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STUDIES ON THE CATABOLISM AND DISTRIBUTION OF FIBRINOGEN IN RATS. APPLICATION OF THE IODOGEN® LABELLING TECHNIQUE.

Willem Nieuwenhuizen[1], Jef J. Emeis[1], Anton Vermond[1]
Piet Kurver[2] and Daan van der Heide[3]

[1]Gaubius Institute, Health Research Organization TNO, Herenstraat 5d, 2313 AD Leiden, The Netherlands

[2]SSDZ, Pathological Laboratory, Reynier de Graefweg 7, Delft, The Netherlands

[3]Department of Endocrinology, University Medical Centre, Wassenaarseweg, Leiden, The Netherlands

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Summary. The iodogen® labelling technique has been applied to rat fibrinogen. Catabolism and body distributions of fibrinogen in the rat have been studied with fibrinogen labelled with 0.5 iodine atoms incorporated/mole of fibrinogen and biologically screened for 48 hours. Seventy-seven percent of fibrinogen body pool appeared to be localized intravascularly and a plasma half-life time of 23 hours has been found. The fractional catabolic rate was found to be 0.96/day under steady-state conditions.

Introduction

For our studies on fibrin(ogen)olysis and disseminated intravascular coagulation we have chosen the rat as an experimental animal.
Relatively little is known about the in vivo behaviour of fibrinogen in rats as compared with other species such as man and rabbit. Investigations in the rat have mainly focussed on determinations of plasma half-life times and values ranging from 24 to 41 hours have been reported (1-7).

For studies on fibrinogen catabolism and distribution a satisfactory preparation of tracer-labelled fibrinogen is required. The quality of labelled fibrinogen is determined by two steps in its preparation i.e. fibrinogen purification and the labelling procedure. In the former step care must be taken to avoid denaturing conditions and conditions which cause partial proteolysis of fibrinogen. In the rat this is a special problem as we have

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described in detail (8). Also the labelling procedure is a possible source of denaturation. A number of radioiodination methods have been proposed, including iodine monochloride (9), chloramine T (10), lactoperoxidase (11) and an electrolytic method (12). For fibrinogen the iodine monochloride method is generally used as it is thought to be the least harmful method. The number of iodine atoms incorporated per fibrinogen molecule is usually taken as 0.5 or less since McFarlane (9) has demonstrated that a higher number results in damage to the molecule which is evident from the abnormally rapid removal from the circulation following injection.

Recently, Fraker and Speck (14) proposed a sparingly soluble chloroamide, 1,3,4,6 tetrachloro-3α,6α-diphenylglycoluril (iodogen®) as a relatively harmless solid-phase reagent for iodination of proteins. They investigated the extremely simple iodination procedure with this reagent as a possible alternative for the iodine monochloride method for the iodination of IgG's. As the labelling procedure applied to rat fibrinogen gave excellent preparations in our pilot experiments, we chose this method for the planned study of rat fibrinogen catabolism and distribution.

We have determined plasma half-life time of rat fibrinogen, distribution over intra- and extravascular space, fractional catabolic rate and flux constants. The results of these determinations are reported in this paper.

Materials and methods

$Na^{125}I$ was purchased from The Radiochemical Centre Ltd. (Amersham, England) and 1,3,4,6 tetrachloro-3α,6α-diphenylglycoluril (iodogen®) from Pierce Chemical Company (Rockford, Illinois, USA). Rat fibrinogen, more than 95% clottable, free from factor XIII and plasminogen, was prepared as described before (8).

Iodination: Fibrinogen was iodinated essentially as described for rabbit IgG by Fraker and Speck (14). Two hundred µl of a solution of iodogen (2 mg/-100 ml methylenechloride) were transferred to each of six conical glass 3 ml-vessels (Reacti-vials®, Pierce, Rockford, Ill., USA). Methylenechloride was evaporated at room temperature under a stream of dry nitrogen. The vessels were closed and kept at room temperature until used on the same day. In a separate vessel were mixed: 200 µl rat fibrinogen solution (11.3 mg/ml of 0.15 M NaCl, i.e. approximately 7 nmoles of fibrinogen) 1.7 ml of borate buffer (0.125 M borax, 0.075 M NaCl, pH 8.2), 100 µl potassium iodide (5.5

mg/l of borate buffer solution) to give an iodine-to-fibrinogen ratio of 0.5, and 20 µl $Na^{125}I$ solution containing about 0.1 µg iodide (70 mCi/ml, 16.6 mCi/µg I).

