CELL STRUCTURE AND FUNCTION 16: 17–22 (1991)

Target Size for a Fibronectin-Cell Adhesion System Determined by the X-ray Inactivation Method

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Key words: fibronectin/target size/X-ray inactivation method/cell adhesion

ABSTRACT. **In order to elucidate the mechanism of cell adhesion, the size of the functional site, both in the fibronectin molecule and in the mouse fibroblast cell, responsible for cell adhesion activity, was determined. The size was assumed to be equivalent to the target size, that can be determined from the X-ray inactivation dose. The target size of the cell-binding site in the fibronectin molecule was 32 kdalton. The molecular weight was much larger than that of the tripeptide, which has been reported to be the minimum peptides having a cell-binding activity. This suggests that submolecular regions in fibronectin other than the tripeptide are necessary for cell adhesion. The target size in the cell responsible for the adhesion to the fibronectin-coated surface was 4300 kdalton. The large molecular weight of the target could be explained by assuming that a complex protein system is involved in the cell-adhesion process in the cell.**

Interaction between cells and extracellular matrices are known to play indispensable roles in both cell motility and cell migration, as can be observed during embryonic development (23) or the healing of wounds (3). Cell adhesion to extracellular matrices would be an initial step in these inter-cellular interactions. Among the molecules included in extracellular matrices, fibronectin, a glycoprotein, has been intensively studied and well characterized in view of the relationship between the cell-adhesion function and the molecular structure. This molecule is reported to have several submolecular domains (1, 13, 24), each of which can bind specifically with extracellular matrices or to cells (6, 25). Recent studies on the adhesion of the cells to fibronectin have revealed two important facts. The first is that there is a fibronectin receptor located on the cell surface. It was identified and isolated to be a glycoprotein having a molecular weight of about 140 kdalton (21). The second fact is that a tripeptide (Arg-Gly-Asp, RGD) in fibronectin is necessary for binding to the cells (18). Although these approaches have provided knowledge concerning the necessary elements involved in the system, there still remains much to be studied from various aspects in order to understand the whole process. For instance, the roles of the submolecular regions in fibronectin other than the RGD sequence, as well as the molecules in the cell other than the receptor, need to be clarified since the cell-adhesion activity of fibronectin is known to be modified by other molecules when bound to fibronectin (10). Our previous report showed that there exists an inter-domain interaction in the fibronectin molecule by the fluorescent depolarization method (14), suggesting the occurrence of both intra- and intermolecular interactions in the adhesion system.

In our recent work we have determined, by inactivating cell adhesion with X-rays, the target sizes of the functional site in the fibronectin molecule and of the molecular system in the cell, both of which are indispensable for the fibronectin cell-adhesion activity. This method has been used since the 1950's to measure the molecular weight of enzymes (20). One of the features of this method is that the target weight can be obtained irrespective of the biochemical nature of the target: namely, the submolecular domain, or multi-molecular system. This method is therefore the best (and perhaps only) method for determining the size of a system whose components have not been completely identified.

We have examined the inactivation of fibronectin under both dry and wet conditions, and estimated, by comparing the results of the two cases, the contribution of the indirect action of radiation through water radicals. Under wet conditions, including living cells, most of the radiation energy is absorbed by water molecules, and the produced radicals contribute greatly to the inactivation of biological functions. The molecular nature of the target for the cell-adhesion activity in the fibronectin molecule and of the target in the cell responsible for adhesion to the fibronectin-coated surface is dis-

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cussed.

MATERIALS AND METHODS

Preparation of fibronectin. Fibronectin was purified from a porcine plasma according to a method proposed by Hayashi and Yamada (5). Briefly, the plasma was applied to a Sepharose CL-4B column; the flow-through fraction was then applied to a gelatin-affinity column. Fibronectin trapped in the column was collected with 4M urea; then, the urea was removed by dialysis. The molecular weight of the obtained protein was determined by SDS-pôlyacrylamid gel electrophoresis. It coincided with the reported value of the fibronectin monomer (230 kdalton) (6). Contamination was less than 3%. The concentration of the fibronectin fraction was determined by assuming the absorption coefficient of fibronectin to be 1.28 ml/mg/cm (15). Fibronectin, thus prepared, was suspended in 0.13 M NaCl, 10 mM Tris HCl buffer, pH 7.0 (buffer A), at the concentration of 1 mg/ml and stored at −20°C until use.

