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Adhesion strength of individual human bone marrow cells to fibronectin. Integrin β_1-mediated adhesion

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The purpose of this work was to study the adhesion strength of individual bone marrow cells, using a micropipette aspiration technique. The adhesion strength of the primary human bone marrow cells to fibronectin-coated substrate, by blocking the β_1 integrin with and without antibodies, was also determined. Human bone marrow stromal cells of the second passage were seeded at a density of 500 cells/cm^2 on two different substrates: plastic culture dish (PCD) and PCD coated with fibronectin. In short adhesion times (15–180 min) the cells attached without spreading and remained almost spherical. A negative pressure of about 3500 Pa was applied, through the micropipette, on individual bone marrow cells and the detach process was recorded. The tip of the micropipette was bent at an 130° angle to the corpus of the pipette and it was manipulated to be on the upper side of the cell and vertically to the bottom of the plate. It was observed from the experiments that the cells exhibited smaller adhesion strength at early adhesion times (30–85 min). After 85 min the adhesion strength increased abruptly and remained relatively constant for the adhesion period from 85 to 180 min for all substrates. Monoclonal antibodies against integrin subunit β_1 were used for integrin blocking experiments. The data suggested that the attachment of osteoblasts to a plastic culture dish without fibronectin coating occurred earlier than to the one coated with fibronectin PCD. In longer adhesion time the coating with fibronectin increased the adhesion strength at 107%. Blocking of integrin β_1 with monoclonal antibody resulted in decrease of the adhesion strength at 49%.

1. Introduction

Cell adhesion is a basal and ultimate cellular process and the extracellular matrix (ECM), apart from the support to the cell to sustain its structure, fundamentally influences cell growth, differentiation and migration as well as tissue morphogenesis, integrity and repair. Bone cell adhesion to extracellular matrix influences directly the cell spreading and growth, the expression of osteoblast phenotype and bone tissue formation [1–7].

Adhesion of cells to proteins adsorbed onto material surfaces is particularly important to host–implant interactions in biomaterial and tissue engineering applications. Immediately upon contacting physiological fluids, many proteins adsorb onto implant surfaces. In combination with proteins produced by the cell, depending on the substratum properties, they determine the strength and the type of adhesion. Adsorption of adhesion proteins from serum containing solutions mediates cell adhesion *in vitro*. Among serum proteins, fibronectin and vitronectin have been found to promote cell adhesion and reorganization of the actin microfilaments [8–11].

Fibronectin mediated cell adhesion has been studied by several researchers. It was found that precoating of bioactive and non-reactive glasses with fibronectin resulted in higher cell detachment strength [12]. Preadsorbed fibronectin to glass slides resulted in a greater number of bonds between the surface and the fibroblasts, increasing the adhesive strength of the cell [13].

Cell attachment to ECMs is primarily mediated by integrins, a family of heterodimeric surface receptors [14]. Various integrin subunits are expressed by osteoblasts cultured on implant materials coated with different ECM molecules [15–17]. The integrin subunit β_1, along with α_3, α_4, α_5, and β_3, was expressed by primary human osteoblasts cultured on tissue culture polystyrene, coated with fibronectin [18].

Quantitative analysis of cell adhesion is essential in understanding tissue–biomaterial interactions. Various experimental methods for quantification of adherent cell detachment have been employed. They are generally classified according to the type of force applied and are divided into categories of micromanipulation, centrifugation and hydrodynamic shear force [14, 19–23].

