Physical Stability of Cell Culture Procoagulants Detectable in First-Stage Coagulation Factor Assays (36222)

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(Introduced by G. H. Mudge)

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A potent procoagulant is synthesized by cells cultured *in vitro* (1). Of several cell types thus far studied, highest levels have been observed in cultures of human skin fibroblasts. This procoagulant is rapidly evolved following passage of cells in fresh medium. Activity is low at beginning of culture but rises several hundredfold after 12 to 24 hr incubation. Activity is readily demonstrable in cell culture homogenates by means of specific assays for first-stage coagulation factors (VIII, IX, XI, XII) (2).

Considerable effort has been made to characterize this procoagulant and determine its physiologic significance (if any) (2). It seemed logical to assume that multiple procoagulant substances might exist within living cells. This proved to be the case. We have demonstrated (2) activity in cell homogenates resembling phospholipid, which is capable of substituting for platelets. In addition, the presence of tissue factor (thromboplastin) was demonstrated by its ability to bind factor VII.

The presence of tissue factor complicates interpretation of first-stage factor assays. Tissue factor may account for all or only part of the procoagulant activity observed. Its presence may obscure the activity of one or more specific coagulation factors (e.g., factor VIII).

Several methods can be employed to resolve this problem. For example, Dodds has recently shown (Dodds, W. J., personal communication) that procoagulant activity, as judged by the one-stage assay for factor IX, was labile to repeated freeze-thawing while that detected by the factor VIII assay was stable. This suggested that multiple procoagulants might account for the results of first-stage coagulation factor assays and that these might vary in physical stability. Thus, the effects of heat, sonication, and repeated freeze-thawing on the procoagulant of cell homogenates and brain tissue factor were evaluated and are reported below.

Materials and Methods. Reagents were prepared, cells cultured, and homogenates were prepared for assay as described previously (1, 2). Stock brain thromboplastin was obtained from Ortho Pharmaceuticals. Plasmas congenitally deficient in factors IX, XI, and XII were provided by Dr. Anthony F. H. Britten of Boston. Freezing was carried out on the surface of solid carbon dioxide; and thawing, at 37°. Samples were heated in a water bath for 30 min at 56°. Fibroblast homogenates in 9-ml samples, which had not previously been frozen, were sonicated. Sonication (Model W140 D, Heat Systems, Plainview, NY) was performed with output control arbitrarily adjusted such that a midscale reading was obtained on the output control indicator. Similar experiments were performed on samples of normal fresh plasma and brain thromboplastin.

Prior to, and after, each procedure, homogenate or plasma was assayed for factors VIII, IX, XI, and XII by the one-stage method described by Simone *et al.* (3). The procoagulant properties of brain thromboplastin were similarly assessed by determining the clotting time of a mixture of 0.01 ml of stock thromboplastin, 0.1 ml of imidazole buffered saline, 0.1 ml of oxalated normal plasma and 0.1 ml of 0.2 *M* $CaCl_2$.

Results. Following heating of the homogenate to 56° for 30 min, the clotting time (judged by the one-stage assay for factors VIII, IX, and XI) increased 16, 16.5 and 20.6 sec, respectively. By contrast, heating

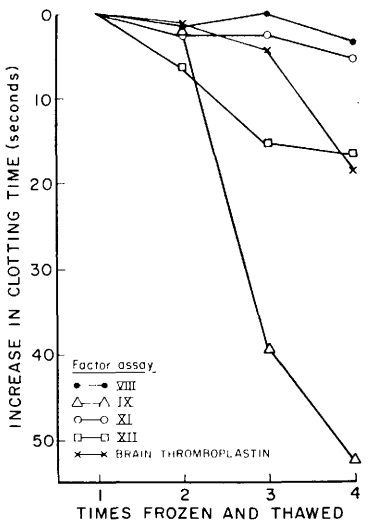

FIG. 1. Effect of freeze-thawing on procoagulant activity of fibroblast homogenates detected by assays for factors VIII, IX, XI, and XII.

prompted a 40-sec prolongation in the factor XII assay and a 40.3-sec prolongation in the test on brain tissue factor.

The results of repeated freeze-thawing on homogenate and tissue factor are illustrated in Fig. 1. Activity in the factor IX assay showed marked lability. Little change was observed in assays for factors VIII and XI. Activity in the factor XII assay and the effect on thromboplastin were similar and demonstrated an intermediate lability.

The effect of sonication is illustrated in Fig. 2. Sonication resulted in enhancement in activity which was marked in the factor IX assay and moderate (but virtually identical) in the VIII and XI assays. Little effect was observed in the XII assay and in tests on brain tissue factor.

