



# Malignant hypertension, fibrinoid deposition, and fibrinogen electrophoresis

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■ Electrophoretic profiles of the molecular weight distributions of fibrinogen derivatives in blood provide a tool for combined assessment of coagulation and fibrinolysis in the course of vascular disease. Profiles obtained in studies on an experimental model of hypertension and in humans with occlusive vascular disease are discussed. In the experimental studies elevations in the level of cross-linked dimers provided a more reliable means for predicting development of malignant hypertension than did many other criteria, especially near the outset when blood pressure changed to similar degrees in rats with malignant and benign hypertension. Similarly, we find that levels of dimeric and occasionally trimeric forms of fibrinogen are more prominently elevated than degraded forms of fibrinogen in patients with occlusive vascular disease.

□ INDEX TERMS: FIBRIN FIBRINOGEN DEGRADATION PRODUCTS; FIBRINOLYSIS; HYPERTENSION, MALIGNANT □ CLEVE CLIN J MED 1989; 56:451-456

**P**ROGRESS in recognizing and treating arterial hypertension has reduced the occurrence of some of its usual complications enormously, particularly the malignant phase. Although risk of transition to the malignant phase is virtually eliminated with adequate control of blood pressure, the principal factors contributing to the pathogenesis of necrotizing lesions in the untreated hypertensive population remain obscure.

Fibrin deposition in the vascular system, considered to be the hallmark of malignant hypertension, is also observed in diseases such as scleroderma, polyarteritis

nodosa, and lupus erythematosus; all of these include hypertension and widespread vascular disease among their secondary complications. It has been suggested<sup>1,2</sup> that the fibrinoid deposits may be caused by infiltration and deposition of circulating fibrin-fibrinogen-related products into the vascular wall.

Gavras et al<sup>2</sup> observed that patients with malignant hypertension had increased fibrinogen levels and fibrin degradation products in blood. The findings implicate a form of chronic, disseminated intravascular coagulation in the pathogenesis of this disease. As recounted in a review in this journal,<sup>3</sup> numerous laboratory tests have recently been developed to detect products of coagulation and fibrinolysis in blood. Since all these tests provide only partial information, it becomes difficult to decide how many and which ones should be used.

The answer to that question cannot as yet be given, but we suggest that still another recently devised method, electrophoretic profiling of the molecular weight distribution of fibrinogen and its derivatives in

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blood, provides a means of holistically assessing the nature and levels of both coagulation and fibrinolysis products in blood. This commentary summarizes our experience in applying an earlier version of the technique in studies on an experimental model of hypertension and provides examples of the differing profiles obtained in normal human subjects and subjects with both severe and moderate occlusive vascular disease.

#### FIBRIN COMPLEXES IN THE GENERALIZED SHWARTZMAN REACTION

A direct and quantitative relationship between fibrinoid deposition and intravascular coagulation was established by measuring fibrinopeptide-release<sup>4</sup> and formation of circulating fibrin-complexes<sup>5</sup> in the course of the generalized Shwartzman reaction in rabbits. This reaction is produced by intravenous injection of endotoxin on two consecutive days. Fibrinoid deposition occurs only infrequently after the first injection, but at high frequency after the second. The fibrinoid deposition is widespread and is associated with bilateral renal cortical necrosis. Blood taken from the rabbits after either the first or second injection was found to contain a cryoprecipitable form of fibrinogen that was subsequently identified as a fibrinogen/fibrin complex. It was then found that complexing the molecular form of fibrin (fibrin monomer) with fibrinogen provided a means to maintain fibrin in a soluble form in blood. Production of the complexes at levels exceeding the threshold for solubility coincided with the deposition of fibrin within the glomeruli and with associated bilateral renal cortical necrosis. The fibrin complexes were not degraded or cross-linked but were products of direct interaction of intact fibrin monomers with intact fibrinogen. They rose to maximal concentrations within two hours after injection of endotoxin and were cleared from the circulation within six hours as blockade of the RES by the endotoxin subsided. The fate of fibrin before its deposition appeared critical. There is reason to believe that clearance was mediated largely by endocytotic uptake of the circulating fibrin.<sup>6-9</sup>