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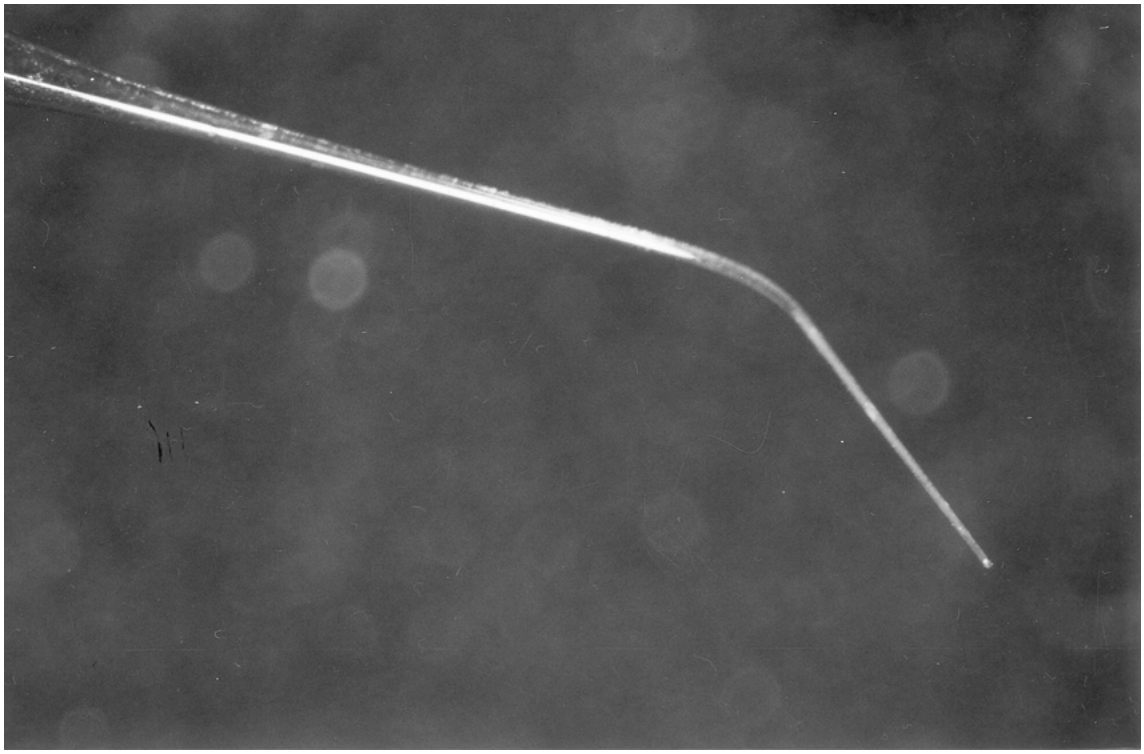

Figure 1 The micropipette with its tip bent at 135° to its corpus.

In the present work, a micropipette aspiration technique was established to study the adhesion strength of individual bone marrow cells. The adhesion strength of the primary human bone marrow cells to fibronectin coated substrate, by blocking the β_1 integrin with and without antibodies, was also determined.

2. Materials and methods

2.1. Cell culture

Human bone marrow stromal cells, obtained by aspiration from the femoral diaphysis of four healthy male and female patients, 50–70 years old, undergoing hip surgery after trauma, were used for these experiments. From each donor, a single-cell suspension was prepared by repeatedly aspirating the cells successively through 19 gauge and 21 gauge needles. The cell suspension was cultured until confluence in α-MEM (Gibco, Life Technologies GmbH, Carlsruhe, Germany) with 10% fetal calf serum (FCS) supplemented with 2.5 μg/ml fungizone (Gibco), 50 μg/ml gentamicin (Gibco), 10^{-8} M dexamethasone (Sigma, Aldrich chemie GmbH, Germany), 1.5 mM β-glycerol phosphate (Sigma) and 50 μg/ml ascorbic acid (Sigma) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Positive identification of the cultured cells as osteoblasts was based on alkaline phosphatase activity. Staining for alkaline phosphatase activity (Sigma diagnostic kit, 85L-2, St. Louis, MO), showed extensive dark purple areas and granules which indicate the presence of the enzyme activity. The flasks with the most intense alkaline phosphatase activity (4+, according to Kaplow [24]) were seeded on the biomaterials.

Cells of second passage were seeded, at a population density of 500 cells/cm^2, on three different culture substrates: (a) polystyrene culture dish (PCD) (Costar Europe Ltd, The Netherlands); (b) PCD coated with fibronectin (Fn); and (c) PCD coated with Fn and monoclonal antibody anti-β_1 added in the medium together with the seeded cells. The cells were allowed to attach to the substrates at least for 30 min at 37 °C, up to the time of the experiment.

