Clinical Chemistry 42:9
1537-1541 (1996)

Oak Ridge Conference

Thrombus precursor protein (TpP™): marker of thrombosis early in the pathogenesis of myocardial infarction

David G.M. Carville,[1]* Nikola Dimitrijevic,[1] Mark Walsh,[2] Tamara Digirolamo,[2] Elaine M. Brill,[1] Natasha Drew,[1] and Paul E. Gargan[1]

We have developed an enzyme-linked immunosorbent immunoassay for quantifying the immediate precursor proteins to intravascular thrombi. This thrombus precursor protein (TpP™) assay identifies active thrombosis in several clinical conditions, including early myocardial infarction (MI). In a study of patients recruited for the GUSTO intervention study, MI patients had concentrations of TpP 4–20-fold that of controls; patients diagnosed without MI had concentrations similar to the control subjects. In a separate study of subjects presenting at the emergency room with chest pain, MI patients who presented early after the onset of chest pain had TpP concentrations significantly (P <0.01) higher than controls. Patients presenting late or diagnosed with other chest pain had concentrations within the reference range. The potential utility of the TpP assay as an aid for the diagnosis of thrombotic MI and other thrombotic conditions is described.

INDEXING TERMS: coronary thrombosis • fibrin polymers • enzyme immunoassay • reference values

Thrombosis plays a key role in the pathogenesis of many disease states, including myocardial infarction (MI), stroke, unstable angina (UA), deep vein thrombosis (DVT), and pulmonary embolism *[1, 2]*.[3] Thrombosis occurs in both the venous and arterial systems and can be precipitated by various predisposing clinical conditions, including vascular disease (atherosclerosis), surgery, trauma, malignancy, prosthetic vascular devices, anesthesia, pregnancy, oral contraceptives, systemic lupus erythematosus, and bacterial infection *[2]*. Congenital deficiency in certain anticoagulant proteins (e.g., protein C, protein S, and antithrombin III) can also predispose individuals to thrombosis *[3-5]*. Coronary artery thrombosis is the leading cause of mortality in the US, with 1.5 million cases of MI each year resulting in >700 000 deaths *[6]*. In addition, >1 million cases of UA present to the emergency room (ER) each year in the US, of which 10–15% progress to MI *[7]*.

A fundamental biochemical process in thrombosis is the conversion of circulating soluble fibrinogen to the insoluble fibrin matrix that serves as a structural "glue" that consolidates and gives mechanical rigidity to the cellular components of the blood clot *[8]*. The principal cellular components of blood clots are activated platelets and erythrocytes. Venous clots traditionally form in areas of stasis and are composed mainly of erythrocytes and fibrin. In the arterial circulation, clots form under conditions of high flow and shear and are composed mainly of platelets held together by fibrin strands *[8]*.

In almost all cases, acute MI occurs by occlusive thrombosis after rupture of atherosclerotic lesions in the coronary arteries *[9, 10]*. This coronary thrombosis in stenosed vessels leads to total or partial occlusion of the vessel, resulting in ischemia, injury, and eventually necrosis of the myocardial tissue *[10]*. The duration of the ischemia and the size of the area involved determine the extent of necrosis and consequently the extent of loss in ventricular function *[11]*. The early dissolution of the thrombotic occlusion by thrombolytic therapy can dramatically limit necrosis, thereby preserving ventricular function and substantially reducing morbidity and mortality from acute MI *[12, 13]*.

Irreversible necrosis of myocardial tissue generally occurs between 4 and 6 h after the onset of chest pain and is associated with the release of cardiac muscle cell proteins, including creatine kinase (CK) and its MB isoenzyme (CKMB), troponin,

[1] American Biogenetic Sciences & Lobund Laboratory, University of Notre Dame, Notre Dame, IN.
[2] The Department of Emergency Medicine and Care and the Office of Medical Education, St. Joseph's Medical Center, South Bend, IN.
*Address correspondence to this author, at: American Biogenetic Sciences 1539, N. Ironwood Dr., South Bend, IN 46635. Fax 219-271-3422; e-mail Paul.E.Gargan.1@nd.edu.

