A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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EFFECT OF GAMMA IRRADIATION ON BLOOD PLASMA FIBRINOGEN

The effect of gamma irradiation on clotting of plasma fibrinogen has been investigated. Critical alterations of mechanical properties of the fibrin clot formed both from blood plasma and isolated fibrinogen (^{60}Co-source), were identified by thrombelastography. The dose-response (0-3 KGy) analysis of the polypeptide composition of purified fibrinogen showed the gradual disappearence of the Aα chain. This polypeptide was the most sensitive one to ionizing irradiation and was probably directly involved in the aggregation of fibrinogen molecules. This was not the case when whole plasma was subjected to irradiation.

Introduction

Irradiated fibrinogen exhibits the enhanced tendency to form insoluble aggregates and this phenomenon is probably associated with the loss of its clottability [1-5].

The studies reported here have been directed towards an evaluation of the nature of post-irradiation alterations of fibrinogen structure and properties. We attempted to correlate alterations of fibrinogen subunits of fibrinogen and fibrin with the loss of clotting properties of blood plasma and purified fibrinogen after irradiation.

Materials and methods

Human citrated plasma was employed. Freshly collected by venipuncture, 9 parts of blood were drawn into tubes containing 1 part of 3.8% sodium citrate.

Fibrinogen was isolated from fresh human plasma by the cold ethanol precipitation technique [6] and was further purified by precipitation with ammonium sulfate at 26% of saturation. Fibrinogen preparations dissolved in 0.14 M NaCl containing 0.01 M phosphate buffer, pH 7.1 (PBS), were 97% coagulable by thrombin and did not show any contamination with other plasma proteins as judged by SDS polyacrylamide gel electrophoresis [7].

Treatment of blood plasma and fibrinogen. Aliquots of the same human plasma fibrinogen solutions (5 mg/ml, 10 mg/ml, 15 mg/ml) in PBS were subjected to irradiation. The irradiation was carried out in a Gamma Cell at different doses (0.25, 0.50, 0.75, 1.0, 2.0 and 3.0 KGy) at a dose rate of 0.90 KGy per hour while keeping the temperature at 4°C. The tubes were kept under the ^{60}Co source with air as gas phase.

Thrombelastography. The Hellige thrombelastograph was used in these experiments. Three-tenths of citrated plasma was pipetted into the cells and after 5 min. (for temperature equilibration) 0.06 ml of $CaCl_2$ solution at concentration 1.29% was added. The reagents were mixed by rapidly moving the piston up and down six times before finally lowering it into clotting mixtures. The thrombelastographic recordings were allowed to proceed for 90 min. Although the results were quite reproducible, one of the three cells always contained a control clotting mixture (unirradiated sample of the same blood plasma) in each experiment. Thrombelastographic tracings were recorded and the following parameters were determined: reaction time (r), clot formation time (k), maximal amplitude (MA) and elasticity of fibrin gel (ME) [8].

Fibrinogen concentrations of plasma samples before and after irradiation were determined. Plasma was clotted by thrombin. Fibrin clots were then exhaustively washed out with PBS and dissolved in 0.1 M NaOH. The content of clotted protein in both cases was estimated by a microbiuret method [9].

Thrombin clotting time of irradiated fibrinogen was determined spectrophotometrically at 600 nm [10].

Preparation of fibrin from irradiated plasma. Noncrosslinked fibrin was formed by thrombin action on blood plasma in the presence of excess EDTA. One hundred µl of plasma was mixed with 100 µl of 0.5 M EDTA, pH 7.1 and then 10 µl of thrombin (40 NIH-u/ml) was added. After the clot was formed it was washed with PBS and

dissolved in 0.1 M phosphate buffer, pH 7.1 containing 8 M urea and 1% SDS [11]. Polyacrylamide gel electrophoresis was performed in 5% and 7% gels in the presence of 0.1% SDS [7]. Samples of fibrinogen and fibrin were reduced with 2% 2-mercaptoethanol for 3 hours at 37°C. Gels were stained with Coomassie Brillant Blue.