2.2. Fn adsorption

PCDs were coated with Fn by incubating them in Fn diluted in PBS, at a concentration of 0.2 mg/ml [25, 26], for 30 min at 22 °C and then in 1% bovine serum albumin (BSA) for 30 min to block non-specific adhesion. The Fn coating the dishes were allowed to air dry and were washed with PBS. Just before cell plating, the dishes were equilibrated in serum-free medium.

2.3. Blocking integrin β_1 by antibodies

A monoclonal antibody directed against integrin β_1 (MAB 1981, Chemicon, Inc., CA) was used for integrin blocking experiments. It was reconstituted in deionized water and diluted to 1/100. The monoclonal antibody was incubated with bone marrow cells for 30 min at 37 °C in α-MEM with 1 mg/ml of bovine serum albumin. Cell suspensions previously incubated with the antibody were incubated for an additional minimum 30 min at 37 °C on Fn-coated PCD at a density of 500 cells/cm^2.

2.4. Cell detachment assay – micropipette manipulation and aspiration of the cell

A micropipette aspiration technique was used to aspirate bone marrow cells from a surface and measure the forces required to detach the cells from the above described

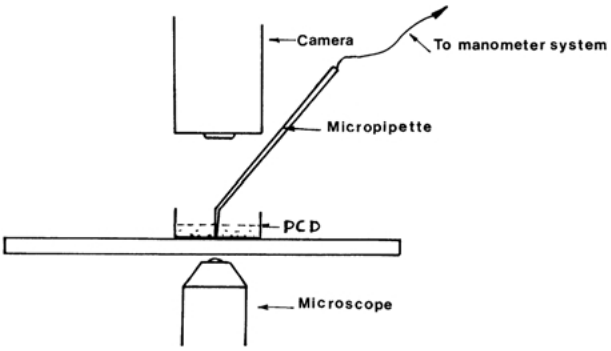

Figure 2 Schematic of the micropipette system and the PCD in which bone marrow cells were adhered.

substrates. This technique was able to apply forces vertically to the surface (like a vacuum cleaner).

The micropipettes with an internal diameter of 14–16 μm were prepared by pulling borosilicate glass tubes (I.D.: 0.50 mm) with a micropipette puller (Model P-87, Sutter Instrument Company, Novato, CA). The tip of the micropipette was bent at an 130° angle to the corpus of the micropipette (Fig. 1). The diameter of the micropipette was measured with a video microscaler (FOR A IV 550, Japan) which placed two vertical lines on the television screen and gave a digital readout of the distance between them.

The PCD (Fig. 2) was placed directly into the microscope stage. The micropipette was manipulated to enter the open side of the PCD. The tip of the micropipette and the aspirated osteoblast were observed through an inverted microscope (Olympus IM, Japan) and were viewed through a black and white video camera and monitor and images were recorded on a videotape recorder (J.V.C.-Japan). The measurements were carried out at room temperature (about 21 °C). The micropipette was filled with phosphate-buffered saline (P.B.S.-30: 6.375 g/l NaCl, 3.143 g/l Na_2HPO_4, 0.738 g/l KH_2PO_4) under boiling at 60 °C and connected to a hydrostatic pressure measuring system. A pressure transducer (DP-103, Validyne, USA) gave a digital readout for every applied negative pressure through the micropipette on the osteoblast, PC recorded. A timer gave a digital readout during the process of the osteoblast detachment, which was recorded continuously. The tip of the micropipette was manipulated to be on the upper side of the osteoblast and vertically to the bottom of the PCD. In this way, a vertical detach force to the adhesion area of the osteoblast was applied.

The negative suction pressure was applied just at the moment when the micropipette tip made contact with the cell. For static situation, the attachment force (adhesion strength) F of the cell is equal to $F = P\,S$ (1) [20], where P is the applied suction negative pressure and S is the cross-sectional area of the tip of the micropipette equal to $\pi d^2/4$ where d is the diameter of the micropipette tip. The present experimental data were taken with a micropipette tip diameter $d = 14.5$ μm.