In some experiments, the ratio of iodine to fibrinogen was chosen as 1 or 8. All steps were carried out between 0°C and 4°C. The mixture was transferred to the first iodogen-coated vessel and stirred. After ten minutes a 50 µl sample was taken for clottability and iodine incorporation assays. This was repeated after another ten minutes. Then the reaction mixture was transferred to the second iodogen-coated vessel and the same process of sampling and transfers was repeated through the sixth vessel. Then the mixture was dialysed against four changes of non-radioactive sodium iodide (10 grams per liter of 0.15 M NaCl) and finally against 0.15 M NaCl.

Clottability: The ten-minutes samples were diluted each with two milliliters of non-labelled fibrinogen solutions (1 mg/ml 0.15 M NaCl) and divided into two equal portions; to one was added 1 ml 20% (w/v) trichloroacetic acid, to determine the percentage of protein-bound iodine, to the other 100 µl thrombin solution (100 NIH units/ml) was added and it was allowed to clot for 30 minutes at 37°C. The clot was collected on a glass rod, washed and the percentage clottable radioactivity determined.

In vivo experiment:

Biological screening. One ml of a solution of iodinated fibrinogen (0.5 atoms of iodine/mole fibrinogen; 0.5 mg fibrinogen per ml, 55 µCi per mg fibrinogen) was injected into each of two male Wistar rats weighing approximately 350 grams. These rats were given 1% (w/v) potassium iodide in their drinking water for two days before the start of the experiment onwards. Fourty-eight hours after injection the animals were bled as described (8), blood was collected in plastic syringes containing a citrate solution (0.13 M, 1 volume per 9 volumes of blood). Plasma was prepared as quickly as possible, divided into portions and injected into the experimental rats.

Measurement of the time course of plasma radioactivity: Into each of 15 male Wistar rats weighing between 200 and 250 grams, 0.5 ml (about 790,000 cpm) of biologically screened fibrinogen was injected into the dorsal vein of the penis, under Nembutal anaesthesia. These rats had been given 1% potassium iodide in their drinking water starting 48 hours before injection and this was continued until the end of the experiment. Ten minutes after injection a 150 µl sample of blood was collected from each animal by aspiration in a calibrated capillary glass tube after making a very small incision in one of the tail veins. A separate sample was used to determine the hematocrit value. From the amount of radioactivity in these samples per ml blood and the hematocrit values the plasma volume was calculated. Blood sampling at 10 minutes intervals was continued in three animals for the first hour. Two more samples were taken under ether anaesthesia: one sample at t = 3 hours and one at t = 6 hours and the animals were bled. Every 24 hours, blood samples from three out of the remaining animals were taken and these three animals bled. This was repeated until the last group of three animals at t = 96 hours. Plasma-fibrinogen was determined by radial immunodiffusion.

Treatment of experimental data: The blood radioactivity at 10 minutes was taken as unit and the activities found at the other times of sampling were expressed as fractions of this first sample. For every group of three animals the averages of these fractions were used for analysis. The tracer data were analyzed using a two-compartment mathematical model as derived by Atencio et al. (16) and as applied by Collen et al. (17) to the human system. Data were fitted with the function applying to this system:

$$x(t) = C_1 e^{-a_1 t} + C_2 e^{-a_2 t}$$

with a DEC PDP 11-34 computer using the method described by Bevington (18) for an iterative non-linear least square fitting. Iteration was stopped when chi-squared and all curve characteristics differed less than 0.001% from the

previous set. Radioactivity distribution ratio between the extravascular (EV) and intravascular (IV) pools (EV/IV), the fractional transcapillary efflux constant (k_{12}), the fractional transcapillary reflux constant (k_{21}) and the fractional catabolic rate constant (k_{10},p) were calculated from C_1, C_2, a_1 and a_2 (Collen et al., 17).

Results and discussion

Labelling procedure: When the amount of iodine incorporated in fibrinogen was followed with time, it appeared that the iodogen-induced reaction levelled off after 10 minutes and stopped completely after 20 minutes. At that time 16-22% of the added iodine appeared to be incorporated in the TCA-precipitable protein. With each transfer to a freshly iodogen-coated vessel approximately another 20% was incorporated after 20 minutes, until finally, after the sixth transfer, nearly 100% incorporation was achieved. The final radioclottability and the TCA-precipitable radioactivity after dialysis were at least 95%. These values did not change significantly with the iodine-to--fibrinogen ratio, since 8 iodines incorporated per fibrinogen molecule gave the same values for radioclottability and TCA-precipitable radioactivity as a 1 or 0.5. SDS-PAGE electrophoresis showed the normal pattern of undamaged Aα, Bβ and γ-chains (8).