Non-covalent coating of glass slide with fibronectin. The fibronectin solution (50 μg/ml) with a volume of 40 μl in buffer A was applied over an area of 15 mm × 15 mm on a glass slide, and then dried in air. Slides coated with bovine serum albumin (BSA), which does not show any cell-adhesion activity, were prepared for a comparison. Fibronectin-coated slides stored in a desiccator (humidity less than 5%) for 24 hrs were also prepared in order to estimate the effect of the remaining water molecules in samples during X-ray irradiation.

Cell lines. Mouse embryo fibroblast C3H 10T1/2 cells, originally developed by Reznikoff *et al.* (22), were kindly provided by Dr. Terashima. These cells were cultured in Eagle's basal medium containing a 10% fetal bovine serum (M. A. Bioproducts, USA) supplemented with 40 μg/ml kanamycin. The cells were inoculated into 25 cm^2 culture flasks at 5×10^4 cells/flask and cultured at 37°C in a CO_2 incubator for 10 days until the confluent stage was achieved.

X-ray irradiation. X-ray irradiation was performed with an X-ray generator (RU-200, Rigaku) operating at 30 kV (Cu target), 150 mA. The distance between the Be window and the sample was 33 cm. Although no filter was used, the soft X-ray component was reduced by the air in the beam path. The half-value layer in aluminum was 0.043 mm at the sample position, meaning that the effective energy of the X-rays irradiated onto the sample was 7.5 keV (half value layer in water, 0.58 mm). The exposure rate was 6.1 kR/min (1.6 C/kg/min).

The fibronectin-coated slides, thus prepared, were placed in an irradiation field and irradiated in air. The thickness of the fibronectin on the slide was about 15 μm; this value was larger than the range (about 1 μm) of 7.5 keV electrons (8). A secondary-electron equilibrium was achieved within the sample. The solution of fibronectin was irradiated in a sample chamber made of acryl plates with a thin film window (5 μm thick mylar). The depth of the chamber was 1 mm and the transmittance of 7.5 keV X-ray in 1 mm of water layer was 0.29. Hence, 58% of the exposure at the surface was used as the effective exposure in the sample. The remaining activities were plotted against this value. For the cell-irradiation experiments, 1.3×10^4 plateau-phase cells attached to 35 mm diameter culture dishes were irradiated and used for a cell-adhesion assay.

Cell-adhesion assay. A measurement of the cell-adhesion activity of fibronectin was performed by a method reported by Hahn and Yamada (4). Both irradiated and non-irradiated cells were separately trypsinized, washed twice with phosphate buffer saline (calcium and magnesium free, pH 7.4) supplemented with 2% (w/v) BSA, and resuspended in this medium at a concentration of 1.5×10^4 cells/ml. The cell suspension (100 μl) was applied onto the fibronectin-coated slides, and incubated at 37°C, under 5% CO_2. After incubation for 2 hours, the cells were observed under a microscope and classified into two types: 1) cells which attached to and spread on the fibronectin-coated slide with an obscure and rough boundary, and 2) round cells which did not spread, looking as they did when floating in the suspension. More than 500 cells were morphologically classified and counted in each experiment.

When no X-rays were irradiated onto either fibronectin or the cells, more than 90% of the cells attached to and spread on the fibronectin-coated slide, while less than 1% of the cells became attached to the BSA-coated slide. About 3% of the cells became attached to the non-coated slide. These results showed that fibronectin coated noncovalently on the slide surface keeps its specific activity for cell adhesion. This activity can be quantitatively measured by counting the cells which became attached to and spread on the fibronectin-coated slide. By this method, the adhesion activities between fibronectin and cells were measured under various conditions.

