT = portal venous thrombosis; ROC = receiver operating characteristic; VTE = venous thromboembolism

The liver is involved in the synthesis and degradation of most coagulative and fibrinolytic factors and their inhibitors. Thus, patients affected by LC normally present a complex hemostatic dysfunction with impaired clotting factors synthesis, prolongation of bleeding time, chronic coagulation activation, and hyperfibrinolysis.¹⁻³

PVT may complicate the clinical course of LC⁴ and have detrimental effects including the occurrence of intestinal ischemia and bleeding.⁵⁻⁶ The prevalence of PVT in LC is unclear, as values ranging from 0.6% to

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The entire sample was included in class A (n = 68), and C (n = 29) according to the CP classification. The entire sample was then divided into 2 groups: PVT− group, including patients without PVT, and PVT+ group, composed of patients with PVT. PVT was detected by CPDU (Advanced Technology Laboratories [Bothell, Wash], Apogee 800, equipped with a sector 3.5-MHz Anular Array scanner and 5-MHz convex scanner with Pulsed, Color and Power Doppler), performed by the same expert physician. On the same day, patients underwent CPDU of the liver and blood sampling for laboratory measurements.

FVIIIc was also measured in samples taken from 38 healthy blood donors matched for age (58.7 ± 10.1 years) and sex (24 men, 63.1% of the entire group) with PVT+ and PVT− subgroups. All healthy donors had D-dimer values lower than 0.250 μg/mL.

The study protocol conformed to the guidelines by the Institutional Ethical Committee. All patients gave their informed consent to be included in the study. The research was carried out according to the principles of The Declaration of Helsinki.

Blood coagulation study. Blood sample mixed with 3.8% sodium citrate (ratio 9:1) was taken from each patient, who had fasted for at least 12 hours. Sample was centrifuged for 10 minutes at 2000 g, and the supernatant was stored at −80°C until use.

D-dimer was measured by automated latex enhanced immunoassay in citrated plasma on Instrumentation Laboratory (IL, Lexington, Mass) Coagulation Systems ACL 9000. FVIIIc was assayed by the activated partial thromboplastin time test, with use of FVIII-deficient plasma provided by IL on IL Coagulation Systems ACL 9000.

Statistical analysis. Because FVIIIc and D-dimer values were not normally distributed, Kruskall-Wallis and Mann-Whitney U test analyzed changes with the worsening of LC as assessed by CP classification. Other comparisons of FVIIIc and D-dimer values between groups were equally performed by Mann-Whitney U test. To investigate to what extent FVIIIc and D-dimer changed between PVT+ and PVT−, ROC curves were constructed. Sensitivity was plotted on the y-axis and 1-specificity on the x-axis for different cutoff values. The AUC, which provides the global assessment of the performance of the test, was calculated. An AUC < .05 indicated that the cutoff of the test significantly discriminated between PVT+ and PVT− patients. An ideal test with a sensitivity and a specificity of 100% generates an AUC of 1, whereas the null hypothesis is an AUC of .5. A P value lower than .05 was considered significant.

RESULTS

Because only 1 patient was excluded owing to difficulties in performing CPDU (.007%), our consecutive sample was fully representative of the cirrhotic population followed in our Unit. Clinical and laboratory characteristics of the 2 groups of LC patients with and without PVT are reported in Table I. Incomplete or complete thrombosis of the portal system (main portal vein, right portal vein, left portal vein, superior mesenteric vein, splenic vein) was detected in 33 patients (24.2% of the entire population). The cryptogenic origin was nearly 3 times more prevalent in the PVT+ as
compared with the PVT− group, but the difference was not significant because of the small size of this origin subgroup. There was no other statistically significant difference between the 2 groups with the exception of D-dimer, which was higher in patients with PVT (P < .05). No difference in FVIIIc values was also detected between the control group (79.9 ± 24.6) and either PVT− or PVT+ groups of LC patients (Table I).

In PVT− patients, FVIIIc increased in class C (P < .05 with Kruskall–Wallis test) with significantly higher values compared with class A (P < .05) and B (P < .02), whereas no change with the increasing severity of the liver disease was observed in PVT+ patients. However, PVT+ patients of class C had lower FVIIIc than the corresponding patients without PVT, with a statistically significant difference (P < .05) (Table II).

In both groups, D-dimer was higher in class C with significant differences compared with class A (P < .03 in PVT− and P < .01 in PVT+) and B (P < .03 in PVT− and P < .01 in PVT+); patients of class C with PVT had significantly higher values of D-dimer than the corresponding PVT− patients (P < .05) (Table III).

We also compared FVIIIc and D-dimer levels of the cryptogenic subgroup with values observed in both the alcoholic and the viral origin subgroups, but no statistically significant difference was observed (data not shown).