In vivo experiments: In preliminary experiments it was found that the initial phase of clearance of labelled fibrinogen (0.5 I/fibrinogen) that had not been screened biologically differed somewhat from that of screened fibrinogen. Material screened for 48 hrs did not differ from 24 hrs-screened material.

A complicating factor appeared to be the fact that fibrinogen is an acute--phase reactant, i.e. its concentration in blood increases greatly upon tissue damage e.g. by operation. In our initial experiments we took blood samples from the same animals over a period of 96 hours by cutting small pieces from their tail-ends. It was observed that the fibrinogen concentration in plasma increased from 2.5 to 4 mg/ml in 96 hours. As such changes in fibrinogen pool may interfere with calculations on fibrinogen behaviour in blood, it was decided to take blood from groups of animals which were damaged only

Table 1

Rat fibrinogen system parameters

(Average of five independent determinations ± SD)

parameter	value found ± SD	parameter	value found ± SD
C_1	0.75 ± 0.05	$k_{12}(h^{-1})$	0.056 ± 0.014
C_2	0.25 ± 0.03	$k_{21}(h^{-1})$	0.22 ± 0.06
$a_1(h^{-1})$	0.030 ± 0.002	$k_{10,p}(h^{-1})$	0.039 ± 0.013
$a_2(h^{-1})$	0.29 ± 0.07	EV (%)	77 ± 3
$T_{\frac{1}{2}}(h^{-1})$	23 ± 1	IV (%)	23 ± 3

once (at t = 10 minutes) by a small incision in their tail veins. Under those conditions the fibrinogen level remained constant (2.5 ± 0.3 mg/ml). The fibrinogen system parameters found with the finally chosen experimental set-up (48 hrs screened fibrinogen, 0.5 I/fibrinogen and groups of animals bled at timed intervals) are summarized in the table.

Preparations labelled at a ratio of 1 or 8 iodine atoms incorporated per molecule had a good clottability (95%) and an approximate T 1/2 of 24 h. Closer investigation of the ln x_t versus t curves, however, showed that these curves were not linear for the preparations with 1 or 8 atoms iodine incorporated per molecule of fibrinogen. We concluded from this that a good clottability and a "correct" half-life time are not enough to qualify rat fibrinogen preparations as satisfactory. A minimum deviation from linearity of the ln x_t versus t plot after 24 hours seems to be required together with a minimum value for EV/IV.

Our results show that the fractional catabolic rate of fibrinogen in the rat is 0.039 ± 0.003/hour i.e. 0.96 ± 0.07/day. This indicates that in the rat under steady-state conditions about 95% of the amount of fibrinogen present in the plasma pool is catabolized per day and also replaced by newly synthesized fibrinogen. We calculated that in our rats the average blood

volume is 6.7 ± 0.3 ml per 100 grams body weight. The average hematocrit values were 41 ± 2%. Thus, the plasma fibrinogen pool is 105 ± 15 mg/kg body weight since the plasma fibrinogen concentration appeared to be 2.5 ± 0.3 mg/ml. Our values are compatible with data of Owens and Miller (19). From these data a synthesis rate of about 100 mg/day/kg body weight can be calculated. Heyes et al. (4) found a value of 78 mg/day/kg body weight. Our data are somewhat higher than synthesis data by Jejeebhoy et al. (20) for isolated liver cells. They found a rate of 64 mg/day/kg body weight. Ruckdeschel et al. (7) found a value of 65 mg/day/kg body weight.

The plasma half-life time of our preparations is in agreement with those reported by Heyes et al. (4), Hilgard et al. (5) and Ruckdeschel (7) but is shorter than those reported in refs. 1, 2 and 3. As pointed out by McFarlane (13) this discrepancy may be due to re-utilization of the isotope, when ^{14}C labelled amino acids are used (1) or to the presence of small amounts of longer-lived material.

It must be noted that the radioclottability of fibrinogen after 96 hours in the rat-circulation had decreased from 95% to 90%. This might indicate the presence of non-clottable fibrin or fibrinogen degradation products gradually formed in the circulation.

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