RESULTS

Cell adhesion activity of fibronectin irradiated in dry condition. Fibronectin was irradiated at various exposures of X-rays after being dried on slides. Unirradiated cells were dropped on the irradiated slides and inactivation of adhesion activities were measured. The remaining activity was defined as being the fraction of cells which showed some adhesion activity, and normalized to the value in the experiment with an unirradiated slide. The remaining activity decreased exponentially with an increase in the X-ray exposure (Fig. 1 (a)). E_{37}, the exposure giving 37% of the remaining activity, was 3300 kR. Fibronectin-coated slides dried in a desiccator were also tested. The remaining adhesion activities were the same as those samples dried in air. Adhesion inactivation in dry fibronectin was not affected by the drying conditions.

Cell adhesion activity of fibronectin irradiated in solution. Solutions of fibronectin irradiated with various X-ray exposures were dropped and dried on the slides; the remaining activities were measured with unirradi-

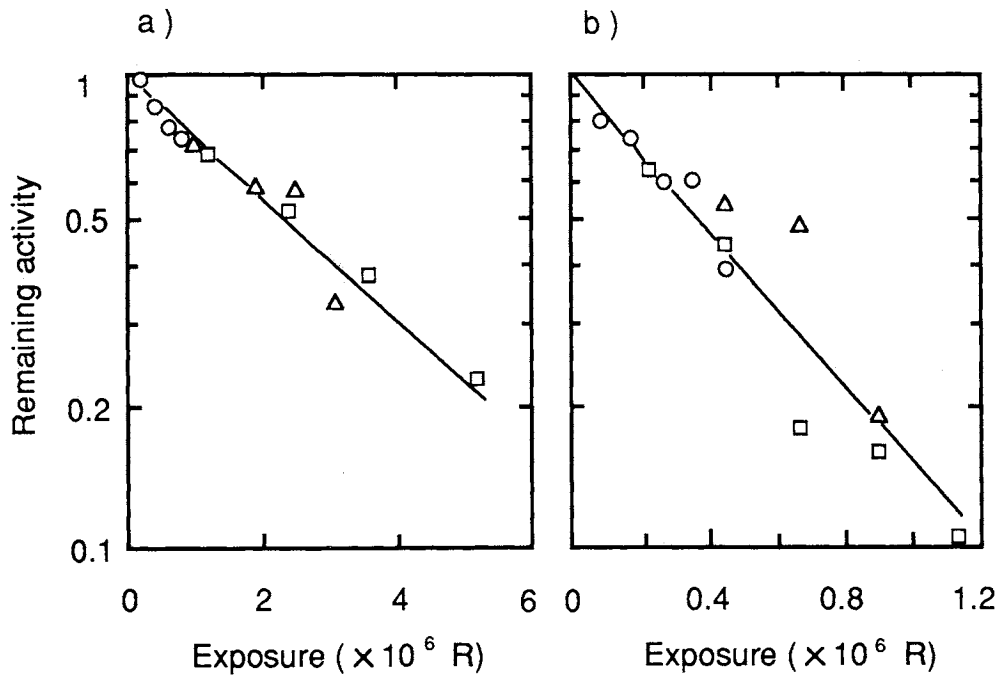

Fig. 1. Inactivation of the cell-adhesion activity of fibronectin irradiated under dry conditions (a) and in the solution (b). Three experiments, designated by different symbols, were carried out: E_{37} in (a) and in (b) were 3300 kR and 533 kR, respectively.

ated cells, and were found to decrease exponentially with an increase in the X-ray exposure (Fig. 1 (b)). E_{37} was 533 kR. This value was about one-sixth of the value obtained when fibronectin was irradiated under dry conditions.

Adhesion activity of X-ray irradiated cells to fibronectin-coated surface. The cells were irradiated at various X-ray exposures, and the remaining adhesion activity of the cells to the surface of a slide coated with unirradiated fibronectin was measured. The remaining activity decreased exponentially with an increase in the X-ray exposure (Fig. 2). E_{37} was 53.1 kR.