As patients of class C with PVT had higher D-dimer and lower FVIIIc than patients of class C without PVT, we analyzed the sensitivity, specificity, positive, and negative predictive values for PVT of D-dimer and FVIIIc in patients of this class (Table IV). Because all 7 PVT+ and 10 out of the 21 PVT− patients had D-dimer values above 0.55 μg/mL, this cutoff provided a sensitivity and a negative predictive value of 100% but low specificity (52%) and positive predictive value (63%). In patients of class C, 16 out of the 21 PVT− also had FVIIIc higher than 80%, with a specificity and a negative predictive value of 76% and 84%, respectively. With these cutoff values, both D-dimer and FVIIIc had significant AUC (P < .05), thus significantly discriminating between PVT+ and PVT− patients (Table IV).

In patients of class B, 17 out of the 19 PVT+, but also 39 out of the 48 PVT− patients, had D-dimer above 0.225 μg/mL, with a sensitivity and a negative predictive value of 89% and 82%, respectively. With this cutoff value, the AUC was statistically significant (P < .05) (Table V).

**DISCUSSION**

The prevalence of PVT in the cirrhotic population was variable in previous studies, but values ranging from 10% to 20% were usually reported.4–12 Our series of cirrhotics without overt clinical signs of PVT suggests that as many as 24% of patients may have asymptomatic PVT.

We confirm that FVIIIc levels raise in advanced LC without PVT, whereas patients with severe disease complicated by PVT show significantly lower values, likely as a result of a consumption during the process of thrombosis. In addition, we provide preliminary evidence that D-dimer testing may have clinical usefulness as a highly sensitive and nonspecific marker of underlying PVT in patients affected by moderate-to-severe LC.

FVIII is mainly produced by hepatic sinusoidal en-

| Table I. Clinical and laboratory characteristics in the whole series of 136 cirrhotic patients with and without PVT |
|---|---|---|
| Males n (%) | PVT− (n = 103) | PVT+ (n = 33) |
| Age (years) | 59.6 ± 11.7 | 59.8 ± 9.9 |
| Origin n (%) | HVB | 11 (10.6) | 4 (12.1) |
| | HCV | 56 (54.3) | 17 (51.5) |
| | Alcohol | 26 (25.2) | 7 (21.2) |
| | Cryptogenic | 6 (5.8) | 5 (15.1) |
| Child-Pugh class n (%) | 4 (3.9) |

**Table II. Factor VIIIc (% of activity) values in PVT− and PVT+ patients divided according to Child-Pugh classification**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVT−</td>
<td>n</td>
<td>29</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>85.5 ± 34.4†</td>
<td>82.2 ± 40.6§</td>
<td>128.6 ± 68.3*</td>
</tr>
<tr>
<td>PVT+</td>
<td>n</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>92.2 ± 60.4</td>
<td>89.2 ± 31.1</td>
<td>70.7 ± 39.9†</td>
</tr>
</tbody>
</table>

SD, standard deviation; n, number.
1P < .05 compared with CP class C group (Mann–Whitney U test).
2P < .05 compared with PVT− patients in CP class C (Mann–Whitney U test).
3P < .05 compared with CP class C group (Mann–Whitney U test).
4P < .02 compared with PVT− patients in CP class C group (Mann–Whitney U test).
$^*P < .05$ compared with CP class C group (Mann–Whitney U test).
$^†P < .05$ compared with CP class C group (Mann–Whitney U test).
$^§P < .05$ compared with CP class C group (Mann–Whitney U test).

HVB, hepatitis B virus; HCV, hepatitis C virus; N, number.

Continuous variables are given as mean ± standard deviation.

*Percentage referring to the total number of patients in each CP class.

$^1P < .02$ compared with PVT− group (Mann–Whitney U test).
An alternative FVIII synthesis has also been demonstrated in the lungs, spleen, lymphatic system, and endothelium. The extra-hepatic place of synthesis, the production of an abnormal protein by endothelial cells, and the impaired catabolism of a normal protein are all mechanisms potentially liable for the increased FVIII levels in advanced LC.

Accordingly, we observed a statistically significant rise in CP class C patients without PVT compared with classes A and B. On the contrary, in PVT patients of class C, FVIIIc levels dramatically dropped, with a mean reduction of more than 40% compared with PVT patients of the same class. Therefore, no difference in FVIIIc between PVT− and PVT+ patients was observed in classes A and B; conversely, PVT− cirrhotics of class C had very high FVIIIc levels, which significantly decreased in patients of the same class who had PVT.

One possible explanation for this result may consist in a relative consumption of FVIIIc during PVT. Thus, PVT is most commonly a long-lasting complication, as suggested by the occurrence of compensatory mechanisms like cavernous transformation (the development of new veins bypassing the thrombosed segment), which allows many patients to remain asymptomatic for a long time. It is therefore possible that, in advanced LC with asymptomatic PVT, low FVIIIc levels are the result of an ongoing prothrombotic state with ensuing relative consumptive coagulopathy.

