MACROPHAGES PRODUCE BLOOD COAGULATION FACTORS

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1. Introduction

The vitamin K-dependent coagulation factors are known to be synthesized in the liver [1,2]. We report here that they are also produced by mouse peritoneal macrophages in vitro. This finding suggests a role for mononuclear phagocytes in extravascular fibrin deposition, and may also play a role in thrombosis.

2. Materials and methods

2.1. *Preparation of macrophage cultures*

Macrophages were obtained from hybrid $C_3D_2F_1$ (C3H/Tifo♀ × DBA/2 ♂) mice by peritoneal washing with phosphate-buffered saline without anticoagulant. Cells were routinely cultured in MEM (minimum essential medium with Earle's salts, Gibco Bio-cult, Paisley, Scotland) supplemented with 0.05% (w/v) heat inactivated (100°C, 10 min) lactalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, OH) and 100 IU/ml of penicillin and streptomycin (Gibco Biocult). The cells were either seeded in Costar plastic plates (Costar, Broadway, Cambridge, MA) in 16 mm circular wells on glass coverslips (0.7×10^6 cells seeded/well) or in 100 mm Falcon plastic dishes (20×10^6 cells seeded/dish). After 2 h in culture non-adherent cells were washed away and new medium containing lactalbumin hydrolysate was added. The incubation took place at 37°C in an atmosphere of 5% CO_2 in air. The medium collected after 24 h in culture was centrifuged at 500 × *g* and the supernatant tested for coagulation factors or subjected to chromatography on dextran sulphate–Sepharose for partial separation of the factors.

In the experiments with [^{35}S]methionine, cells were cultures in medium supplemented with 50 μCi [^{35}S]methionine/ml. After 24 h incubation the medium was collected and subjected to chromatography on dextran sulphate–Sepharose column.

2.2. *Dextran sulphate chromatography*

The dextran sulphate column (1.5 × 1.0 cm) was equilibrated with 0.025 M Hepes containing 0.05 M NaCl and 0.4% sodium citrate. After application of the sample, usually 7–10 ml macrophage supernatant, the column was eluted with a linear gradient of NaCl (0.05–0.4 M) in the same Hepes–citrate buffer. One ml samples were collected and tested for clotting activities.

3. Results and discussion

Serum-free medium collected after 24 h macrophage culturing ([3]; see the legend to fig.1 for details) contained significant activities of factors VII, IX, X and prothrombin, as determined by one-stage clotting assays [4]. Provided that factor X was not activated, the concentrations of the various factors remained quite constant from one experiment to another; compared to a reference standard of normal, pooled human plasma the macrophage culture medium contained 8–12% factor VII, 5–8% factor IX, 7–11% factor X and 4–7% prothrombin. However, in some experiments factor X was partially or fully activated to Xa (see below) and in such cultures the level of prothrombin was reduced. In addition to the specific clotting assays the factors were identified by chromatography on dextran sulphate–Sepharose, which yielded the expected elution patterns (fig.1).

To prove that the coagulation factors were synthesized in vitro and not carried over from the animal, cultures were incubated with [^{35}S]methionine in serum free, methionine-free medium. The medium was subsequently fractionated on a column of

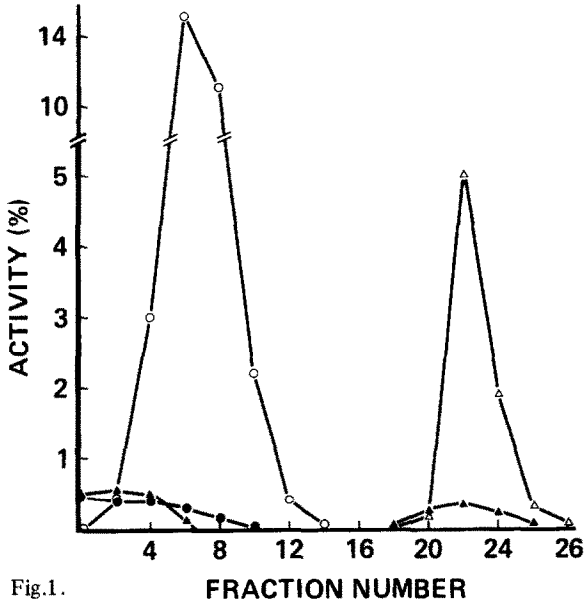

Fig.1.

Fig.1. Chromatography of a macrophage supernatant on a dextran sulphate–Sepharose column. Due to varying amounts of factor Xa in the culture media, antithrombin III, 1 mg/ml final conc., was added and incubated for 60 min at 4°C before assaying for factor IX activity [4]. (○) Factor VII; (●) prothrombin; (△) factor IX; (▲) factor X. Factor X was partially bound to the dextran sulphate-Sepharose as ~70–80% of the activity was recovered in the break-through fraction. All factor Xa together with some native factor X were eluted in fractions 18–25. Prothrombin did not bind as the whole break-through fraction had the same prothrombin activity as the first 4 fractions eluted after applying the NaCl gradient. A normal pooled plasma reference standard was prepared. This was arbitrarily said to contain 100% of factors VII, IX, X and prothrombin.