[3] Nonstandard abbreviations: TpP, thrombus precursor protein; MI, myocardial infarction; GUSTO, Global Utilization of Streptokinase and Tissue plasminogen activator for Occluded coronary arteries; ER, emergency room; UA, unstable angina; DVT, deep vein thrombosis; CK, creatine kinase; CKMB, creatine kinase MB isoenzyme; and ECG, electrocardiogram.

Received May 2, 1996; accepted June 27, 1996.

and myoglobin *[14, 15]*. Detection of these necrotic markers forms the basis of current diagnostic tests for MI. Clearly, earlier markers of thrombotic occlusion and ischemia of cardiac muscle would greatly facilitate the diagnosis of an evolving MI and permit more timely therapeutic intervention. Such therapy would reduce the extent of damage to the cardiac tissue. Considerable effort has been invested by numerous groups in recent years in attempts to develop such diagnostic tests *[16]*.

Even though the role of thrombosis in MI has been understood for some time, there has been little advance in the development of direct diagnostic tests for active thrombosis. Indeed, the development of noninvasive, highly specific tests for the direct measurement of active thrombosis has proven particularly difficult *[17]*.

Several immunoassays measure markers of the activation of the blood coagulation system, which are not necessarily coincident with the extent of thrombosis. Such tests include assays for the measurement of prothrombin fragment F1.2 and for enzyme–inhibitor complexes such as thrombin–antithrombin III and plasmin–antiplasmin. However, because they measure changes in activity of the coagulation system, these tests have not been effective in aiding in the accurate diagnosis of MI *[18]*.

Our approach has been to develop a specific and reproducible test that can measure the concentrations of proteins previously shown to be present in the blood of patients with thrombosis *[1]*. These proteins are high-molecular-weight soluble fibrin polymers, the immediate precursors to the insoluble fibrin found in a thrombus. The immunochemical test for these thrombus precursor proteins (TpP™; American Biogenetic Sciences) uses a monoclonal antibody highly specific for polymeric fibrin *[19]*.

In this study we investigated the diagnostic accuracy of this new direct test for active thrombosis in blood samples from MI and non-MI patients who presented to the ER with symptomatic chest pain.

Materials and Methods

SUBJECTS

Two independent studies were performed in chest pain patients presenting to the ER. The first study included 47 patients who had been recruited specifically for the Global Utilization of Streptokinase and Tissue plasminogen activator for Occluded coronary arteries (GUSTO) intervention study. These patients met all GUSTO entrance criteria, i.e., had symptomatic chest pain, showed classic electrocardiogram (ECG) changes, and presented to the ER within 4 h after the onset of pain *[20]*. Patients were diagnosed with MI, UA, or other chest pain.

In the second study, we included a larger patient cohort, recruiting 300 ER patients. These patients were categorized as MI early presenters (i.e., met GUSTO criteria and had reference range concentrations of cardiac proteins); MI late presenters (who presented to the ER >6 h after the onset of pain or had above normal concentrations of CK); other chest pain; and other conditions in which intravascular fibrin formation was indicated. The diagnosis was made by extensive review of the individual medical records. Both studies were approved by the institutional review board at St. Joseph's Medical Center. All patients' blood samples were drawn at presentation to the ER.

PROCEDURES

Sample processing. Blood samples were drawn by hospital personnel into Vacutainer Tubes (Becton Dickinson, Rutherford, NJ) containing buffered sodium citrate (129 mmol/L final concentration). Within 30 min of collection, the blood was centrifuged at 1500–1800*g* for 15 min, and the plasma was aspirated, aliquoted, and stored frozen at −20 °C until analysis.

Measurement of TpP. We used the microtiter plate ELISA procedure described previously *[21]*. In brief, the anti-TpP antibody was coated onto the wells of a 96-well microtiter plate (Nunc Maxisorb; Fisher Scientific, Glendale, IL). Calibrators, plasma samples (thawed for 15 min at 37 °C), and controls were incubated in the wells. Samples with visible clots and extensive hemolysis or lipemia were rejected from the analysis. Bound TpP was detected after incubation with a nonspecific detection antibody conjugated to horseradish peroxidase and then with 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance of samples was read at 450 nm (Molecular Devices, Menlo Park, CA), and the concentration of TpP in patients' samples was determined by comparison with a calibration curve constructed with a set of TpP calibrators.