D-dimer is a sensitive marker of coagulation and fibrinolysis. Hyperfibrinolysis has been described as a frequent clinical feature of LC, which probably de-

### Table III. D-dimer values (μg/mL) in PVT− and PVT+ patients divided according to Child-Pugh classification

<table>
<thead>
<tr>
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<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVT−</td>
<td>n=32</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.50 ± 0.52†</td>
<td>0.56 ± 0.58†</td>
<td>1.22 ± 1.34*</td>
</tr>
<tr>
<td>PVT+</td>
<td>n=7</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.39 ± 0.25§</td>
<td>0.92 ± 0.97§</td>
<td>2.90 ± 3.38†</td>
</tr>
</tbody>
</table>

SD, standard deviation; n, number.
*P < .05 (Kruskall-Wallis test).
†P < .005 (Kruskall-Wallis test).
§P < .03 compared with CP class C (Mann-Whitney U test).
‡P < .01 compared with CP class C (Mann-Whitney U test).
¶P < .05 compared with PVT− in CP class C (Mann-Whitney U test).

### Table IV. Sensitivity and specificity for PVT of D-dimer (cutoff value: 0.55 μg/mL) and FVIIIc (cutoff value: 80%) in Child-Pugh C cirrhotic patients

<table>
<thead>
<tr>
<th></th>
<th>SE (sensitivity)</th>
<th>SP (specificity)</th>
<th>PPV (positive predictive value)</th>
<th>NPV (negative predictive value)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer (≥0.55 μg/mL)</td>
<td>100 (87.7–100)</td>
<td>52 (33.8–72.5)</td>
<td>63 (44.1–81.4)</td>
<td>100 (87.7–100)</td>
<td>0.75 ± 0.09*</td>
</tr>
<tr>
<td>FVIIIc (≥80%)</td>
<td>57 (37.2–75.5)</td>
<td>76 (55.1–89.3)</td>
<td>44.4 (24.5–62.8)</td>
<td>84 (67.3–95.9)</td>
<td>0.76 ± 0.09*</td>
</tr>
</tbody>
</table>

SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value are presented as percentage (95% confidence limits).
AUC is area under the curve ± standard deviation.
*P < .05.

### Table V. Sensitivity and specificity for PVT of D-dimer (cutoff value: 0.225 μg/mL) in Child-Pugh B cirrhotic patients

<table>
<thead>
<tr>
<th></th>
<th>SE (sensitivity)</th>
<th>SP (specificity)</th>
<th>PPV (positive predictive value)</th>
<th>NPV (negative predictive value)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer ≥0.225 (μg/mL)</td>
<td>89 (79.6–95.8)</td>
<td>19 (10.7–30.8)</td>
<td>30 (20.5–43.8)</td>
<td>82 (70.8–90.3)</td>
<td>0.66 ± 0.076*</td>
</tr>
</tbody>
</table>

SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value are presented as percentage (95% confidence limits).
AUC is area under the curve ± standard deviation.
*P < .05.
pends on primary clotting activation,\textsuperscript{2,14} delayed hepatic clearance of tissue plasminogen activator, and impaired synthesis of inhibitors of fibrinolytic protein by the liver.\textsuperscript{31} Accordingly, D-dimer values are commonly high in LC and increase in severe disease, but previous studies did not distinguish between patients with and without PVT.\textsuperscript{2,14} We measured D-dimer in a large sample of stable LC patients after a rigorous ultrasonographic diagnosis of potential underlying PVT. In accordance with previous findings, in class C we observed a significant increase in both PVT+ and PVT− groups. In addition, patients with PVT had even higher D-dimer values compared with PVT− patients, which suggests that both liver failure and PVT contribute to increased D-dimer values.

Because of its very high sensitivity and negative predictive value, a D-dimer level below 0.2 μg/mL safely excludes VTE in patients presenting with suggestive symptoms.\textsuperscript{13} In this study we planned to evaluate the value of D-dimer measurement as an exclusionary test for asymptomatic PVT in LC. We analyzed the discriminatory performance of D-dimer in classes B and C separately, because in these 2 groups, PVT+ patients had different mean D-dimer levels. In both classes, D-dimer was increased in PVT+ patients and significantly discriminated between PVT+ and PVT− cirrhotics. In particular, all 7 class C and PVT+ patients had D-dimer levels higher than 0.55 μg/mL, a cutoff value offering a sensitivity and a negative predictive value of 100%. According to these data, in patients of class C, a D-dimer testing lower than 0.55 μg/mL may safely rule out the possibility of an underlying PVT. Furthermore, because in this class PVT+ patients had decreased FVIIIc, we calculated that a value lower than 80% provides a negative predictive value of 84%, which may add to the exclusionary capacity of D-dimer. Thus, a D-dimer lower than 0.55 μg/mL and a FVIIIc higher than 80% may identify a patient in which PVT is highly unlikely.

In class B, we obtained a cutoff value of 0.225 μg/mL, with a sensitivity and a negative predictive value of 89% and 82%, respectively. Therefore, in this class, the capacity to exclude asymptomatic PVT is still acceptable, but additional diagnostic strategies are necessary. As expected, because D-dimer tends to increase in patients with moderate-to-severe LC even without PVT,\textsuperscript{2,14} the specificity for PVT was rather low (52% in class C and 19% in class B).