dextran sulphate–Sepharose, and the fractions containing Xa-activity were analysed further by SDS–polyacrylamide gel electrophoresis [5]. As shown in fig.2, radioactive peptides were found corresponding to M_r 27 000 and 17 000. These two polypeptides, obviously synthesized by the macrophages in vitro

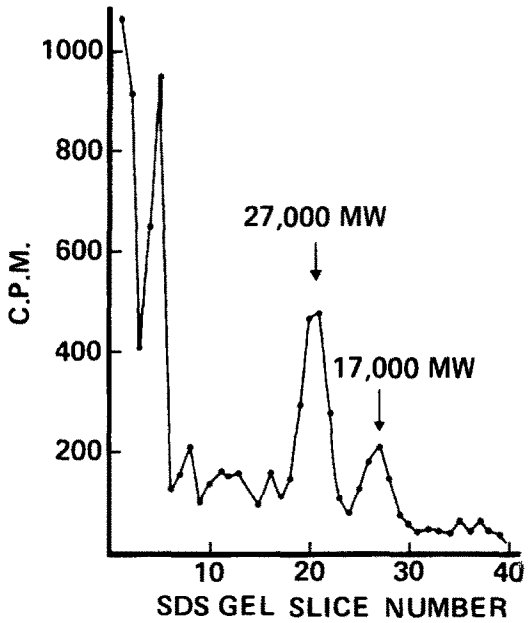

Fig.2. SDS–polyacrylamide gel electrophoresis on factor Xa fractions isolated from macrophage supernatants after incubating the macrophage cultures with [^{35}S]methionine in serum-free, methionine-free medium. Factor Xa was isolated from the macrophage supernatant by utilizing the dextran sulphate–Sepharose column as in fig.1. Fractions containing factor Xa were pooled, concentrated and subjected to SDS–polyacrylamide gel electrophoresis in the presence of a reducing agent, mercaptoethanol, as reported earlier [5]. The gels, 7 cm long were cut into 40–45 slices, and each slice was tested for ^{35}S.

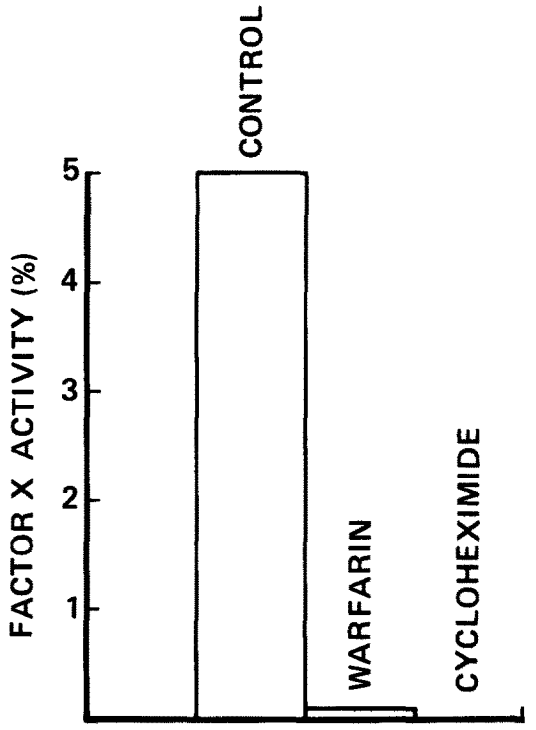

Fig.3. The effect of cycloheximide and warfarin on the synthesis of factor X in the macrophages. The cycloheximide was added to the cultures at 5 μg/ml final conc. Warfarin was added at 12.5 μg/ml final conc. After culturing the macrophages for 24 h, the medium was collected and tested for factor X activity.

are identical in size with the heavy and light chains of the human factor Xa [9].

Coagulation factor activity in macrophage medium was also assayed in cultures with added cycloheximide and warfarin. Fig.3 demonstrates that both cycloheximide and warfarin, a vitamin K antagonist, prevented the appearance of factor X in the culture medium. Similar results were obtained with the other vitamin K-dependent coagulation factors (not shown).

The amounts of activated factor X (Xa) in relation to native factor X varied. This variation appeared to be correlated to the degree of contamination of the cultures with lymphocytes. Pure lymphocyte cultures did not generate X nor Xa activity. Macrophage cultures containing 10% non-adherent peritoneal cells gave total activation of X to Xa, whereas cultures containing ~1% non-adherent cells showed only ~10% conversion of X to Xa. The activation of factor X could be reduced to ~60% by the use of anti-theta antibody and complement [8], control cultures showing complete activation. It thus appeared that normal lymphocytes produced tissue factor or stimulated macrophages to produce such factor. The formation of Xa is then explained as a result of the action of factor VII and tissue factor.

It seems justified to conclude that mouse peritoneal macrophages can produce all the vitamin-K dependent coagulation factors. Together with tissue factor, these represent all the essential factors required to initiate coagulation via the 'extrinsic system'.

Our results explain the finding of procoagulant activity in mononuclear cell suspensions [10]. Here it was demonstrated that stimulated lymphocytes caused a tremendous increase of procoagulant activity when coupling with the macrophages in macrophage cultures.

Acknowledgements

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