Results

Irradiation of purified fibrinogen. The isolated fibrinogen dissolved at different concentrations in PBS was exposed to gamma irradiation. The induced alterations of its subunit structure were controlles by the analysis in SDS polyacrylamide gels. Electrophoretic patterns of irradiated fibrinogen are presented in Fig. 1.

When unreduced irradiated fibrinogen was run, high molecular weight aggregates appeared at the top of the gels. These were not dispersed even in the presence of 8 M urea and 1% SDS. The quantity of aggregated proteins increased with the dose up to 3 KGy. However, no obvious changes in the fibrinogen solution could be observed. After reduction of disulfide bonds in fibrinogen with 2-mercaptoethanol there was a gradual loss of the Aα chain in the total composition. Following exposure of fibrinogen to 3 KGy there was almost a complete lack of the Aα chain in the molecule and only the Bβ and γ chains were detectable in the gels. Since SDS gel electrophoresis of the same but unreduced samples of fibrinogen, has not revealed the presence of plasmic fragments, this may suggest that a disappearence of the Aα chain is not caused by proteolytic degradation. Therefore we presumed that Aα polypeptide may be directly involved in the formation of fibrinogen aggregates which are well visible on gels (Fig. 1) even after reduction with 2-mercaptoethanol. Alterations in subunit structure of the molecule were associated with the loss of polymerizing activity of fibrinogen. Figure 2 shows a plot of the polymerization activity against the time of incubation of fibrinogen with thrombin. Fibrinogen was irradiated in the range 0.75-2.0 KGy. The dose above 3.0 KGy caused a complete loss of the ability of fibrinogen to polymerize.

Irradiation of fibrinogen in human plasma. The effect of gamma irradiation on clotting of fibrinogen in human plasma by recalci-

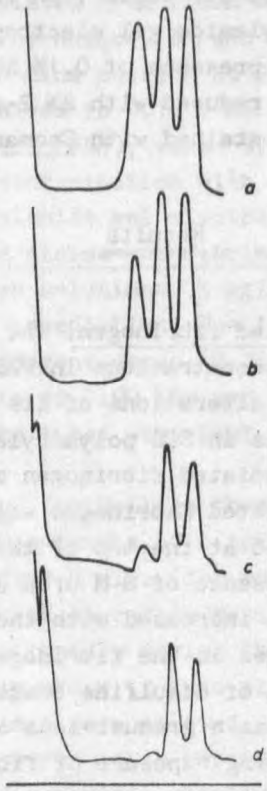

Fig. 1. Densitometric scans of SDS polyacrylamide gels of control fibrinogen (a) and fibrinogen exposed to 1 KGy (b), 2 KGy (c) and 3 KGy (d). Gels were stained with Coomassie Brillant Blue and after destaining they were scanned at 560 nm

Wykresy densytometryczne żelów poliakryloamidowych po rozdziale kontrolnego fibrynogenu (a) oraz fibrynogenu napromienionego dawką 1 KGy (b), 2 KGy (c) oraz 3 KGy (d). Żele barwiono błękitem Coomassie a po odbarwieniu densytometrowano przy długości fali 560 nm

Денситометричные графики полиакрыламидных гелей после разделения контрольного фибриногена (а) и фибриногена облученного дозой 1 KGy (b), 2 KGy (c) и 3 KGy (d). Гели крашеные лазурью Coomassie а после обесцтветения денситометрированы при длине волны 560 nm

fication, as recorded by thrombelastographic measurements, is shown in Fig. 3 and Tab. 1.