To sum up, our study suggests that, in patients with severe liver failure, normal values of D-dimer and FVIIIc should exclude the presence of asymptomatic PVT. Conversely, PVT cannot be suspected in case of elevated D-dimer or low FVIIIc values, because of the unsatisfactory specificity of these variables. This finding is in accordance with previous data indicating that D-dimer is a sensitive, but non-specific, marker of VTE and is essentially used by physicians for its exclusionary power.\textsuperscript{13}

CPDU is much more sensitive than contrast computed tomography in detecting PVT.\textsuperscript{5,6} However, this technique is operator-dependent, and some institutions may lack physicians with sufficient experience in diagnosing PVT in cirrhotic patients. Thus, if confirmed in a validation sample, our preliminary results may be useful to decide in which patient a well-performed CPDU can be safely postponed.

Although the role of PVT in the natural history of LC remains to be clearly understood, it indicates a poor prognosis and an increased risk of complications because of portal hypertension, in particular hemorrhage from esophageal varices.\textsuperscript{5,6} Management of PVT in LC is still unclear, and no trial has been planned to assess whether an antithrombotic therapy may arrest its progression without increasing bleeding risk. At this regard, recent reports suggest the potential usefulness of low-molecular-weight heparin, even though further studies are necessary to explore the cost-benefit ratio of this treatment.\textsuperscript{5,6}

In conclusion, our study shows that asymptomatic PVT may occur in 24% of cirrhotic patients; FVIIIc increases in advanced LC, but it tends to be consumed when PVT complicates the disease; conversely, D-dimer raises in cirrhotic patients with PVT; measuring D-dimer and FVIIIc may be a simple and helpful tool to rule out the presence of an underlying PVT in severe LC.

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Influence of tissue fixation on the microextraction and identification of amyloid proteins

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In surgical pathology, correct immunohistochemical identification of AL amyloidosis poses a particular problem. Immunostaining for λ- or κ-light chains is commonly encountered even in non-immunoglobulin-derived amyloidoses, which leads to a false-positive classification as AL amyloidosis. In this respect, microextraction of amyloid proteins from surgical pathology specimens and their subsequent biochemical characterization may prove useful in reaching the correct diagnosis. In this study, we investigated systematically the influence of fixation on the extraction of amyloid proteins from amyloid-containing tissue samples. Tissue samples were obtained from a patient with generalized AA amyloidosis and from a second patient with generalized AL amyloidosis. The samples were stored either unfixed or fixed in phosphate buffered 4% p-formaldehyde, methacarn, or Bouin for 3 days, 1 week, or 1 month. Thereafter, proteins were extracted according to the procedure of Layfield et al., separated by SDS-PAGE and subjected to Western blotting, using antibodies directed against AA amyloid and immunoglobulin-derived λ-light chain. Following this procedure, a variety of differently sized AA amyloid or λ-light chain immunoreactive protein bands were found in both patients, which is typical for amyloid proteins. Fixation time did not per se prohibit the extraction of these amyloid proteins from tissue samples, which remained detectable irrespective of fixation time. Although all three fixatives impaired the resolution of some, but not all, individual amyloid proteins, this procedure may help to confirm or reject a diagnosis of AL amyloidosis, because detection of several λ- or κ-light chain immunoreactive protein bands in the low-molecular-weight range (<20 kDa) is a common characteristic of their amyloid nature. (J Lab Clin Med 2005;146:244–250)

Abbreviations: AA amyloid = amyloid A protein-derived amyloid; AL amyloid = immunoglobulin light-chain-derived amyloid; anti-AL1 antibody = antibody directed against λ-light-chain-derived amyloid proteins; anti-κ-light chain(DAKO) antibody = commercially available antibody directed against immunoglobulin-derived κ-light chain; ApoAI = apolipoprotein AI; B = Bouin; M = methacarn; MIDD = monoclonal Immunoglobulin deposition diseases; pFA = phosphate-buffered 4% p-formaldehyde; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

A amyloid defines a heterogeneous group of diseases of diverse origin characterized by proteinaceous tissue deposits that show green birefringence in polarized light after Congo red staining.1,2 After the histological diagnosis of amyloid, it is mandatory to classify the amyloid protein(s) to assess patient prognosis and treatment, which may include chemotherapy or organ transplantation.2,3 A variety of different procedures are used by physicians for the classification of amyloid. Tissue specimens submitted to histology are usually fixed in alcoholic or aldehyde-
based fixatives and embedded in paraffin. Immunohistochemistry is applied to classify amyloid in these specimens.\(^2\,^4\) Despite significant technical and methodological improvements and its broad application in surgical pathology, immunohistochemical classification of amyloid proteins still poses several problems and requires experience.\(^2\,^4\) It can produce ambiguous or misleading results and correct classification of AL amyloid and hereditary amyloidoses is a serious obstacle and sometimes even impossible.\(^5\) Thus, additional biochemical analyses may be required for the identification of amyloid proteins. Several reports have shown that biochemical analyses can be applied to formalin-fixed tissue samples. It is achieved by isolating amyloid fibrils with denaturing solvents\(^6\,^9\) and requires relatively small amounts of amyloidotic tissue,\(^6\) such as those provided by biopsies. However, different fixation protocols are used in surgical pathology and no systematic study has yet been undertaken to investigate the influence of the fixative used by the physicians, as well as the duration of fixation, on the extraction and biochemical classification of amyloid proteins. In this study, we investigated the influence of fixation on the extraction of amyloid proteins from amyloid-containing tissue samples and can finally show that amyloid proteins can be extracted from tissue samples, which have been fixed with fixatives common in surgical pathology for various periods of time.

