PRODUCTION OF BLOOD COAGULATION FACTOR V AND TISSUE THROMBOPLASTIN BY MACROPHAGES IN VITRO

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Received 27 February 1981

1. Introduction

Reports in the literature have indicated that mononuclear phagocytes may induce coagulation of blood [1,2]. We have demonstrated the production of the vitamin K-dependent coagulation factors, VII, IX, X and prothrombin by mouse peritoneal macrophages in vitro [3]. This study shows that these cells also synthesize factor V and thromboplastin (tissue factor), and thus all the essential proteins of the extrinsic coagulation pathway.

2. Materials and methods

2.1. *Cell cultures*

Peritoneal cells from mice were obtained and cultured as in [4]. In some cultures non-adherent cells (mainly lymphocytes) were removed and the remaining cells were washed carefully. After this procedure the cultures contained <5% lymphocytes, as judged by fluorescent microscopy with anti-theta and anti-Ig [5]. In other cultures the non-adherent and adherent cells were incubated together for 20 h, after which non-adherent cells (>95% lymphocytes) were removed and tested for thromboplastin activity. In some experiments T lymphocytes were specifically destroyed by the use of a rabbit anti-theta antibody and guinea-pig complement [5]. The anti-theta antibody was added to the cells immediately after extraction from the peritoneal cavity, and the suspension was incubated for 60 min at 0°C. After washing, guinea-pig complement was added and the cells were incubated for another 45 min at 37°C. Control cells were incubated with only anti-theta antibody or with only guinea-pig complement. The effectiveness of the anti-theta antibody and complement was tested by their effect on thymus cells and spleen cells from mice [5].

The experiments carried out to study thromboplastic activity were performed with culture media containing warfarin (12.5 μg/ml) to avoid production of factor VII and factor X. In some experiments endotoxin (*E. coli* lipopolysaccharide 0.26:B6, Boivin preparation, Difco Labs., Detroit MI) was added to the tissue culture medium at 10 μg/ml final conc.

2.2. *Factor V assays*

Factor V was assayed in either a one stage clotting assay as in [7] or in a coupled amidolytic assay. The coupled amidolytic assay was based on the requirement for factor V in the conversion of prothrombin to thrombin by factor Xa in the presence of phospholipid. The generated thrombin was quantitated by the use of a chromogenic substrate, S-2238 (KabiVitrum AB, Stockholm). The assay was carried out by incubating 50 μl factor Xa ([8], 0.4 U/ml), 50 μl prothrombin (1.5 U/ml), and 50 μl cephalin [9] at 37°C for 1 min. After the addition of 50 μl test sample, and 20 μl 50 mM $CaCl_2$ the incubation was continued for another 3 min. The reaction was stopped by adding 10 μl 0.3 M Na_2-EDTA. An aliquot of 100 μl incubation mixture was mixed with 600 μl 0.05 M Tris–HCl (pH 7.4) containing 0.1 M NaCl, and 100 μl S-2238 (1 mg/ml). The amount of thrombin generated was determined by measuring the rate of increase of absorbance at 405 nm. Prothrombin was purified by methods similar to those used for the purification of factor X [8] with one modification. The pooled prothrombin fractions from the preparative polyacrylamide gel electrophoresis were diluted 1:3 with water and applied to a dextran sulphate–Sepharose column (1.5 cm × 10 cm). The column was eluted with a gra-

dient of 0–0.05 M NaCl in 0.01 M Hepes (pH 7.4). Prothrombin obtained by this method gave a single band on SDS–polyacrylamide gel electrophoresis and contained no other coagulation factor activities.

2.3. *Thromboplastin assay*

Thromboplastin was quantitated in a two-stage assay based on the principle that thromboplastin is the limiting factor in the activation of factor X in a system consisting of purified factor X, factor VII, and Ca^{2+}. The assay was initiated by incubating 25 μl factor X ([8], 2.7 U/ml) and 25 μl factor VII ([10], 0.7 U/ml) at 37°C. After 1 min 100 μl of test sample and 20 μl 50 mM $CaCl_2$ were added, and the incubation was continued for 10 min. An aliquot of 50 μl was then removed, and the amount of factor Xa generated was assayed by utilizing the chromogenic substrate S-2222 (KabiVitrum AB). Rabbit anti-human thromboplastin [6] was kindly provided by Dr E. Bjørklid, Tromsø.

3. Results

Spent media from cultures of adherent cells contained significant amounts of factor V when tested in a one stage clotting assay. With media collected after 24 h cell culture a clotting time of 65 s was obtained as compared to 135 s with a control medium, incubated without cells. A similar sample, analyzed by a coupled amidolytic assay, showed a factor V activity corresponding to 0.5% of the activity in normal plasma (fig.1). In this procedure the addition of factor Xa is a prerequisite to the formation of thrombin. Accordingly, no significant amounts of thrombin could be detected in the absence of this factor, thus excluding the presence of any agent capable of activating prothrombin directly. As predicted, the production of factor V was unaffected by the presence of warfarin in the culture medium, but was greatly reduced in the presence of cycloheximide. Contrary to the formation of thromboplastin (see below) the production of factor V was unaffected by the addition of endotoxin, nor did it require the presence of non-adherent cells (not shown).