The results obtained in tests on cell homogenates bore little resemblance to results of tests on circulating first-stage coagulation factors. Thus, plasma factor IX was relatively stable to the effects of freeze-thawing, heating, and sonication; whereas, factors VIII, XI, and XII were unstable to these influences.

Discussion. We have demonstrated activity in cell culture homogenates, which resembles tissue factor in that it complexes factor VII (2). Green and associates (4) also presented evidence for tissue factor in cell culture homogenates. Their results are difficult to compare with ours in some respects, however, since cells were assayed after trypsinization, a procedure which we have shown markedly reduces activity demonstrable in the factor VII assay (5). Furthermore, maximum levels of procoagulant obtained in their system were approximately one hundredfold lower than levels we have achieved.

Aside from phospholipid and tissue factor activity, the effects of procoagulant generated by cultured cells appears restricted to the first stage of coagulation (2). Thus, specific assay for the first-stage coagulation factors VIII, IX, XI, and XII is a sensitive method for detecting this procoagulant. Activity may be entirely or partially due to the presence of tissue factor. We have shown (2) indirectly that tissue factor does not entirely account for the procoagulant activity observed in these specific factor assays. Thus, while activity in assays for each of the 4 factors rises with incubation of cells at 37°, levels of activity detected in the factor VIII and XI assays greatly exceeded levels obtained in assays for factors IX and XII. Furthermore, factor VIII activity rises as determined by both the one-stage assay method, based upon the activated partial thromboplastin time; and the two-stage method, based upon the thromboplastin generation test. Procoagulant activity was observed to increase dramatically as judged by

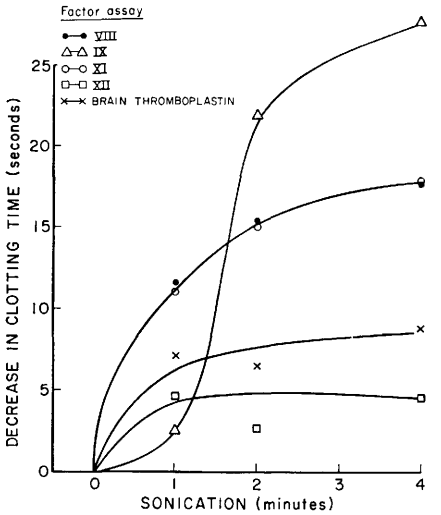

FIG. 2. Effect of sonication on procoagulant activity of fibroblast homogenates detected by assays for factors VIII, IX, XI, and XII.

the factor XI assay during contact activation in the presence of Celite. Serial dilutions of homogenate produced a different procoagulant effect on the activated partial thromboplastin time of normal plasma than did brain thromboplastin. Trace quantities of homogenate accelerated the coagulation of factor VII-deficient plasma more efficiently than did brain thromboplastin, suggesting that factor X activation was indeed occurring by way of the intrinsic pathway.

Assessment of the physical stability of this procoagulant lends further support to the hypothesis that multiple procoagulants account for activity detected in first-stage coagulation factor assays. The effect of heat, sonication, and freeze-thawing on brain tissue factor most closely resembled activity arising in the factor XII assay. The responses in assays for factors VIII and XI was similar. These responses did not resemble those observed in tests on plasma factors VIII, IX, XI, and XII.

Summary. The physical stability of the potent procoagulant(s) synthesized by cultured fibroblasts was studied. Variation in stability of procoagulant under the influence of heat, sonication, and freeze-thawing suggested that multiple procoagulants account for activity observed in specific assays for the first-stage coagulation factors (VIII, IX, XI, XII) rather than a single procoagulant (such as tissue factor). The stability of brain tissue factor most closely resembled activity observed in the factor XII assay. Stability patterns observed in assays for factor VIII and XI were similar.

1. Zacharski, L. R., Bowie, E. J. W., Titus, J. L., and Owen, C. A., Jr., Mayo Clin. Proc. 44, 784 (1969).

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3. Simone, J. V., Vanderheiden, J., and Abildgaard, C. F., J. Lab. Clin. Med. 69, 706 (1967).

4. Green, D., Ryan, C., Malandruccolo, N., and Nadler, H. L., Blood 37, 47 (1971).

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Erratum

Vol. **138**, No. 2 (1971), in the article, "The Effects of Age and Sex on Glycolic Acid Metabolism in Rats," by Thora J. Runyan, pp. 671–673:

Page 671, second column, line 17, "0.5 *M* sodium benzoate" should be "0.05 *M* sodium benzoate."