It can be seen that gamma irradiation reduces significantly the thrombelastography maximum amplitude. This suggests that there is a significant reduction of clot strength when plasma is exposed

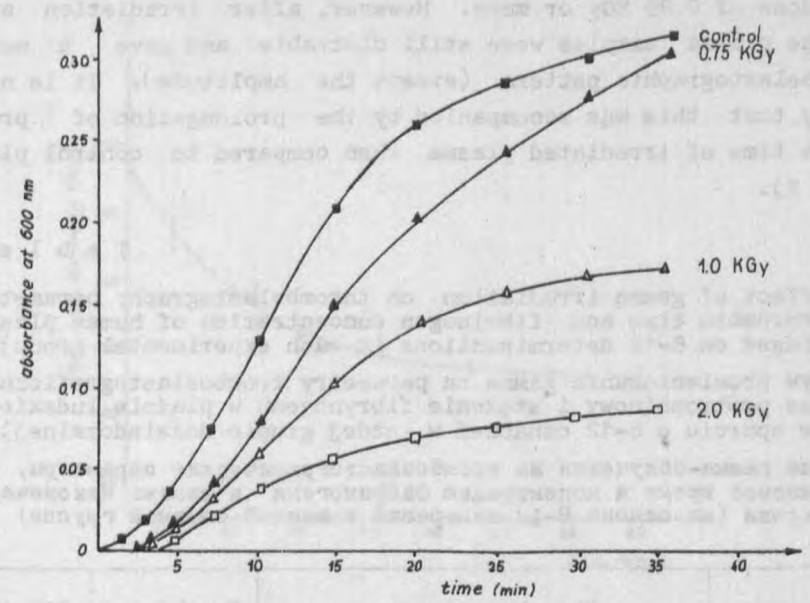

Fig. 2. The polymerization of irradiated fibrinogen induced by thrombin action

Polimeryzacja napromienionego fibrinogenu indukowana działaniem trombiny

Полимеризация облученного фибриногена индуцирована действием тромбина

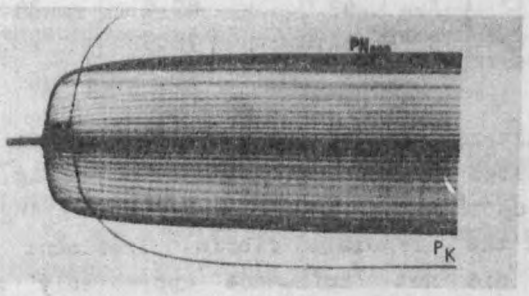

Fig. 3. A typical thrombelastograpny recording of control plasma (P_K) and after its exposure to 3 KGy (P_{300})

Zapisy tromboelastograficzne wykrzepiania kontrolnego fibrynogenu (P_K) oraz napromienionego dawką 3 KGy (P_{300})

Тромбоэластографичные высвёртывания контрольного фибриногена ($P_к$) и облученного дозой 3 КGy (P_{300})

to a dose of 0.25 KGy or more. However, after irradiation at 3 KGy the plasma samples were still clottable and gave a normal thrombelastographic pattern (except the amplitude). It is noteworthy that this was accompanied by the prolongation of prothrombin time of irradiated plasma when compared to control plasma (Tab. 1).

Table 1

The effect of gamma irradiation on thrombelastography parameters, prothrombin time and fibrinogen concentration of human plasma (based on 8-12 determinantions in each experimental group)

Wpływ promieniowania gamma na parametry tromboelastograficzne, czas protrombinowy i stężenie fibrynogenu w plaźmie ludzkiej (w oparciu o 8-12 oznaczeń w każdej grupie doświadczalnej)

Влияние гамма-облучения на тромбоэластографические параметры, протромбиновое время и коцентрацию фибриногена в плазме человеческой крови (на основе 8-12 измерений в каждой опытной группе)

Dose	Thrombelastogram				Prothrombin time	Fibrinogen mg/100 ml
	r	k	MA	ME		
Control	12	2	68.5	217.5	16.5	257
0.25 KGy	12	2	64.0	117.7	20.9	255
0.75 KGy	12	2	58.0	114.0	21.5	260
1.0 KGy	12	2	53.0	112.7	23.0	260
2.0 KGy	12	3	47.0	88.6	24.0	257
3.0 KGy	12	3	44.0	78.5	28.0	260

Critical alterations of mechanical properties of the fibrin clot of irradiated plasma are domenstrated in Fig. 4. The exposure to doses up to 1 KGy brought about a striking loss of the elasticity of the irradiated fibrin. Treatment of plasma with higher doses did not influence appreciably the mechanical strength of the resultant gels. The elasticities remained practically unchanged (Fig. 4).