**MATERIALS AND METHODS**

**Materials.** Unfixed spleenic and renal tissue containing amyloid was obtained from 2 patients. The first patient had suffered from generalized AA amyloidosis\(^10\) and the second patient from primary systemic AL amyloidosis.\(^11\) The primary structure of the amyloid proteins of both patients had been characterized previously by amino acid sequencing or mass spectrometry.\(^10\,^11\) Tissue specimens had been stored unfixed at −80 °C until required.

**Histology.** Tissue specimens were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin. The presence of amyloid was demonstrated by the appearance of green birefringence from Congo red staining under polarized light.\(^1\)

**Immunohistochemistry.** Immunostaining was performed with monoclonal antibodies directed against AA amyloid (1:400; DAKO, Hamburg, Germany), and with polyclonal antibodies directed against amyloid P-component (1:1600), fibrinogen (1:1000), lactoferrin (1:800), lysozyme (1:3000), transthyretin (1:600), \(\alpha\)-light chain (anti-\(\alpha\)-light chain, DAKO); (1:10,000; all DAKO), anti-AL1 antibody (1:10,000\(^{11}\)), \(\kappa\)-light chain (1:10,000; DAKO), and ApoAI (1:1500). Before immunostaining for amyloid P component and transthyretin, the specimens were pretreated with 10-mM ethylenediaminetetraacetic acid (2 × 10 min, 450-W microwave oven). Immunoreactions were visualized with the avidin–biotin complex method, which applies a Vectastain ABC alkaline phosphatase kit (Biogene-Alexis GmbH, Grünberg, Germany) or iVIEW DAB Detection kit (Ventana, Illkirch, France). Neufuchsin and 3,3-diaminobenzidine-tetrahydrochloride, respectively, served as chromogens. The specimens were counterstained with hematoxylin. The specificity of immunostaining was verified with specimens containing known classes of amyloid (AA amyloid, ApoAI, transthyretin, \(\alpha\)-light chain), with positive controls recommended by the manufacturers (remaining antibodies) and by omitting the primary antibodies.

**Fixation protocols.** Small tissue specimens (approximately 5 × 5 × 5 mm) were cut from frozen tissue blocks, quickly thawed, and placed in the following fixatives, respectively: (1) pFA, pH 7.4; (2) B (150-mL picric acid, 50-mL 37% formalin, 10-mL acetic acid), or (3) methanol (60-mL M, 30-mL chloroform, 10-mL acetic acid). The tissue samples were fixed for 3 days, 1 week, or 1 month at 4°C. Unfixed tissue samples served as a control.

**Specimen processing.** The preparation of amyloid fibril proteins from fixed specimens was performed as described by Layfield et al.\(^7\) Briefly, the fixed tissue was incubated overnight in deionized water and subsequently homogenized in 5 volumes (w/v) of 50-mM Tris-HCl, 1-mM dithiothreitol, pH 7.5, for 2 min with a rotor-starter homogenizer. The homogenate was centrifuged at 11,000 rpm for 15 min. The pellet was resuspended in 2.5 volumes of electrophoresis sample buffer [150-mM Tris-HCl, 8-M urea, 2.5% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 10% (v/v) 2-\(\beta\)-mercaptoethanol and 3% (w/v) dithiothreitol, pH 6.8], heated to 90°C for 20 min and again centrifuged at 11,000 rpm for 15 min. The supernatant containing the extracted protein was resolved by SDS-PAGE. Gel loading was assessed empirically, as the Layfield et al technique does not allow quantification of the protein concentration. Two to 3 μL were loaded per lane.

**SDS-PAGE and Western blotting.** Proteins were resolved in polyacrylamide gels, according to Schägger and von Jagow\(^12\) (4% stacking gel, 10% and 16.5% resolving gels) and visualized by Coomassie blue staining. For Western blotting, proteins on unstained polyacrylamide gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P\(^{\text{TM}}\); pore size 0.1 μm; Millipore, Bedford, Mass), with the tank-blotting system from Bio-Rad Laboratories (Munich, Germany) according to the manufacturer’s instructions. Immunoblotting of the proteins was performed with anti-AA amyloid antibodies (monoclonal; clone mc1; dilution 1:100; DAKO, Glostrup, Denmark), anti-AL1 antibody (polyclonal; dilution 1:10,000\(^{11}\)) or anti-\(\alpha\)-light chain\(_{\text{DAKO}}\), antibody (polyclonal; 1:10,000; DAKO). Free binding sites of the membrane were blocked overnight at room temperature with Tris-buffered saline supplemented with 5% milk powder (Roth, Karlsruhe, Germany) in 0.05% Tween-20 (TBST). Incubation with the primary antibody was performed at room temperature for 60 min. After extensive washing in TBST, the membrane was incubated with the secondary biotinylated rabbit anti-mouse or goat-anti-rabbit antibody for 60 min at room temperature (dilution 1:1000; both DAKO). Immunoblotting was visualized with BCIP/NBT (Pierce, Rockford, Ill).
Assurances. This study was carried out in accordance to the guidelines of the Ethics Committee of the University of Magdeburg and the Declaration of Helsinki. Data were encoded to ensure patient protection. Informed consent was obtained.