Since a mechanism exists for nondegradative solubilization of fibrin through formation of soluble complexes, and since the soluble complexes are rapidly cleared from the circulation by the RES, removing fibrin by the fibrinolytic pathway might not be critical except in abnormal circumstances predisposing fibrin deposition. However, we know this perspective on hemostasis—that it depends largely on the balance between low-level production and rapid clearance of soluble complexes—can apply only to healthy subjects. Deposition of fi-

brinogen-derived protein accompanies many diseases, such as atherosclerosis,<sup>10-13</sup> that chronically afflict the mature human population. As summarized by Bang,<sup>14</sup> fibrin complexes and degradation products with very differing properties, stabilities, and circulatory half-lives can be produced depending on the order and extent to which fibrinogen is acted upon by thrombin, fibrin stabilizing factor, plasma, and leukocyte proteases. Also, the possibility exists that many of the derivatives found in blood may be products of enzymes released by injured cells within the vasculature.<sup>15</sup>

#### FIBRINOGEN IMMUNOELECTROPHORESIS

To approach full assessment of the changes occurring in fibrinogen, we devised a means to profile the molecular weight distributions of the protein by SDS-electrophoresis (SDS = sodium dodecyl sulfate) and subsequent immunochemical probing of the electrophoregrams with specific antibodies. This type of electrophoresis uses a novel supporting medium, glyoxyl agarose,<sup>16,17</sup> which can be used interchangeably as a separation medium and a protein-immobilizing medium. It is an aldehydic derivative of agarose that interacts so weakly with proteins that it has virtually no effect on their electrophoretic mobility at neutral pH, but rapidly forms covalent linkages with protein amino groups when a selective Schiff-base reductant such as NaCNBH<sub>3</sub> is added. Thus, following electrophoresis, the protein can be chemically attached to the gel matrix by simply immersing the gel in amine-free buffer containing NaCNBH<sub>3</sub>, either 0.1 mol/L at neutral pH or 0.02 mol/L at pH 10. With the protein covalently linked to the gel, it can be probed repeatedly with specific antibodies without risk of losing it through dissolution and/or through formation of soluble immune complexes. Even small peptides such as fibrinopeptide A are fixed by this procedure so long as they contain at least one amino group.

One of the principal advantages of the medium is that it eliminates the need for Western blotting to probe the electrophoregrams; they are probed directly. With recent development of a removable polyacrylamide filler that can be polymerized within the gel and subsequently removed after electrophoresis and fixation of the protein, the methodology offers the same resolving power as conventional polyacrylamide gel electrophoresis (PAGE), but with the added capability to probe the gels directly with antibody. Polyacrylamide gels are just too impermeable for direct probing with antibodies. But when proteins are immobilized within the polyacryl-