Since the mechanical strength of the fibrin gel following irradiation at 1 KGy reached only about 50% of the maximum observed for control plasma, experiments were attempted to explain this phenomenon. First of all, there was no evidence for varia-

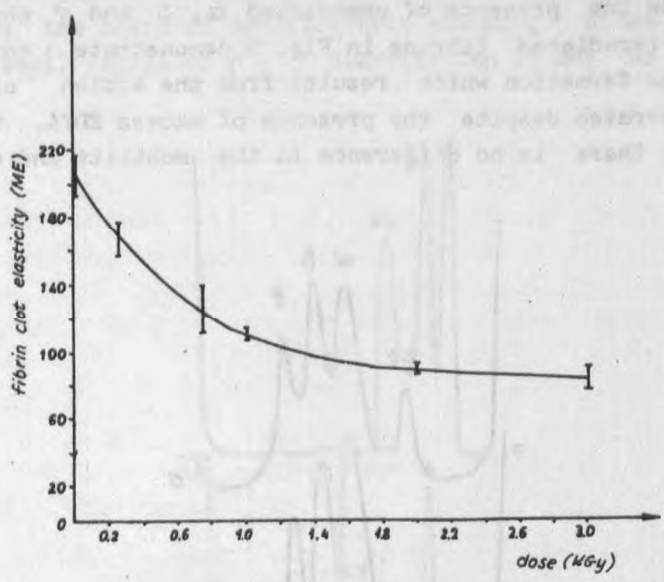

Fig. 4. The effect of gamma irradiation on the elasticity of fibrin gels formed from plasma exposed to doses of 0.25-3 KGy. The elasticity was calculated as standard parameter ME from thrombelastographic recordings (mean values were calculated from 10 determinations)

Wpływ promieniowania gamma na elastyczność żelu włóknika utworzonego z osocza krwi napromienionego dawkami 0.25-3 KGy. Elastyczność żelu wyliczano jako parameter ME z zapisów tromboelastograficznych (są to wartości średnie 10 oznaczeń)

Влияние облучения гамма на эластичность геля фибрина возникшего из плазмы крови облученной дозами 0,25-3 КGy. Эластичность геля высчитано как параметр ME из тромбоэластографичных записей (это средние величины 10 обозначений)

tions in fibrinogen concentration of irradiated blood plasma (Tab. 1). This seems to indicate that changes in the quantity of coagulable protein do not provide a basis for explaining the large differences observed in fibrin gel rigidity. To study this further, cross-linked and noncross-linked fibrin were prepared from control and irradiated plasma. These were next analysed by SDS polyacrylamide gel electrophoresis to characterize the dose--dependence of the changes in subunit structure of the molecule. The results of these analysis are presented in Fig 5. The gels for noncross-linked fibrin obtained from irradiated and from control

plasma show the presence of unmodified α, β and γ chains. The scans for irradiated fibrins in Fig. 5 demonstrate considerable γ - γ dimer formation which results from the action of Factor XIIIa, generated despite the presence of excess EDTA. This also shows that there is no difference in the mobility and concentra-