We estimated a reference interval by assaying TpP in 45 healthy volunteers (men and women, ages 22–70 years) without suspected intravascular thrombosis.

Determination of cardiac proteins. CK was determined in all chest pain patients and CKMB isoenzymes were tested in those patients with confirmed MI. Both CK and CKMB were measured with test kits from Abbott (Abbott Park, IL). The reference interval for CK is <150 U/L; for CKMB, it is <6% of total CK.

Statistical methods. For statistical analysis of the results for the samples from patients recruited for the second study, we used the Statview software application for the Macintosh (Abacus Concepts, Berkeley, CA). The four patients' categories were subjected to ANOVA.

Results

A typical calibration curve for the TpP assay (Fig. 1) was generated by using a preparation of TpP calibrator that consistently demonstrates linearity to 40 mg/L. The TpP calibrator has demonstrated stability for >12 months with no significant loss in immunoreactivity when stored at −20 °C, 4 °C, room temperature, or 37 °C. The concentration of TpP in the healthy controls ranged from 1 to 6 mg/L, and normal control plasma was used to validate the assay. The processing of samples was validated by testing TpP concentrations in fresh samples (n >50) and again after storage at −20 °C. TpP demonstrated stability when stored under the conditions described. In independent studies, the TpP concentrations in an age- and sex-matched population (including smokers and nonsmokers) <50 years old was <4.0 mg/L (data not shown).

Initially, we studied patients categorized into three groups: MI (n = 15), UA (n = 18), and other chest pain (n = 14)

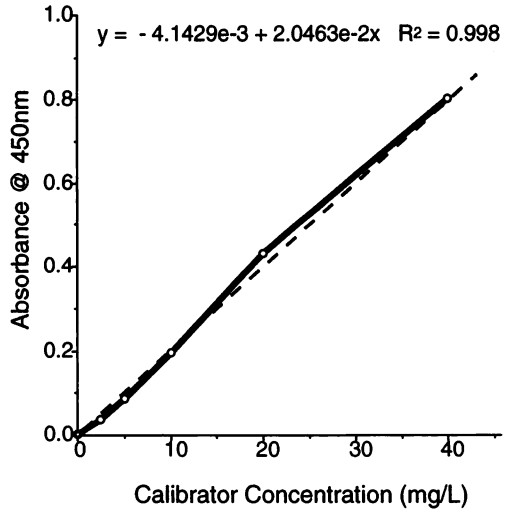


Fig. 1. Dose–response calibration curve for the TpP immunochemical assay.

Comparison of the concentrations of TpP in these three groups with those in healthy controls (n = 13) is summarized in Fig. 2 (left). In this early chest-pain study, TpP was expressed in ELISA units because a well-defined formulation of the calibrator had not yet been developed. In patients diagnosed with MI, the TpP concentrations were 4- to 20-fold those of healthy controls. In contrast, 15 of the 18 UA patients had TpP concentrations approximating that of the controls, as did the patients diagnosed with other chest pain (e.g., angina pectoris, pulmonary edema, anxiety, and indigestion). Of the 3 UA patients who had increased TpP, all progressed to MI within 48 h, as indicated by the diagnosis on discharge.

In the second study, the patients were categorized into four separate groups: MI patients who presented to the ER <6 h after the onset of chest pain (n = 21); MI patients presenting to the ER >6 h after the onset of chest pain, most of whom had CK >150 U/L (n = 33); patients diagnosed with other chest pain (n = 130); and patients diagnosed with other conditions associated with intravascular thrombosis (n = 24), e.g., DVT, pulmonary embolism, and congestive heart failure. Individual data points for the MI patients, those patients diagnosed with other chest pain, and healthy controls (n = 45) are summarized in Fig. 2 (right). By the time of this study, reference calibrators were available for quantification of TpP concentrations.