A, B

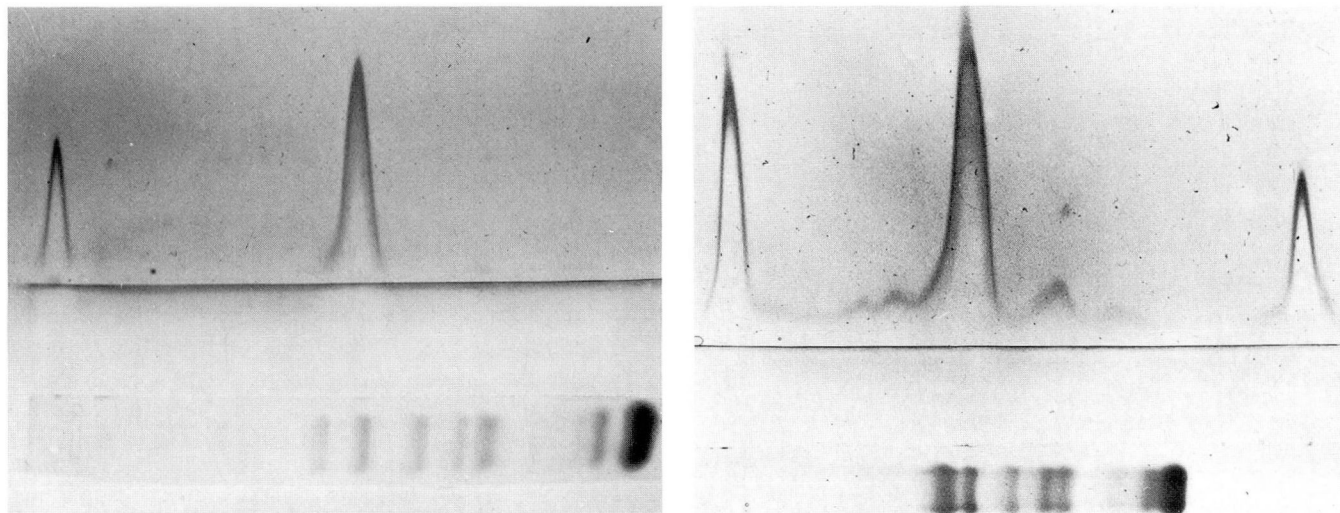


FIGURE 1. Results of crossed immunoelectrophoretic analysis of the distribution of fibrinogen-related antigens in SDS/glyoxyl agarose electrophoregrams of plasma from a normal (A) and a malignant hypertensive (B) rat. The primary, SDS-glyoxyl agarose electrophoregram (located horizontally in the gel) was exposed to 1° antibodies (rabbit anti-fibrinogen antibodies), and retained 1° antibody was desorbed electrophoretically and electrophoresed (vertically) into an agarose gel containing 2° antibody for immunoprecipitation (pig anti-rabbit-IgG antibodies). The “rocket” profile portrays the distribution of fibrinogen-related antigens in the electrophoregram. The large peaks at the extreme right and left sides of the electrophoregrams are immunoprecipitation “rockets” formed by nonimmune rabbit IgG applied for calibration. The large peak in the center of each of the electrophoregrams coincides with the position of undegraded fibrinogen. The smaller peaks to the right of the central peak display the concentrations of degradation products in the specimen, and those to the left are from cross-linked polymeric derivatives of fibrinogen. Polymers and degradation products were not detected in the normal specimen (A), but high concentrations of both were present in the abnormal specimen (B). The photographs are reproduced from ref. 17 with permission.

amide composite, they become linked only to the highly porous glyoxyl agarose component of the matrix. Apart from electrophoretic applications, the medium has been employed in novel chromatographic<sup>18</sup> and blot-dot analyses<sup>19</sup> for screening and affinity separations with both polyclonal and monoclonal antibodies. The medium has recently become commercially available as NuFix® (FMC BioProducts, Rockland, ME 04841), and a manual detailing its application is in press.<sup>20</sup>

While no disadvantages arise from the medium itself, an important disadvantage arises from use of SDS to promote separation of the protein according to molecular size. SDS is a potent chaotropic agent that obliterates protein/protein interactions. Thus, the analysis of molecular weight distributions of fibrinogen by SDS-electrophoresis does not provide any indication of the interactive properties of the protein. Fibrin monomer migrates with the same mobility as parent fibrinogen because the molecular weights differ by only a little more than 1%. However, dimers produced by cross-linking and degradation products produced by fibrinolytic enzymes have mobilities that differ greatly from the parent protein and are readily resolved. The slight differ-

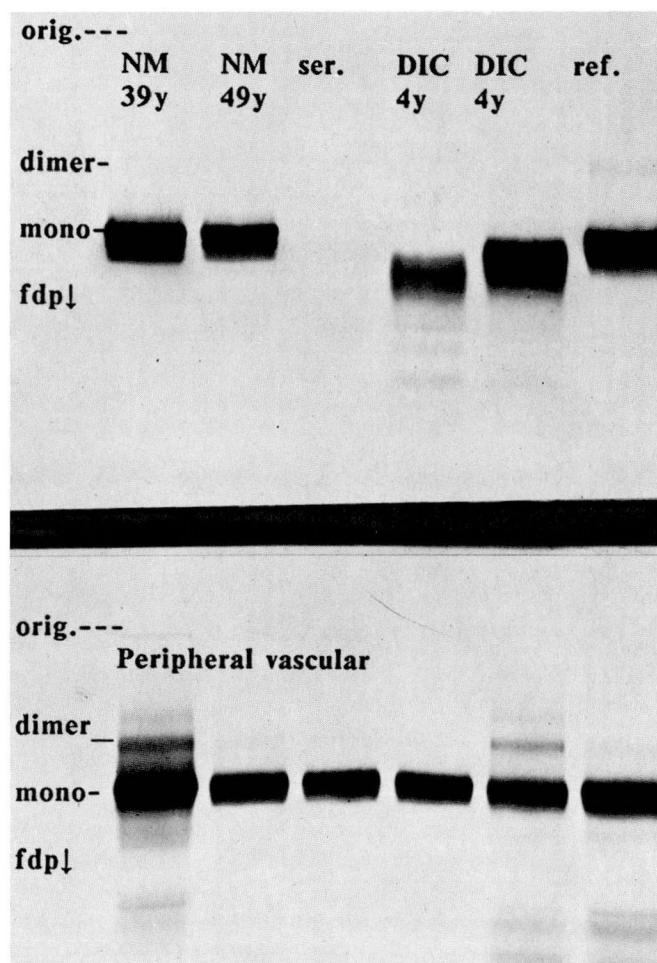
ences in composition that distinguish fibrin monomer from fibrinogen will ultimately become resolvable as monoclonal antibodies capable of recognizing the slight chemical differences between the proteins are developed, as discussed at the conclusion of this paper.