Adhesion activity when both fibronectin and the cells were irradiated. The adhesion activities of the irradiated cells to the irradiated fibronectin in solution were also measured in order to examine whether or not an inactivation of the adhesion activity with X-rays occurs independently in the fibronectin molecule and in the cell. If such inactivation occurs independently in irradiated fibronectin and in the irradiated cell, the adhesion activity between them is equal to the product of the remaining activities of the irradiated fibronectin and of the irradiated cells. The remaining activities were measured at different activity levels. First, fibronectin and the cells were irradiated separately with an exposure giving 70% remaining activity: namely, 20 kR for the cells and 1800 kR for fibronectin, as can be seen in Figs. 1b and 2. The obtained adhesion activity between them

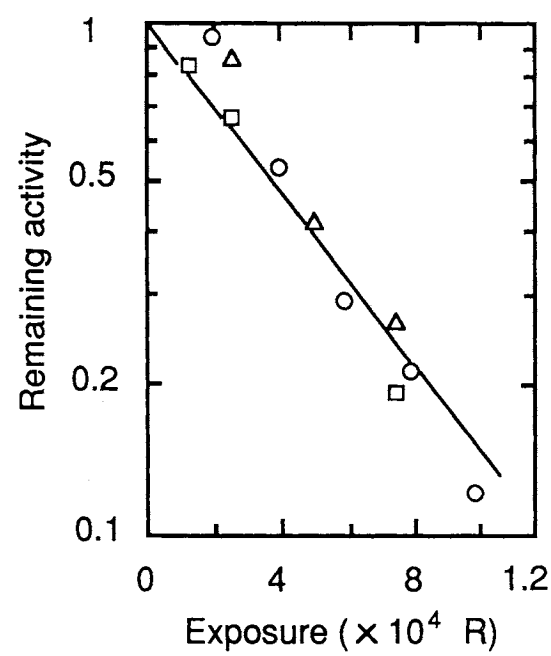

Fig. 2. Inactivation of adhesion activity of a cell to fibronectin-coated glass. Three experiments, designated by different symbols, were carried out. E_{37} was 53.1 kR.

Table I. REMAINING ADHESION ACTIVITY OF THE IRRADIATED CELLS TO THE SLIDE COATED WITH IRRADIATED FIBRONECTIN.

Remaining adhesion activity when the cell or fibronectin was irradiated	Exposure on the cell / Exposure on fibronectin	Observed remaining adhesion activity when both the cell and fibronectin were irradiated	Expected remaining adhesion activity from independent irradiation experiments
0.70	20 kR / 1800 kR	0.50	$0.70\times0.70=0.49$
0.45	44 kR / 4000 kR	0.25	$0.45\times0.45=0.20$

was 0.50. This value agreed with the product of the two survival activities, 0.49 ($=0.7\times0.7$). Secondly, fibronectin and the cells were irradiated at exposures of 44 kR and 4000 kR, respectively, which would give the remaining activities of 45% (Figs. 1b and 2). The obtained adhesion activity was 0.25; this value agreed with the expected value, 0.20 ($=0.45\times0.45$). These results are shown in Table I.

DISCUSSION

We succeeded in quantitatively measuring the inactivation of the fibronectin-cell adhesion activity with X-rays. The obtained inactivation curves were reproducible. The results indicate that the inactivation processes of the adhesion activity of fibronectin and of the cell are independent and, therefore, that the two processes can be discussed independently. In wet systems, such as in a fibronectin solution and in a living cell, it is necessary to consider the contribution of indirect action through water radicals in the method adopted in this paper.