Compared with control subjects, MI patients presenting within 6 h of the onset of pain had significantly (P <0.01) increased TpP; MI patients presenting late and patients diagnosed with other chest pain did not. Patients diagnosed with other conditions of active fibrin formation also demonstrated significantly higher TpP concentrations than the healthy controls (data not shown).

In several cases, patients diagnosed with UA or noncardiac chest pain were later diagnosed with MI. Among these was a 53-year-old woman who presented at the ER with chest pain. A nonspecific ECG led to her being diagnosed with UA and she was discharged. This patient's TpP concentration (30.0 mg/L), however, was much higher than that for a control sample (4.0 mg/L) and clearly indicated a highly increased extent of thrombosis. Returning to the ER the next day, the patient was diagnosed with MI. In another patient who presented at the ER with chest pain, the ECG showed nonspecific ST depression of <1 mm on initial presentation; 20 h later, however, T wave inversion was detected in the ECG profile, and both CK and CKMB were increased to 437 U/L and ~10% of total CK, respectively. The TpP in this second patient was >40 mg/L (off scale), and the patient progressed to transmural MI.

In 8 of the 33 MI patients presenting within the 6-h window after the onset of chest pain, TpP and CK were measured in serial samples collected at 12 and 24 h after admission. Fig. 3 shows the temporal profile of these analytes. In all MI patients, TpP was increased at presentation and returned to normal values at 12 h (Fig. 3, top). The converse was observed for CK: i.e., MI patients presenting early to the ER after the onset of chest pain with normal concentrations of CK had increased CK at the second time point.

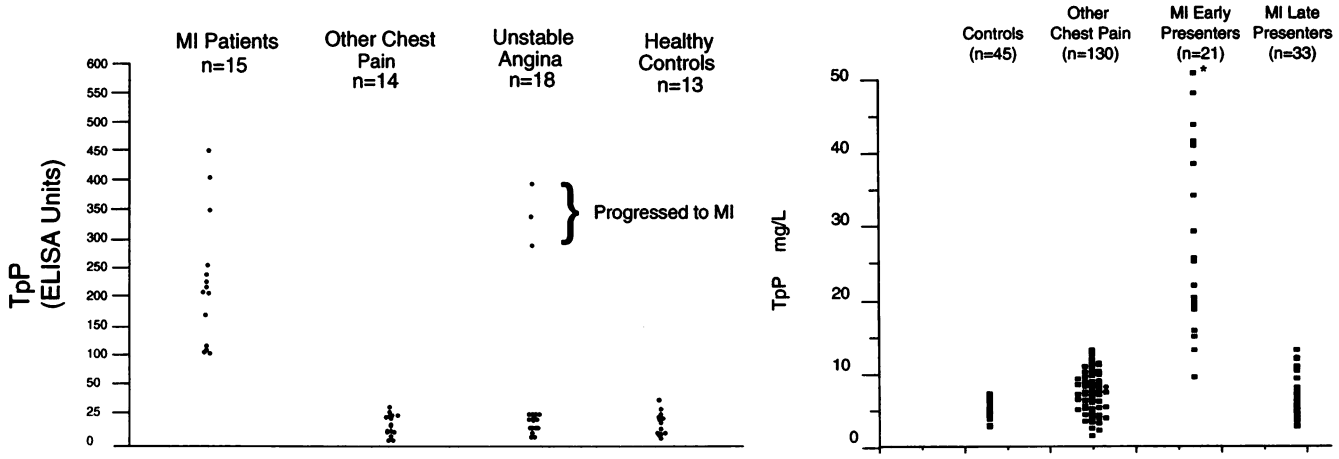


Fig. 2. TpP data for (*left*; initial study) patients recruited for the GUSTO intervention study and (*right*; second study) patients presenting to the ER with chest pain symptomatic of MI.