3. Results

Fig. 3 shows four successive stages of the detachment process of a bone marrow cell from Fn-coated PCD. As it can be observed, the detachment of cells took place in different stages. In the beginning, the cell was deformed and entered into the micropipette, without detachment from the surface. Afterwards, the pressure increased in a maximum value and the process of the detachment started. This maximum recorded pressure value was used in the relationship (1), to calculate the adhesion strength. The total time of the detachment process varied with attachment time and substrate type. Because of the preliminary nature of the experiments, this dependence was not studied in this work

The results indicated that the adhesion strength increased with the adhesion time in all substrates and the cells exhibited smaller adhesion strength at early adhesion times (30–85 min). After 85 min the adhesion strength increased abruptly and remained relatively constant for the adhesion period from 85 to 180 min for all the substrates.

Table I summarizes the adhesion strength F of the individual bone marrow cells adhered on three different substrates: (a) PCD, (b) PCD Fn-coated and (c) PCD Fn-coated in the presence of monoclonal antibodies, for two adhesion periods 30–85 min and 85–180 min. Statistical comparison of the measured quantities ($F_{\text{adh.time85}}$, $F_{\text{adh.time180}}$) on the three substrates (PCD, PCD + Fn, PCD + Fn + ab) was performed by ANOVA. Table II summarizes the p-values for all the possible comparisons.

Fig. 4 displays the adhesion strength on the three different substrates for two adhesion periods. The adhesion strength to PCD was 2.57×10^{-2} dynes for adhesion period of 30–85 min. At longer adhesion times (85–180 min) the adhesion strength increased to 3.84×10^{-2} dynes, indicating an increase of 49% ($p < 0.001$). The adhesion strength to Fn-coated PCD for the initial adhesion period decreased to the value 1.11×10^{-2} dynes. At longer adhesion times, the Fn coating increased the bone marrow cell's adhesion strength, to 7.96×10^{-2} dynes, indicating an increase of 107% ($p < 0.001$).

The data suggested that the adhesion of bone marrow cells without Fn coating, occurred earlier than to Fn-coated PCD. Blocking of integrin β_1 with a monoclonal antibody, resulted in decrease of the bone marrow cell's adhesion strength at 49%, than that on Fn-coated PCD for both adhesion periods ($p < 0.01$).

4. Discussion

This work aimed at the establishment of a method to study the adhesion strength between individual bone marrow cells and the substrate by the use of a micropipette aspiration technique. The first goal was to establish an accurate and repeatable experimental method, able to calculate the adhesion strength, vertically to the surface of individual osteoblast cells on different substrates

The role of integrin β_1 subunit in mediating the attachment of bone marrow cells to Fn-coated polystyrene surfaces was also studied by use of a blocking monoclonal antibody to β_1 subunit.

The adhesion strength of bone marrow cells onto different substrates and the research of the influence of