RESULTS

AA amyloid (patient 1). Histology of formalin-fixed and paraffin-embedded tissue samples obtained from patient 1 shows extended amyloid deposits in the kidney, which strongly immunoreacted with antibodies directed against the AA amyloid and amyloid P component (Fig 1). No other antibody reacted with the amyloid deposits, including in particular the polyclonal antibody directed against AA amyloid (f, insert). Hematoxylin and eosin-stain (a, d); Congo red staining in polarized light (b, e); anti-AA amyloid antibody (e), (f, insert); anti-AL1 antibody (f), (c, insert); original magnifications 200×.

We next extracted amyloid proteins from the corresponding unfixed amyloid-laden tissue, with the extraction procedure described by Layfield et al.7 After gel electrophoresis and Western blotting with a monoclonal antibody directed against AA amyloid (anti-AA amyloid antibody, clone mc1), at least 4 bands were detectable at around 6.5 kDa, which represents AA amyloid proteins (Fig 3, upper panel). Three bands were found just below 14.2 kDa probably representing intact SAA and a few amyloid proteins larger than 6.5 kDa. Multiple bands were finally detectable between 14.2 kDa and 45 kDa, and they may represent dimers and oligomers of amyloid proteins, which were not denatured by the extraction procedure (Fig 3).

After fixation of amyloid-laden tissue samples with pFA, M, or B, protein smearing already occurred after 3 days of fixation and did not significantly worsen when the fixation time was extended to 1 week or 1 month. Smearing was most prominent in the upper half of the immunoblot, starting at the upper margin of the running gel. Interestingly, smearing was most prominent after fixation with pFA and B. Several bands seemed not to be resolvable anymore after fixation in pFA and B: 2 of the 3 bands between 6.5 and 14.2 kDa were still resolved after 1 month of fixation with M. Fixation also leads to the occurrence of additional, strongly immunoreactive bands between 14.2 kDa and 66 kDa.

No immunostaining of any AA amyloid protein was found after probing the blotted membranes with an anti-AL1 antibody (Fig 3). Only a single band occurred between 20 kDa and 29 kDa, which probably represents an intact immunoglobulin light chain, which was coextracted with AA amyloid proteins.

AL amyloid (patient 2). Spleenic tissue samples from patient 2 showed a complete obliteration of the red and

Fig 1. Specimens obtained from the kidney of patient 1 (a)–(c) and from the spleen of patient 2 (d)–(f) showed extended amyloid deposits. In patient 1, the amyloid deposits stained strongly with a monoclonal antibody directed against AA amyloid and failed to stain with an anti-AL1 antibody (c, insert). In patient 2, the amyloid deposits showed a strong immunoreaction with an anti-AL1 antibody and did not immunoreact with an antibody directed against AA amyloid (f, insert). Hematoxylin and eosin-stain (a, d); Congo red staining in polarized light (b, e); anti-AA amyloid antibody (e), (f, insert); anti-AL1 antibody (f), (c, insert); original magnifications 200×.

Fig 2. Specimens obtained from the kidney of patient 1 enclosed λ-light chains in the lumina of renal tubuli (asterisks). Note lack of immunostaining of the glomerular amyloid deposits. Anti-AL1 antibody; original magnification 200×.
white pulp by amyloid deposits and almost complete atrophy of the lymphatic tissue (Fig 1). The amyloid deposits strongly immunoreacted with the anti-AL1 antibody and the anti-λ-light chain (DAKO) antibody. The anti-AL1 antibody had been obtained by immunizing rabbits with amyloid proteins obtained from this patient. The amyloid deposits were also stained with an anti-amyloid P component antibody, but not with any other antibody.

Amyloid proteins were extracted from unfixed amyloid-laden spleenic tissue. After gel electrophoresis and Western blotting with the anti-AL1-antibody, 3 faint bands were detectable at around 14.2 kDa (Fig 3, bottom panel). Four bands were found between 14.2 kDa and 20 kDa, which shows a stronger immunoreaction. These bands below 20 kDa were interpreted as differently sized AL amyloid proteins. One single strongly immunoreactive band was found above 20 kDa, which probably represented the intact immunoglobulin light chain, and at least 5 distinct bands were detectable between 29 kDa and 66 kDa. Minor smearing was observed in the molecular weight range above 45 kDa.

When amyloid-laden tissue samples were fixed in pFA, M, or B and then subjected to the extraction procedure, protein smearing worsened and already occurred after 3 days of fixation, starting at the upper margin of the resolving gel. Fixation time had little influence on protein smearing. Several bands did not seem to be resolvable after fixation; eg, 1 band at around 14.2 kDa and between 2 and 5 bands in the molecular weight range of 29 kDa to 66 kDa were no longer apparent. The resolution of the 4 bands between 14.2 kDa and 20 kDa varied considerably. Only the single, strongly immunoreactive band just above 20 kDa was detectable in all 3 fixatives for all fixation times.