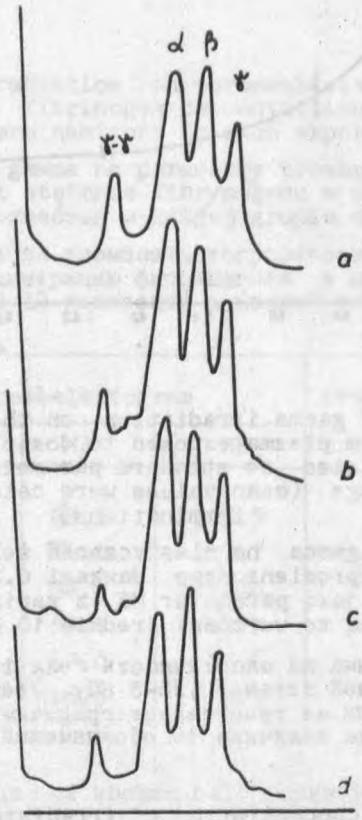

Fig. 5. Densitometric scans of SDS polyacrylamide electrophoresis gels of reduced, noncross-linked fibrin isolated from control human plasma (a) and from plasma irradiated at 1 KGy (b), 2 KGy (c) and 3 KGy (d)

Wykresy densytometryczne żelów poliakrylamidowych po rozdziale zredukowanego włóknika niestabilizowanego otrzymanego z ludzkiego osocza kontrolnego (a) oraz z próbek osocza napromienionych dawkami 1 KGy (b), 2 KGy (c) oraz 3 KGy (d)

Денситометричные графики полиакрыламидных гелей после разделения сокращенного нестабилизованного фибрина полученного из человеческой контрольной плазмы (a) и из пробы плазмы облученной дозами 1 KGy (b), 2 KGy (c) и 3 KGy (d)

tion of constituent chains of fibrin of irradiated plasma as estimated on the basis of densitometric scanning. Since these gels showed a small degree of γ - γ dimerization it may be suggested

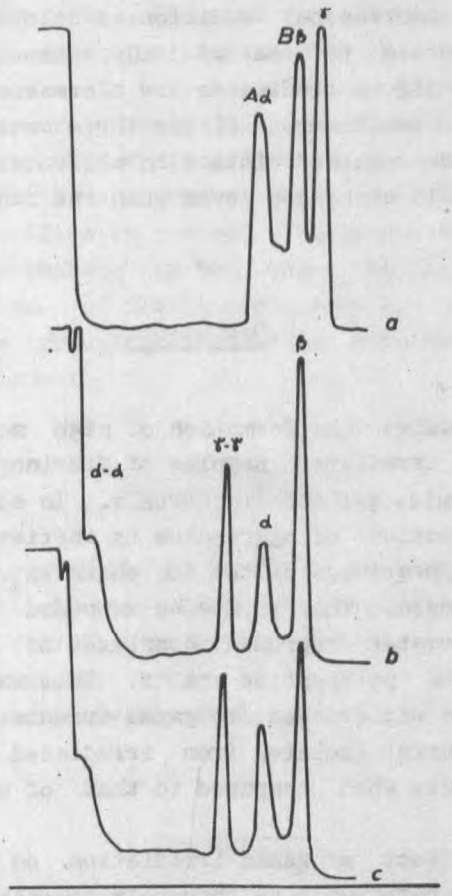

Fig. 6. Densitometric scans of SDS polyacrylamide gels of reduced human fibrinogen (a), reduced, cross-linked fibrin prepared from control plasma (b) and from irradiated plasma at 3 KGy (c)

Wykresy densytometryczne żelów poliakrylamidowych po rozdziale zredukowanego fibrynogenu ludzkiego (a), zredukowanego włóknika stabilizowanego otrzymanego z osocza kontrolnego (b) oraz z osocza napromienionego dawką 3 KGy (c)

Денситометричные графики полиакрыламидных гелей после разделения сокращенного человеческого фибриногена (a), сокращенного стабилизованного фибрина полученного из контрольной плазмы (b) и из плазмы облученной дозой 3 KGy (c)

that gamma irradiation even at as high a dose as 3 KGy does not damage Factor XIIIa nor impair its function.