In [3] we found that factor X produced by cultured mouse peritoneal macrophages occurred partially in activated form (Xa) and that the extent of activation varied with the number of lymphocytes present [3]. These results could only be explained by

Table 1
Tissue thromboplastin activity in peritoneal cells

Culture conditions	Factor Xa formed (%)
Adherent cells	2.7 ± 0.7
Adherent cells + endotoxin	14.7 ± 1.9
Adherent cells + αθ + C	0.5 ± 0.2
Adherent cells + αθ + C + endotoxin	0.9 ± 0.3
Non-adherent cells	<0.1 ± 0
Non-adherent cells + endotoxin	0.1 ± 0

Peritoneal cells were obtained as in section 2. After 2 h in culture, non-adherent cells (mainly lymphocytes) were removed by washing, and the remaining adherent cells (mainly macrophages) were cultivated separately overnight and thereafter lysed by repeated freezing and thawing and thromboplastin was determined in a coupled amidolytic assay. Results are expressed as the amount of Xa formed relative to a fully activated pooled plasma standard ±SD. The anti-theta antibody (αθ) and complement (C) were added immediately after extraction of the cells from the peritoneal cavity, whereas endotoxin was added after separation of the adherent and non-adherent cells. The adherent cells represented >95% macrophages, with a <5% contamination by T and B lymphocytes as judged by fluorescence microscopy. For additional details see section 2

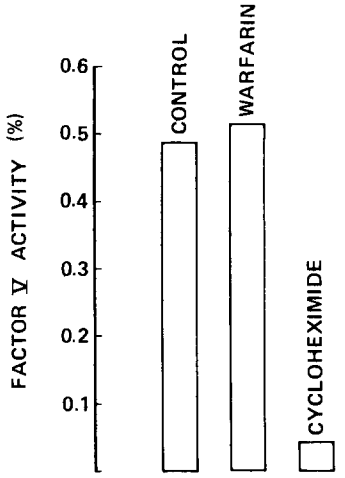

Fig.1. Adherent cells (>95% macrophages) were cultured for 24 h in media containing either 12.5 μg warfarin/ml, or 5 μg cycloheximide/ml, or no added inhibitor (control). The cells were centrifuged, and the resulting supernatants were tested for factor V as in section 2. The results are expressed as the amount of factor V activity relative to pooled, normal human plasma. Media from cultures of non-adherent cells (>95% lymphocytes) grown in the absence of any inhibitor contained 0.01% factor V activity.

assuming the production of thromboplastin (tissue factor) in the cell cultures. Such production was demonstrated here, using a specific, reproducible and highly sensitive, coupled amidolytic assay. Table 1 shows that adherent cells (>95% macrophages) produced thromboplastin whereas non-adherent cells (>95% lymphocytes) did not. However, the few adherent lymphocytes were apparently essential to the production of thromboplastin, as shown by the effects of adding anti-theta antibody and complement to the cultures. Moreover, the formation of thromboplastin was greatly enhanced by the presence of endotoxin; this effect was also dependent on lymphocytes.

The thromboplastin activity in the cell cultures could be blocked by rabbit anti-human thromboplastin. In a representative experiment, 28% factor Xa (table 1 legend) was generated in a culture stimulated by endotoxin, whereas only 0.3% factor Xa was generated in a parallel incubation with anti-thromboplastin antibody. This experiment provides conclusive evidence that the factor measured in the coupled amidolytic assay employed is indeed thromboplastin and not some other component capable of activating factor X.

4. Discussion

These results, along with those in [3], show that mouse peritoneal macrophages produce all the proteins that constitute the extrinsic blood coagulation system. The activation of this system, expressed by the formation of factor Xa from endogenous factor X [3], requires the presence of thromboplastin. Since factor Xa is secreted to the culture medium [3] whereas the thromboplastin is confined to the cells, it appears likely that the interaction between factor X and thromboplastin is an intracellular process.

Thromboplastin activity has been shown in monocytes [11,12] and pro-coagulant activity in spleen macrophages [2]. Our results agree with [2] in showing a requirement for lymphocytes and a stimulatory effect of endotoxin. However, there is one notable difference. Here, a drastic increase in thromboplastin activity was obtained on adding endotoxin to macrophage cultures containing <5% lymphocytes. By contrast, in [2] showed no stimulatory effect of endotoxin at lymphocyte:macrophage ratios <3:1. While the reason for this discrepancy is unknown, it may be relevant to point out that the sensitivity of our thromboplastin assay exceeds that used in [2] by ~2 orders of magnitude.

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