#### EXPERIMENTAL MALIGNANT HYPERTENSION

The electrophoresis method was applied initially to address the question of whether fibrinogen alterations occurred early or late in the course of development of malignant hypertension<sup>21</sup> in an experimental model in which rats were made hypertensive by complete ligation of the aorta between the renal arteries.<sup>1,22</sup> Approximately 30%–46% of the rats made hypertensive by this means develop malignant hypertension, whereas the rest of the animals develop benign hypertension.<sup>23</sup> The study was carried out as a part of a more comprehensive work, detailed previously.<sup>23–27</sup>

Matched sets of animals were randomly divided into two subgroups, one for study of pathology and direct measurement of blood pressure, and the second for sequential blood sampling. Animals were killed at three-





**FIGURE 2.** Profiles of size-distribution of fibrinogen-related protein in specimens from human subjects. Plasma samples (diluted 5× with 4% SDS) were applied (1  $\mu$ L) at position labeled "orig.," electrophoresed (direction: top to bottom) on the glyoxyl agarose gels, and stained with peroxidase-labeled antifibrinogen antibodies after fixing the electrophoregrams with buffer containing NaCNBH<sub>3</sub>. The fibrinogen in normal plasmas is usually devoid (<<1% of total of fibrinogen) of polymers and low molecular weight degradation products, as represented by the samples from normal males (NM) in the upper electrophoregram. It migrates as three closely spaced bands with mobilities corresponding to molecular weights ranging from  $3.4$  to  $3.0 \times 10^5$  Daltons. Serum (ser.) from normal subjects is usually devoid of fibrinogen-related protein. Two sequential specimens from a leukemic patient showed considerable degradation of the fibrinogen, as indicated by shifts in mobility of the major band and presence of very fast migrating degradation products (fdpP). A reference fibrinogen containing approximately 2% cross-linked dimer forms is shown in the specimen labeled DIC in the electrophoregram. The lower electrophoregram illustrates specimens in which both polymers and degradation products are elevated to varying degrees (lanes 2, 5, and 6). The specimens in lanes 2–3 show elevation of dimeric fibrinogen in absence of degradation products.

day intervals between three and 24 days after aortic ligation. Based on pathological assessments of animals killed at either 12 or 24 days postligation, they were characterized as either benign (BHYs) or malignant (MHYs) hypertensives.<sup>25</sup> Immunoprobings of electrophoregrams at the earliest sampling (3 days) regularly showed, as in *Figure 1A*, high concentrations of both polymeric and degraded forms of fibrinogen in the plasma of rats that were subsequently found to be MHYs.<sup>25</sup> By contrast, these derivatives were seldom found (one of 11 animals) at resolvable levels (>1% of the total fibrinogen) in the three-day BHYs and not at all in sham-operated controls. Usually, the levels of both polymers and degradation products dropped substantially over days 6–9. The fall in level of the polymers and degradation products coincided with a diminution in the abundance of fibrinoid observed in the parallel matched series of animals.<sup>25</sup> However, the polymers rose again during days 12–24, and this secondary rise appeared to coincide with glomerular fibrinoid deposition.<sup>22</sup>

The findings provide evidence that the development of malignant hypertension was either directly or indirectly associated with the appearance of high levels of cross-linked forms of fibrinogen (ranging from 3%–9% of the total fibrinogen) in blood shortly after ligation. Intravascular coagulation was also indicated by the appearance of elevated levels of degradation products along with the fibrinogen/fibrin polymers in blood. Although degradation products and cross-linked polymers seldom rose to impressive levels (>1% of the fibrinogen), the method of analysis was not designed to detect fibrin complexes that are not cross-linked, because these complexes are fully dissociable by the SDS used for electrophoretically profiling the molecular weights of the protein. As described,<sup>25</sup> we know that fibrinoid deposition occurred at the outset in both BHYs and MHYs, but the extent of deposition was much smaller and the deposits disappeared almost entirely by the ninth day in BHYs. Fibrin monomer without cross-linkages is cleared from the circulation very rapidly.<sup>28</sup>