Figure 6 shows the electrophoretic patterns on SDS gels of clots formed from plasma following gamma irradiation. The clots were made by the simultaneous addition of calcium ions to the plasma samples exposed to doses of 3 KGy. The control pattern shown in the same figure indicates the formation of cross-linked γ-γ dimers and α-multimers. Of the three constituent chains, only β polypeptide remained intact in all cases. The ability to form α-polymers is unchanged even when the dose 3 KGy was applied.

Discussion

In present studies the formation of high molecular weight aggregates in the irradiated samples of fibrinogen was evidenced by SDS polyacrylamide gel electrophoresis. In addition it was observed that formation of aggregates is initiated at the same dose as the disappearence of the Aα chain in reduced samples of irradiated fibrinogen. Thus it may be supposed that the irradiation-induced aggregates represent complexes of fibrinogen molecules linked by Aα polypeptide chains. This was not the case when whole plasma was exposed to gamma irradiation. Polypeptide composition of fibrin isolated from irradiated plasma did not show any differences when compared to that of unirradiated plasma.

The initial effect of gamma irradiation on clotting of plasma fibrinogen is detectable by thrombelastography. Since samples of the same plasma were always subjected to irradiation, a large number of factors which can influence the thrombelastographic tracings were in the same concentration. Hence the interpretation of changes in thrombelastography should not be ambiguous. Gamma irradiation of blood plasma caused the modification of thrombelastographic parameters similar to that observed by T y l e r [12] for decreased activity of Factor XIIIa. However, complete γ-dimerization and almost complete α-polymerization could be observed on polyacrylamide gel electrophoresis when sam-

ples of cross-linked fibrin obtained from plasma exposed at 3 KGy were run (Fig. 6). Therefore these modifications of the solidity of the clot after irradiation cannot be explained by an inactivation of Factor XIIIa.

In agreement with others [5] our experiments demonstrated that due to the presence of a large number of disulfide bridges the fibrinogen molecule is very sensitive to irradiation. However damaging effect on structure and biological activity of fibrinogen was much smaller when whole plasma instead of isolated protein was irradiated. It may suggest that some plasma components can somehow nonspecifically protect fibrinogen to gamma irradiation effects. Nevertheless in both cases the general changes in mechanical properties of fibrin clots were the same. The fibrin gels produced from irradiated substrates were more transparent and significantly less rigid.

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WPŁYW PROMIENIOWANIA GAMMA NA FIBRYNOGEN OSOCZOWY

W pracy badano wpływ promieniowania gamma na wykrzepianie fibrynogenu osoczowego. Za pomocą tromboelastografii wykryto duże zmiany we właściwościach mechanicznych skrzepu włóknika, otrzymanego z osocza krwi lub z wyizolowanego fibrynogenu, poddanych przed wykrzepieniem działaniu promieni jonizujących (^{60}Co). Analiza składu polipeptydowego cząsteczki fibrynogenu, napromienionej różnymi dawkami (0-3 KGy), wykazała stopniowy ubytek zawartości łańcucha A alfa. Ta podjednostka okazała się być najbardziej wrażliwą na działanie promieniowania jonizującego i prawdopodobnie bezpośrednio bierze udział w agregacji cząsteczek fibrynogenu.

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ВЛИЯНИЕ ГАММА ОБЛУЧЕНИЯ НА ПЛАЗМАТИЧЕСКИЙ ФИБРИНОГЕН

В настоящей работе исследовано вдияние гамма облучения на свёртывание плазматического фибриногена. Показано, что наиболее чувствительной подъединицей молекулы фибриногена на действие ионизирующего излучения является цепь Aα. Принимает она непосредственное участие в образовании высокомолекулярных агрегатов в облученных препаратах фибриногена. После облучения, фибриноген образует сгусток, которой очень отличается механическими свойствами от сгустка возникшего из контрольного фибриногена.