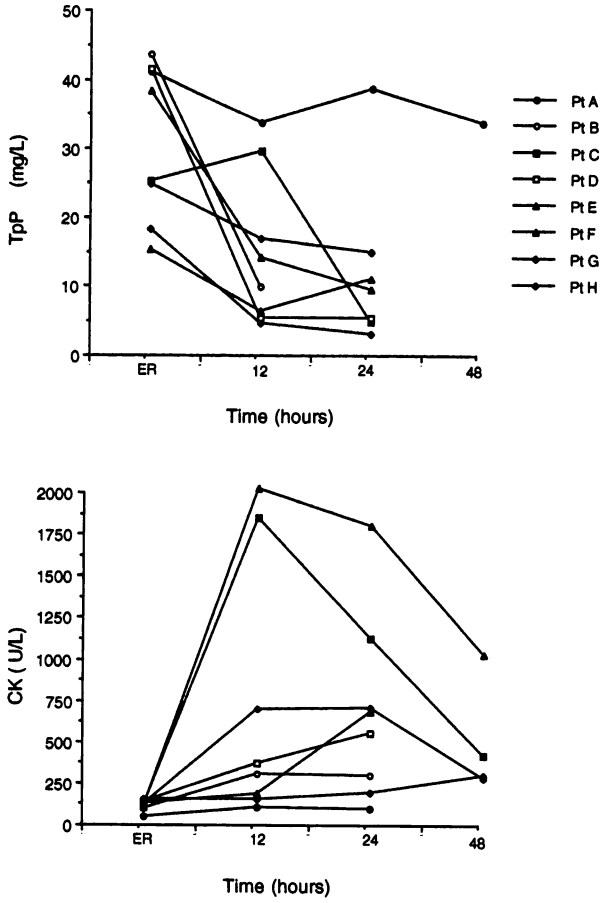


Fig. 3. Concentrations of TpP and CK in eight MI patients presenting to the ER early (<6 h) after the onset of chest pain.

Discussion

Thrombosis remains the leading cause of death in the developed countries *[2]*. Among the reasons for this is that clinical conditions induced by thrombosis are frequently not diagnosed until after acute clinical symptoms become manifest and tissue damage has occurred *[22]*. One such clinical condition induced in most cases by coronary thrombosis is MI. Definitive early diagnosis of MI is difficult because the ECG has a diagnostic sensitivity of only 50–70% *[23]* and the biochemical markers (CKMB, troponin, etc.) are increased only after the death of cardiac tissue.

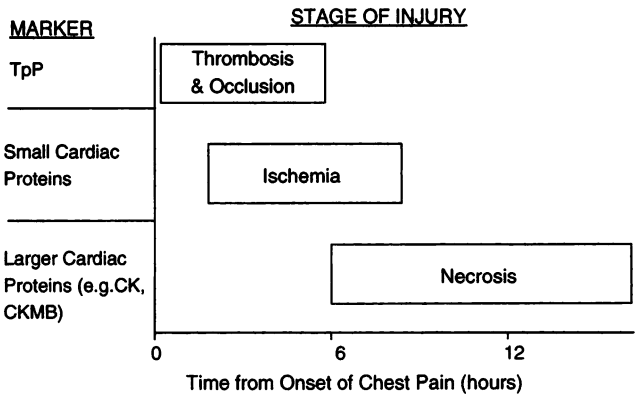
These diagnostic problems in MI patients can result in delays in appropriate intervention and misdiagnosis. As has been conclusively demonstrated in the interventional trials, thrombolytic intervention within 5 h after the onset of chest pain can substantially reduce mortality and salvage cardiac muscle *[13]*. Therefore, there is a clear clinical need for a diagnostic modality that can detect active thrombosis to aid in the administration of appropriate therapies *[13, 23]*. In the current study, we describe how a new diagnostic assay, the TpP test, can measure active thrombosis in MI patients early after the onset of chest pain even though the patients still have no detectable increase in the biochemical markers of cardiac necrosis.

The polymeric fibrin species detected by the TpP test have conditions *[1]*. The generation of highly specific probes (MAbs) for intact fibrin has proven to be technically difficult *[19]* because fibrin is structurally and conformationally similar to its precursor protein (fibrinogen) and to the plasmin-derived degradation products of fibrin, which include D-dimer. To overcome this problem, we used antigen-free technology to generate a fibrin-specific MAb (MH1), which recognizes a conformational epitope found only on the intact polymeric fibrin structure *[19]*. This antibody is used as the specific capture MAb in the TpP test.