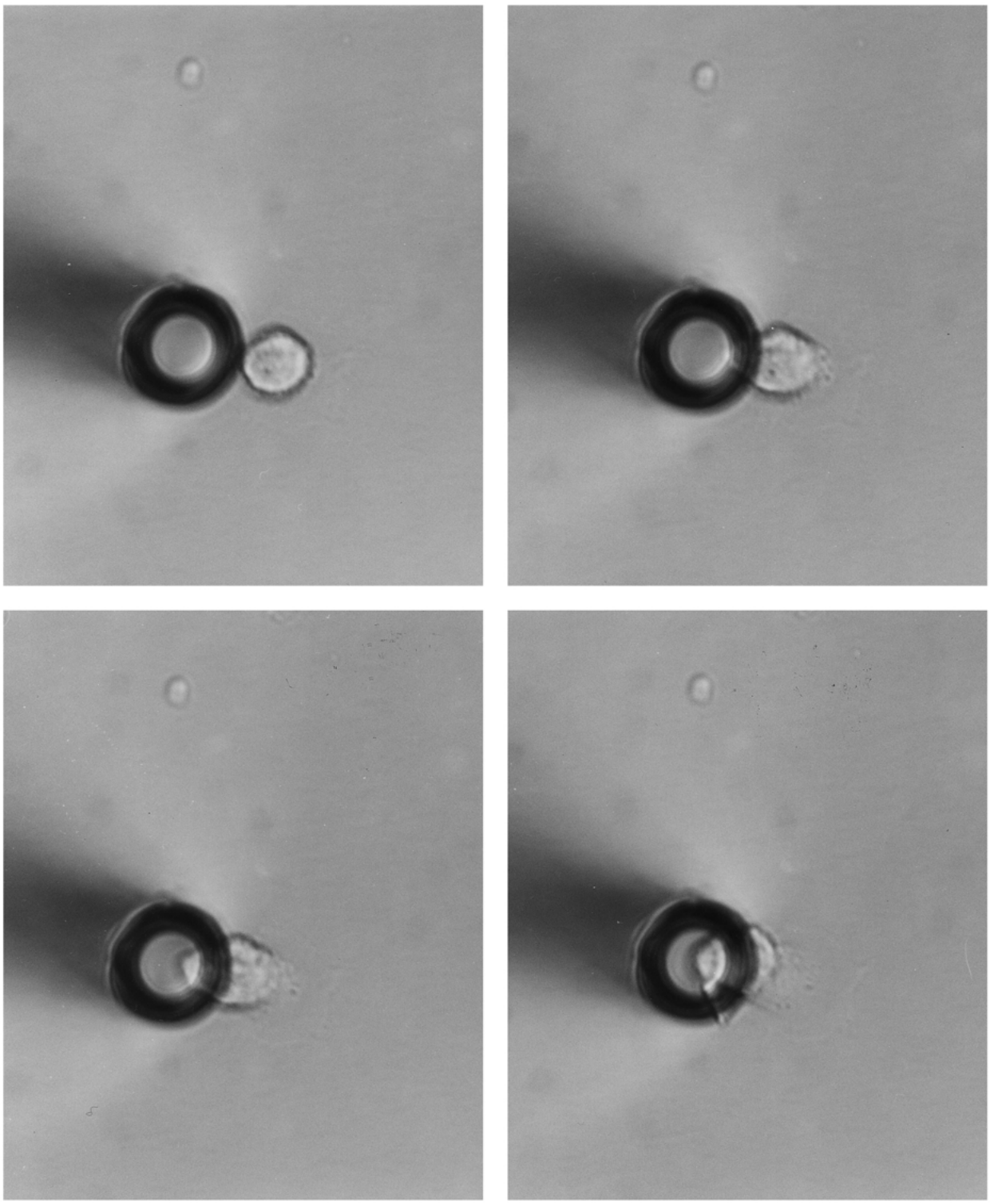

Figure 3 Four successive stages of the detachment process of a bone marrow cell from fibronectin coated PCD. The diameter of the micropipette tip was 14.5 μm and the aspiration pressure was 3500 Pa. The adhesion time of the cell on the surface was 100 min.

adsorbed ECM proteins to the cell-substrate adhesion for the development of successful implants, have been the focus of many studies during the last few years [12, 19, 26–28]. The hydrodynamic flow systems (fluid flow over adherent cells) generate detachment shear forces. These systems allow the application of a range of forces to a large cell population. Values of strength of cell adhesion of a large cell population, have been

TABLE I Adhesion strength F of the individual bone marrow cells to the three substrates

Substrates	Number of cells	Adhesion strength F $(N \times 10^{-7})$ (adh. time: 30–85 min)	Number of cells	Adhesion strength F $(N \times 10^{-7})$ (adh. time: 85–180 min)	p-value
PCD	25	2.57 ± 0.16	20	3.84 ± 0.59	0.001
PCD + Fn	31	1.11 ± 0.25	36	7.96 ± 3.06	0.01
PCD + Fn + ab	28	0.68 ± 0.54	30	4.03 ± 1.25	0.001

TABLE II The probability of variation ($P <$) between different substrates and adhesion times. Statistical comparison of data was performed by ANOVA

Substrates	PCD		PCD + Fn	
	$P(t_{adh} = 85$ min)	$P(t_{adh} = 180$ min)	$P(t_{adh} = 85$ min)	$P(t_{adh} = 180$ min)
PCD + Fn	0.01	0.05	—	—
PCD + Fn + ab	0.05	NS	NS	0.05

reported by different researchers [14, 19, 27, 28]. Shear stresses, for the detachment of 50% of the attached osteoblasts or endothelial cells [14, 19, 28] from fibronectin coated or peptide-immobilized substrates, of the order of 50–100 dyne/cm^2 have been reported for short attachment time (15 min–2 h).