To further test specificity, additional probing was carried out with the commercially available anti-λ-light chain (DAKO)-antibody, with tissue samples that had been fixed for 1 month. Although the immunoreactivity of individual protein bands varied between the 2 antibodies, ie, anti-AL1- and anti-λ-light chain (DAKO)-antibody, the anti-λ-light chain (DAKO)-antibody also showed a strongly immunoreactive protein band just above 20 kDa and multiple bands below and above 20 kDa. Again, strong smearing occurred after fixation with pFA, M, or B and was not significantly different when the protein loading was changed from 2 to 3 μL per lane (Fig 4).

No immunostaining of any AL amyloid protein was

Fig 3. Amyloid proteins were extracted from unfixed (U) tissue samples, or tissue samples that had been fixed for 3 days, 1 week, or 1 month in pFA, M, or B, separated by SDS-PAGE and analyzed by Western blotting. Panel I shows proteins extracted from the amyloid-laden kidney of patient 1, who had suffered from generalized AA amyloidosis. Panel II shows proteins extracted from the amyloid-laden spleen of patient 2, who had suffered from generalized AL amyloidosis. Western blotting was performed with a monoclonal antibody directed against AA amyloid (anti-AA amyloid antibody) and with a polyclonal anti-AL1 antibody. The anti-AL1 antibody detected a single band in the proteins extracted from patient 1 (panel I, Control 1 month), whereas the anti-AA amyloid antibody did not detect any protein in the protein extracts obtained from the amyloid-laden spleen of patient 2 (panel II, Control 1 month).
DISCUSSION

Immunohistochemical classification of amyloid is feasible and can be reliable, when applied cautiously. However, the diagnosis and classification of AL amyloid, which belongs to the family of MIDD, poses a particular problem in surgical pathology. MIDD are a group of disorders, which have in common the deposition of monoclonal proteins (M-protein) in organs and tissue. The proteins deposited are intact immunoglobulins or fragments thereof. Because of the deposition of the variable regions of light chains, almost every patient with AL amyloidosis has a unique amyloid protein and, hence, AL amyloid stains approximately only in 40% of the cases with commercially available antibodies. Negative immunostaining does not exclude AL amyloid. Another problem is that serum proteins, immunoglobulins being among them, commonly contaminate amyloid deposits and positive staining for \( \lambda \)- or \( \kappa \)-light chain does not necessarily indicate AL amyloidosis. Lachmann et al. particularly highlighted the issue of hereditary systemic amyloidoses, which had been misinterpreted as AL amyloid. Among 350 patients initially found to have AL amyloid, 18 (5.2%) were subsequently identified as hereditary AFib amyloidosis, 13 (3.7%) as ATTR\( ^{-} \), 2 (0.6%) as AApOAl\( ^{-} \), and 1 (0.3%) as ALys amyloidosis. The problem is further complicated by the fact that many hereditary systemic amyloidoses may mimick clinically AL amyloidosis by presenting with nephropathy, which makes clinicopathological correlations more difficult. Thus, when amyloid stains immunohistochemically with antibodies directed against immunoglobulins or their respective light chains, one may wish to confirm this by further biochemical analyses, to prevent false positive diagnosis of AL amyloidosis.

In this respect, the development of microextraction procedures from fixed tissue specimens is particularly interesting. We and others have successfully applied this technique to identify amyloid proteins in biopsy specimens. However, until now, no systematic study has been undertaken to investigate the influence of fixative and fixation time on the extraction of amyloid proteins.

Here, we show for the first time that amyloid proteins can be extracted not only from pFA fixed tissue, but also from tissue samples that had been fixed in B and M. These fixatives are common in surgical pathology. Aldehyde fixatives, such as pFA and B, are “cross-linkers,” which are preferred for tissue fixation as they are superior for the preservation of tissue structure for both light and electron microscopy. They are among the most common fixatives worldwide and guarantee the standardization and comparability of morphology, which is particularly important in surgical pathology. Cross-linking joins 2 molecules by covalent bonds. As cross-linking can occur with any protein of any molecular size, this may help to explain the “smearing” across the entire lane in the Western blots. It was interesting to note that smearing was most prominent in the high-molecular-weight range. As amyloid proteins form oligomers, supposedly of many different molecular weight sizes, smearing might represent all different numbers of cross-linked amyloid protein oligomers present in a tissue sample. However, smearing may also result from cross-linking of amyloid proteins with other nonamyloidotic tissue components. It has been hypothesized that amyloid deposits are to some extent resistant to formalin fixation and might explain why monomeric amyloid proteins remained extractable even after 1 month of fixation.

Interestingly, unfixed tissue samples of both patients showed multiple immunoreactive protein bands that were larger than the precursor proteins of AA amyloid.
ie, serum amyloid A (12 kDa), or AL amyloid, ie, immunoglobulin light chain (26 kDa). These high-molecular-weight protein bands may represent dimers and oligomers of amyloid proteins, which were not denatured by the extraction procedure. Differences in the immunoreactivity between the anti-AL1 antibody and the anti-λ-light chain (DAKO) antibody is because the anti-AL1 antibody was obtained by immunizing rabbits with the amyloid proteins extracted from amyloid tissue of patient 2.