In the first study samples, the time from onset of chest pain to ER presentation was <6 h. Specificity and sensitivity were 100% for coronary thrombosis leading to MI. These data indicate that the TpP test has clinical potential in combination with other tests for both rule-in and rule-out early in the clinical event, when intervention with clot-dissolving therapies and (or) angioplasty is beneficial for salvage of cardiac muscle *[13]*.

Results from the second study support the finding from the GUSTO study. In this (second) expanded study (n = 300), the concentrations of TpP in MI patients presenting early after the onset of chest pain were increased by as much as 18 times above that observed in a healthy control population. However, TpP concentrations were not increased in patients presenting >6 h after the onset of chest pain, and who had high concentrations of markers of cardiac damage (total CK). Indeed, in many of the MI patients who were early presenters and had above-normal concentrations of TpP, subsequent samples at 12 and 24 h showed that the TpP concentrations dropped off as the concentrations of other cardiac markers (e.g., total CK) began to increase (Fig. 3). These findings are in agreement with the pathological processes involved in MI, and suggest that TpP has the potential to detect the initial thrombotic occlusive process in MI, which occurs before and overlaps with the ischemic stages of the disease (Fig. 4). A large patient study is currently underway to define the temporal relationship between TpP and the following markers of cardiac necrosis: CK, CKMB, cardiac troponin I, and myoglobin.

In most UA patients, TpP concentrations were within the reference interval, but some who had active thrombosis (as indicated by the TpP test) progressed to MI. Although larger studies are required, these initial observations indicate that the TpP test may have an important clinical role in risk stratification



of UA patients for MI. About 1 million cases of UA are recorded at ERs in the US each year, of whom an estimated 10–15% will progress to MI *[7, 24]*. UA patients with ongoing coronary thrombosis may benefit from thrombolytic intervention with tissue plasminogen activator or streptokinase *[25, 26]*. By detecting active thrombosis, the TpP test has the potential to be a beneficial aid in patient selection for thrombolytic therapy in the acute-care environment of a hospital ER.

MI and UA are just two of the potential clinical conditions in which this test for active thrombosis could have diagnostic utility. The TpP test may also lend itself to application in postoperative thrombosis, a major complication in patients undergoing thoracic, abdominal, and orthopedic procedures *[27, 28]*. Postoperative DVT is responsible for increased mortality in surgical patients, its clinical sequela of pulmonary embolism being the largest single cause of postoperative mortality *[27-29]*. Although great strides have been made in developing interventional and prophylactic therapies for postoperative thrombosis, one of the most important needs for improving the efficacy of these approaches is a specific assay capable of detecting active thrombosis to identify those patients requiring additional anticoagulation *[27]*. Intrasurgical monitoring of active thrombosis is also an important clinical need during such procedures as angioplasty, where thrombus formation at the site of vascular damage can result in abrupt closure of the coronary vessel and lead to periprocedural MI, emergency coronary artery bypass surgery, or death *[30]*.

In conclusion, the TpP test appears to be highly specific for intravascular thrombosis; as such, it has many potential clinical applications. Ongoing large-scale studies include chest pain management, cardiac catheterization, thrombosis as a consequence of trauma, and postsurgical monitoring of DVT.