We suspect that the cross-linking observed in the present study may have contributed important differences by impairing both the clearance and transport of fibrin. Also, direct cross-linking of fibrinogen accompanies cross-linking of fibrin,<sup>29</sup> and it is possible that fibrinogen polymers would not be cleared as rapidly as fibrin polymers by macrophages because macrophages possess receptors that are specific<sup>8</sup> for fibrin. We believe it is significant that the small fibrinoid deposits seen in BHYs were infiltrated with large numbers of macrophages, and the macrophages appeared laden with fibrinoid as

though engaged in its removal,<sup>24</sup> but no such activity was observed in MHYs.

Although many factors such as elevated glucocorticoids<sup>24,27</sup> could have contributed to the differences in macrophage activity,<sup>25</sup> the cross-linking warrants consideration as a potential factor. Many questions remain to be answered concerning underlying reasons for the fibrinogen alterations seen in MHYs. Although the underlying reasons are not known, the fibrinogen alterations provided a useful diagnostic tool in this experimental model; they distinguished MHYs from BHYs, whereas elevations in blood pressure were identical in the two groups.

#### HUMAN PLASMA FIBRINOGEN

Although fibrinogen alterations are known to exist in patients with hypertension,<sup>2</sup> we have not yet applied the electrophoretic method to these patients but have examined a large population of patients with occlusive vascular disease in which high blood pressure was a complication for some. Furthermore, we have just recently simplified the procedure so that it is no longer labor-intensive and can now be applied to large populations. The procedure as used in the study on rats employed crossed immunoelectrophoresis with which we could only analyze one or two blood samples at once. With development of a means for rapid washing of the gels,<sup>19</sup> we can now stain them directly with peroxidase-labeled antibody. Rapid washing is critical because immune complexes are highly dissociable<sup>30</sup>; the considerable loss of bound antibody that accompanies washing is one of the reasons that most immunologic staining procedures require sandwiching techniques for amplification of the staining.

As illustrated in *Figure 2*, a high degree of resolution of fibrinogen derivatives is obtained. Normal fibrinogen is seen to be composed of three fully coagulable components that presumably consist of undegraded protein and derivatives arising from proteolysis of the highly susceptible<sup>31,32</sup> carboxyterminal region of the A $\alpha$ -chain. Dimeric forms and low-molecular-weight degradation

products are not evident in the two normal subjects, but an unphotographable trace of dimer (~0.2% of total fibrinogen) is present in one. In the plasma of a leukemic patient with clinical symptoms of disseminated intravascular coagulation, a pattern of high levels of degradation products with virtually no dimers illustrates that consumptive coagulopathy can occur without cross-linking of fibrinogen and fibrin. Examples are also shown of a converse situation in which plasma from some patients with occlusive vascular disease contains high levels of dimers but no degradation products, whereas other specimens contain high levels of both dimers and degradation products. Trimers and higher oligomers of fibrinogen may also be present. Studies are underway to determine how much correspondence there is between the type of pattern and the nature and severity of vascular disease.

The dimers are not necessarily fibrin dimers, but may in some instances be fibrinogen dimers as shown by staining of gels with antifibrinopeptide-A antibodies.<sup>15</sup> Precise differentiation between fibrinogen and fibrin derivatives by specific staining would require double-staining, one with antibody directed to the fibrinopeptide and another directed to the protein stripped of the peptide.

But how does one stain something that is missing? Sequence-specific monoclonal antibodies have been developed to aid in distinguishing between fibrinogen and fibrin.<sup>33,34</sup> However, the fibrin-specific antibodies currently available react with denatured forms of fibrinogen. Chemically, fibrinogen and fibrin differ with respect to the location of the Gly-Pro-Arg sequence; it occurs as an amino-terminal sequence in fibrin, but is penultimate to fibrinopeptide A in fibrinogen. For specific staining of fibrin monomer in electrophoregrams, antibodies are needed that can specify that the amino acid sequence they recognize does indeed occur at the amino terminus of the protein, and not in a penultimate position as in fibrinogen. To that end, we are preparing antibodies directed to chemically modified peptides derivative amino acid groups, as produced by succinylation.

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