The overall yield of amyloid proteins extracted from fixed tissue seemed to be reasonably good. Nevertheless, the influence of amyloid load on the extraction procedure was not investigated in this study and should be the focus of future investigations. Interestingly, fixation time did not prohibit the extraction of amyloid proteins, and tissue samples can be stored even for a prolonged period of time.

Obviously, the organic solvents enclosed in the extraction buffer extract proteins other than amyloid proteins. A single, λ-light chain immunoreactive protein band was present in the extracts obtained from AA amyloid-laden tissue. However, this band seemingly represents the intact immunoglobulin light chain. Histologically, we observed λ-light chain immunoreactivity in the renal interstitium, tubuluses epithelia, and within the tubuli (Fig 2), which further illustrates that immunoglobulins are commonly present in amyloid-laden tissue without representing amyloid proteins. Similarly, Sakata et al recently reported that intact ApoAI commonly colocalizes with amyloid deposits in various forms of non-ApoAI-derived systemic amyloidoses. However, in AL amyloidosis, the amyloid proteins are commonly truncated, and indeed, our experience has shown that immunodetection of an amyloid protein is to be considered when multiple protein bands are specifically immunoblotted in the molecular weight range below 20 kDa. It is 1 significant advantage of Western blotting over immunohistochemistry. Although immunohistochemistry cannot provide any distinct information about the molecular weight(s) of the antigens detected in a tissue section, extraction and separation by SDS-PAGE can provide valuable information about the size of the respective antigens: Detection of several light chain fragments most likely represents amyloid protein(s), and the presence of only 1 intact immunoglobulin light chain probably represents a “contaminating” immunoglobulin. Immunohistochemical detection of immunoglobulin light chains in the renal interstitium and tubuli of our patient with generalized AA amyloidosis further supports this notion.

**SPECULATIONS**

In summary, we show here that fixation times commonly encountered in surgical pathology do not significantly hamper the extraction of amyloid proteins from fixed tissue samples. Extraction of amyloid proteins from amyloid-laden tissue biopsies may prove useful to either confirm or reject a diagnosis of AL amyloidosis, because detection of several λ- or κ-light chain immunoreactive protein bands in the low-molecular-weight (<20 kDa) range is a common characteristic of their amyloid nature.

The authors thank Antje Ziems for her excellent and skilful assistance.

**REFERENCES**

THE EDIBLE WOLF PEACH

Tomatoes belong to the genus *Lycopersicon*, which means “wolf peach,” from the Greek λυκός (wolf) and περσικός (Persian, which alludes to Persian apple, the appellation Europeans first bestowed on the peach when it arrived from China via Persia). This is a direct translation from another term for the fruit, the German *Wolffpfrisch*. However did such an agreeable fruit acquire such a disagreeable name? It originates in the werewolf myth. Early Renaissance Europeans were suspicious of the tomato upon its arrival from South America because of its striking likeness to deadly nightshade (*Atropus belladonna*—the source of atropine*). German legend claims that witches used nightshade to summon werewolves, and what could be more attractive to a werewolf than nightshade with a berry the size of a peach? Although modern German speakers know tomatoes as *Tomaten* or *Paradiesäpfel*, the earliest German name for tomatoes was *Wolffpfrisch* — “wolf peach.” Sadly, it took years before the tomato was widely recognized as harmless and incorporated into European cuisines. In the 18th century, Carl Linnaeus, noting the werewolf legend, applied his new binomial nomenclature system to name the tomato *Lycopersicon esculentum*, the “edible wolf peach.” Despite the charm of the history behind it, the Linnaean classification may be supplanted by a phylogenetic classification that would group the tomato plant based on its evolutionary relationship to other organisms; the name, *Solanum lycopersicum*, has been suggested.

In contrast to the doubts about the safety of the tomato in centuries past, recent research suggests...
that tomatoes might reduce the risk of heart disease and some cancers. One of the constituents thought responsible for these beneficial effects is the antioxidant red pigment in tomatoes called lycopene, a name that also derives from the werewolf legend. In this issue, researchers from Taipei Medical University in Taiwan asked whether an effect on platelet function might explain an epidemiologic observation — that diets high in lycopene are associated with a low incidence of coronary artery disease. As presented in a paper beginning on page 216, they found that lycopene inhibits platelet responsiveness to a variety of agonists, apparently by preventing activation of Protein Kinase C.

This month’s cover illustration shows two varieties of tomato.** The tomato on the left is a crimson hybrid with double the amount of lycopene compared to the normal variety of tomato on the right.

_Dale E. Hammerschmidt, MD_
_Editor-in-Chief_

_Michael J. Franklin_
_Managing Editor_

**We thank John W. (Jay) Scott, a University of Florida Horticultural Sciences professor, for providing us with the image, and his colleague, John Petti, for photographing the tomatoes.
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