References

1. Francis CW, Conaghan DG, Scott WL, Marder VJ. Increased plasma concentrations of cross-linked fibrin polymers in acute myocardial infarction. Circulation 1987;75:1170–6.
2. Hirsh J, Salzman EW, Marder VJ, Colman RW. Overview of the thrombotic process and its therapy. In: Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia: JB Lippincott, 1994: 1151–63.
3. Clouse LH, Comp PC. The regulation of hemostasis: the protein C system. N Engl J Med 1986;314:1298–304.
4. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med 1984;311:1525–8.
5. Vikydal R, Koringer C, Kyrle PA, Niessner H, Pabinger I, Thaler E, Lechner K. The prevalence of hereditary antithrombin-III-deficiency in patients with a history of venous thromboembolism. Thromb Haemost 1985;54:744–5.
6. National Center for Health Statistics. Publ. No. (PHS) DHHS 1993;94–1232.
7. Cairns JA, Cohen M. Unstable angina—antithrombotics and thrombolytics. In: Fuster V, Verstraete M, eds. Thrombosis in cardiovascular disorders. Philadelphia: WB Saunders, 1992:237–60.
8. Harker LA, Mann KG. Thrombosis and fibrinolysis. In: Fuster V, Verstraete M, eds. Thrombosis in cardiovascular disorders. Philadelphia: WB Saunders, 1992:1–16.
9. Golden MS, Lang T. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. N Engl J Med 1980;303:897–902.
10. Fuster VL, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. N Engl J Med 1992;326:242–50.
11. Reimer KA, Lowe JE, Rasmussen MM, Jennings RB. The wave front phenomenon of ischemic cell death. Myocardial infarction size vs duration of coronary occlusion in dogs. Circulation 1977; 56:786–93.
12. Cannon CP, Antman EM, Walls R, Braunwald E. Time as an adjunctive agent to thrombolytic therapy. J Thromb Thrombol 1994;1:27–34.
13. Roberts R, Kleiman NS. Earlier diagnosis and treatment of acute infarction necessitates the need for a "new diagnostic mind-set." Circulation 1994;89:872–81.
14. Lee TH, Goldman L. Serum enzyme assays in the diagnosis of acute myocardial infarction. Ann Intern Med 1986;105:221–33.
15. Apple FS. Acute myocardial infarction and coronary reperfusion: serum cardiac markers for the 1990's. Am J Clin Pathol 1992; 97:217–26.
16. Apple FS. Glycogen phosphorylase BB and other cardiac proteins: challenge to creatine kinase MB as the marker for detecting myocardial injury [Editorial]. Clin Chem 1995;41:963–5.
17. Hirsch DR, Goldhaber SZ. Blood tests that predict venous thrombosis and the relation of these blood tests to primary (inherited) and acquired risk factors. In: Goldhaber SZ, ed. Prevention of thromboembolism. New York: Marcel Dekker, 1993:3–24.
18. Bauer K, Rosenberg R. The pathophysiology of the prethrombotic state in humans: insights gained from studies using markers of hemostatic system activation. Blood 1987;70:343–50.
19. Gargan PE, Gaffney PJ, Pleasants JR, Ploplis VA. A monoclonal antibody which recognizes an epitopic region unique to the intact fibrin polymeric structure. Fibrinolysis 1993;7:275–83.
20. The GUSTO investigators. An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. N Engl J Med 1993;329:67–82.
21. Gargan PE, Dimitrijevic N, Scheu JD, Walsh M. An immunochemical assay for the measurement of soluble fibrin polymers in plasma [Abstract]. Thromb Haemostas 1993;69:1003.
22. Vogel G, Spanuth E. Predictive value of fibrin monomers in postoperative deep vein thrombosis. Klin Wochenschr 1990;68: 1020–6.
23. Stevenson R. AMI diagnosis could save billions. AACC National Meeting Workshop, Chicago, IL, 1992:28–33.
24. Chesebro JH, Fuster V. Thrombosis in unstable angina. N Engl J Med 1992;327:192–4.
25. Collen D. Fibrin selective thrombolytic therapy for acute myocardial infarction. Circulation 1996;93:857–65.
26. Sobel BE. Thrombolysis in the treatment of acute myocardial infarction. In: Fuster V, Verstraete M, eds. Thrombosis in cardiovascular disorders. Philadelphia: WB Saunders, 1992:289–326.
27. National Institutes of Health Consensus Development Conference Statement. Bethesda, MD: US Govt Printing Office, 1986;491–292:41120.
28. Salzman EW, Hirsh J. The epidemiology, pathogenesis, and natural history of venous thrombosis. In: Colman RW, Hirsh J, Marder VJ, Salzman EW. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia, PA: JB Lippincott, 1994: 1275–96.
29. Weinmann EE, Salzman EW. Deep-vein thrombosis. N Engl J Med 1994;331:1630–41.
30. de Feyter PJ, de Jaegere PP, Suruys PW. Incidence, predictors and management of acute coronary occlusion after coronary angio-