Estimation of the target molecular weights using the inactivation curve. The target mass, M, inactivated by X-ray irradiation, was calculated by applying target theory (19). Briefly, biological functions can be expressed by molecular entities called "targets" and are inactivated by incidents (presumably, ionizations) arising in a target due to radiation. Such incidents arise randomly in a sample located in the radiation field. For simplicity we assume that one incident (called a "hit") arising in the target inactivates its biological function. According to this assumption, the fraction of an unhit target in the sample, S, decreases exponentially with an increase in the absorbed dose, D, (absorbed energy per unit mass). This relationship can be written as

$$S=\exp(-(D/D_{37})),$$

where D_{37} is called the mean lethal (one hit per target on the average) dose and is used as a measure of the inactivation efficiency. At a dose of D_{37} the remaining activity becomes 0.37 ($=1/e$). When one incident occurs in the target at D_{37}, the absorbed energy in the target (weight, m kg), i.e., the absorbed dose in Gy, can be written as

$$D_{37}(\mathrm{Gy})=1.06\times10^{-17}\ (\mathrm{J})/\mathrm{m}\ (\mathrm{kg}),$$

where 1.06×10^{-17} (J) ($=66$ eV) is the energy necessary to produce an incident in the biological system (11). This equation shows the relationship between D_{37} and the target mass. Using experimentally obtained D_{37}, we calculated the target weights under various conditions. The target weights obtained in kg were then converted to the molecular weight, M_0, expressed in dalton,

$$M_0=A\times m\times10^3,$$

where A is Avogadro's number, 6.02×10^{23}.

We converted the exposure E in R to the absorbed dose D in Gy as

$$D(\mathrm{Gy})=8.69\times10^{-3}\cdot\frac{\Sigma\omega_i(\mu_{en}/\rho)_i}{(\mu_{en}/\rho)_{air}}\cdot E(\mathrm{R}),$$

where (μ_{en}/ρ) is the mass energy absorption coefficient and ω_i is the relative mass abundance of the element i in the sample. For 7.5 keV X-rays, $(\mu_{en}/\rho)_{air}$ is 1.13 m^2/kg (9). In the case of a dry sample of fibronectin, the relative mass abundances were C (5.7%), H (1.3%), N (1.7%), O (5.6%), Na (33.7%), Cl (52.1%) and sulfur in the protein was less than 0.001%, which was negligible. The sodium and chlorine came from the buffer solution which was used to dissolve the fibronectin. $\Sigma\omega_i(\mu_{en}/\rho)_i$ of the dry sample and of the fibronectin solution

Table II. TARGET MOLECULAR WEIGHTS ESTIMATED FROM THE INACTIVATION CURVES.

Inactivation activity	Condition of irradiation	D_{37} (kGy)	M_0 (kdalton)	M (kdalton)
Cell adhesion activity of fibronectin	wet	5.2	1240	32
	dry	200	32	
Adhesion activity of cell	wet	0.51	12400	4300

Table III. E_{37} AT DIFFERENT CONCENTRATIONS OF FIBRONECTIN SOLUTION FROM 1 mg/ml TO 0.01 mg/ml.

Concentration of fibronectin solution	E_{37}
1 mg/ml	540 kR
0.5 mg/ml	534 kR
0.01 mg/ml	533 kR

were obtained as 7.79 m^2/kg and 1.26 m^2/kg, respectively. The values of D_{37} obtained from each inactivation curve (Fig. 1 (a), 1 (b) and 2) and the molecular weights of the target, M_0, in dalton, thus calculated, are listed in Table II. The target for cell-adhesion activity of fibronectin under dry conditions was 32 kdalton, smaller than the molecular weight of fibronectin but much larger than that of the RGD tripeptide. The value for fibronectin under wet conditions was 1240 kdalton, about twice the molecular weight of fibronectin. The target for adhesion activity of the cell was as large as 12.4 Mdalton.