Micropipette aspiration techniques have been used to measure forces required to detach cells from different substrates [29]. The advantages of the micropipette technique are its accuracy and the fact that it measures the adhesion strength of an individual cell instead of the distribution of the adhesion strength of a population of cells.

Other investigators used similar micropipette techniques manipulating individual cells by pulling these cells from a surface [29]. Their technique measured detachment shear forces. On the contrary, our technique is based on the application of a force vertical to the substrate to detach the cell. In addition, the micropipette used had a diameter length comparable with the diameter of the cell. In this way, the criticism that for the micropipette method [14] the force applied to a localized region of the cell results in high local stresses which are difficult to measure, is no longer valid. In our experiments, we had a closely fitting cell or one moving freely in a micropipette. In this case, when static, the suction pressure times the cross-sectional area of the pipette equals the attachment force F [20]. The applied force is very easily calculated and the stress field is not complicated.

It has been found [31] that the adhesiveness of human ligament fibroblasts to collagen substrates was dependent on seeding time for all experimental conditions. Adhesion strength decreased with seeding time or remained constant, depending on the origin of fibroblasts. The adhesion strength of human ligament fibroblasts has well-defined functional relationships with the Fn concentration and the seeding time [29]. Forces required for 100% detachment of the fibroblasts were calculated to be from 0.72×10^{-3} and 1.64×10^{-3} dynes, depending on the fibroblast's origin and the cell seeding time.

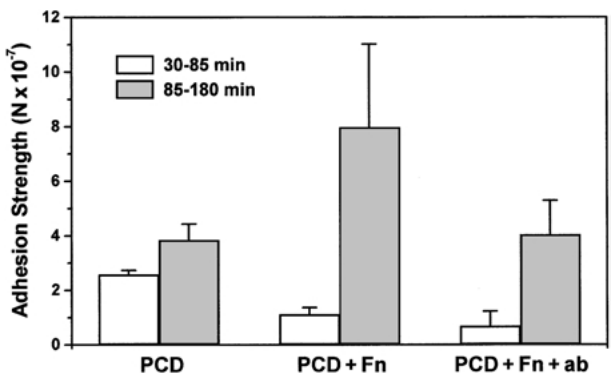

Figure 4 The adhesion strength on the three different substrates for two adhesion periods.

Adhesion forces of bone marrow cells onto Fn-coated glass were measured by Kwon *et al* [30]. They were as high as 20 mdynes after 46–60 min of attachment. Adhesion forces measured by our group were of the same order of magnitude. However, a direct comparison of the results of the different researchers is difficult because of the different substrates, types of cells, times of exposure to detachment force, incubation times for adhesion and stress application methods.

In the present work it was observed that the detachment forces are markedly depended on the time of cell attachment. Our results supported a finding common to all the works concerning cell detachment, that the adhesion strength increases markedly with the adhesion time [14, 19, 27, 29].

The present study showed that Fn mediates adhesion in long adhesion times (85–180 min). The strength of cell detachment from Fn-coated PCD surface was significantly $(p < 0.001)$ lower than that from not coated PCD at attachment times less than 85 min. This means that the initial adhesion $(t < 85$ min$)$ was not mediated through Fn receptors. Anti-β_1 antibody blocked significantly $(p < 0.01)$ cell adhesion to Fn. The β_1 adhesion subunits were the most dominant in mediating bone marrow cell adhesion to Fn and responsible for the 50% of their adhesion strength to Fn. Further experiments with the use of antibodies specific to a panel of integrins would show their contribution to the adhesion strength of bone marrow cells to Fn.

Acknowledgments

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