Under wet conditions, water radicals, such as OH, produced by X-rays around the target, are actively involved in the inactivation processes of biological functions of the target (as indirect action of irradiation). It is also well known that radiation sensitivity of an enzyme irradiated in solution with ionizing radiation depends on the enzyme concentration in the range from μg/ml to mg/ml (17). The number of inactivated enzyme molecules in the solutions at various enzyme concentrations does not change when the same number of water radicals are produced at a certain dose. The remaining enzyme activity, usually defined as the fractional activity of an unirradiated solution, depends upon the total number of enzyme molecules in the solution before irradiation: in other words, the enzyme concentration of the solution. In our study, however, the concentration dependence was not observed within the range from 0.01 to 1 mg/ml, as is shown in Table III. 10 mM Tris in the buffer must have scavenged almost all of the water radicals; a very small fraction of the radicals was used for the inactivation of fibronectin. This fraction is proportional to the concentration of fibronectin in the solution. Therefore, we could determine the M_0 value in the case of a fibronectin solution, independent of the fibronectin concentration.

Exclusion of the water mass in the obtained target weight. The obtained M_0 under wet conditions includes the mass of the water layer surrounding the true target. The water radicals produced by the X-rays in this water layer contribute to the inactivation of the target. We estimated both the mass and virtual thickness of the water layer by comparing the target mass of the fibronectin molecule obtained under dry conditions with the value under wet conditions. The difference between the target values under dry and wet conditions was 1210 kdalton. This difference was attributed to water molecules spherically surrounding the target, i.e., if the target is assumed to be spherical. The thickness of the water layer was calculated to be 5.7 nm. The thickness can be considered as being the effective diffusion distance of the water radicals. Using this value, we have calculated the mass of the site in the cell responsible for adhesion to a fibronectin-coated slide, which does not include the mass of water. The target molecular weight, M, in Table II was obtained through this process. The value was still as large as 4300 kdalton. Hutchinson *et al.* (7) reported that the thickness of the water layer was almost 3 nm in the case of some enzymes in yeast cells. If we use this value, the target molecular weight, M, would become even larger than 4300 kdalton.

The biochemical nature of the targets. The target molecular weight of the cell-binding site in fibronectin obtained under dry conditions was 32 kdalton, far smaller than the molecular weight of fibronectin. This value is about half that of the cell-binding domain, which was 75 kdalton (6). This may support the domain structure model of fibronectin, in which each biological function is undertaken by its corresponding molecular domain.

A tripeptide, RGD, in fibronectin was reported to be a minimum unit which is necessary for the cell-adhesion activity (18). If RGD is the only structure necessary for binding to a cell surface, the target mass could be expected to be equal to the mass of RGD. Our result indicates, however, that regions other than RGD in the fibronectin molecule also play an indispensable role in the adhesion process between fibronectin and the cell. Recently, Obara *et al.* (16) reported that the deletion of the site, which is located more than 20 kdalton polypeptides away from the RGD site, reduces the adhesive activity of fibronectin. This site corresponds to the region at the amino acid repeating units (type III unit 7 and 8 (12)) in the cell-binding domain. The molecular weight of a type-III unit is about 10 kdalton. The obtained target molecular weight under dry conditions might be (partially) explained by summing up the weight of the two type-III units to the RGD site.

The target molecular weight in the cell was calculated to be 4300 kdalton. Since assuming that the target in the cell is spherical gives the least surface area and, hence, the least weight of the water layer, the value of M in Table II might be overestimated. However, the difference between the obtained molecular weight and the molecular weight (140 kdalton) (21) of the fibronectin receptor molecule, integrin, can not be explained by the thickness ambiguity of the water layer surrounding the target alone. The obtained large molecular weight could be explained by assuming that the target comprises a group of proteins that included the fibronectin receptor

and several other proteins, such as talin (2), which are considered to connect the receptor to the cytoskeletal proteins.

Acknowledgements. We would like to express our sincere thanks to Dr. M. Hayashi for teaching us the method to purify fibronectin, and for his valuable advice and suggestions concerning this work. We wish to thank Dr. T. Terashima for providing 10T1/2 cell line and Ms. M. Yasukawa for her technical advices on the preparation of the cell. We also thank Ms. S. Uesaka for reading the manuscript.

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(Received for publication, September 19, 1990
and in revised form, November 20, 1990)