Analysis of the adhesion energy exchanged in CD44-Hyaluronate binding.

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Received: February 2009 – Accepted: April 2009

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Introduction

The hyaluronate receptor is a cell surface glycoprotein of 85 kD, which is responsible for the adhesion of cells to hyaluronate and chondroitin sulfate in the extracellular matrix. This receptor is present on a wide variety of cells including most macrophages, epithelial cells, and some neurons. In the case of epithelia, it is preferentially expressed on the actively proliferating cells as compared to their non-proliferating counterparts. In addition, the hyaluronate receptor appears to be associated with actin filaments, a characteristic that may allow it to mediate transmembrane signaling between the extracellular matrix and the cytoskeleton [1, 2, 3, 4, 5, 6].
Another membrane glycoprotein involved in cell adhesion has been described as being similar to the hyaluronate receptor with respect to molecular weight, tissue distribution and association with actin filaments [7]. This glycoprotein has been variously referred to as Pgp-1, Ly-24, ECMRIII, gp90 or H-CAM [8, 9, 10]. However, subsequent studies have revealed that these proteins are, in fact, closely related or identical to one another, and represent CD44, one of the antigens differentiation clusters in leukocytes [11, 12]. Goldstein et al. [13] and Stamenkovic et al [14] studies first suggested the connection between the hyaluronate receptor and CD44. These researchers independently reported that the extracellular domain of CD44 is homologous to the hyaluronate-binding region of the link protein of cartilage. This finding suggested that CD44 may be able to bind hyaluronate, and thus it closely resembles the hyaluronate receptor.

Investigations concerning blood cell - vascular cell interactions have successively focused on platelet-vessel wall reactions and more recently, on leukocyte – endothelial cell interactions. Major developments have been possible following identification of the molecular bases of these processes, the platelet glycoprotein receptors and the leukocyte selectins and integrins. The molecular bases of the abnormal adhesion of erythrocytes to endothelial cells have been erythrocytes with the endothelium in sickle cell anemia, diabetes mellitus, vasculopathies, hypertension and malaria.
Red blood cells can adhere to a solid surface by means of adhesion molecules that can be linked to erythrocyte membrane receptors, as it is the case of hyaluronate molecules, which bind to the CD44 receptor on the erythrocyte membrane. The study of cell interaction with biomaterials solid surface or with other cells such as vascular endothelium can exert important influence on microcirculatory flow [16, 17].

Different approaches may be used to study erythrocyte - endothelium interactions. Like all other cellular interaction processes, red blood cell adhesion to hyaluronate is produced with energetic exchange. That is why it is very important to determine the energy interchanged in the adhesion reaction, the value of which supplies an estimation of the adhesion strength.
The objective of this work was to measure the specific energy (γ_d) of the erythrocyte hyaluronate receptor (CD44) to its hyaluronate ligand, under dynamic conditions. For this purpose, a controlled flow chamber, installed on the stage of a microscope was used and microscopic images were digitized and processed with a digital image processor (DIP) connected with a charged coupled dense (CCD) camera.

Materials and Methods

- **Suspending medium** (PBS-HSA) was PBS (pH 7.4, 295±5 mOsm/kg) added with 0.25% human serum albumin (HSA).

- **Hyaluronate solution** (Hyaluronic acid sodium salt from human umbilical cord H1876 Lot 77H0544. Sigma) was diluted at 2% (w/v) in PBS (pH 7.4, 295 mOsm/kg). This solution was used to coat the floor of the microchannel, which constitutes the adhesion molecule layer covering the solid surface.

- **Red blood cells.** Blood samples were drawn by venipuncture from 15 healthy donors and anticoagulated with EDTA Na_2. Each sample was centrifuged at 3000 g during 10 minutes. After that the plasma and buffy coat were removed and discarded. Settled red blood cells (RBC) were washed three times in phosphate buffered saline (PBS) (pH 7.4, 295±5 mOsm/kg) and eventually resuspended at a concentration of 0.07% (V/V) in the suspending medium.

Flow chamber:

The experiments were carried out in a transparent parallel plate flow chamber. The geometrically defined microchannel was built by sandwiching a 250 µm thick Teflon film sheet between a thick acrylic cell (6.7cm x 4.2cm x 1.2 cm) and a thin glass sheet (7.8 cm x 5.3 cm x 0.03 cm). The Teflon film (6.7 cm x 4.3 cm) is 4.3 cm long and 1.8 cm side. With the use of vacuum pump, the Teflon sheet was sandwiched between a glass sheet and an acrylic cell. This cell has two rectangular grooves (1.6 × 0.1 cm) 4 cm apart, from each other which have been used as inlet and outlet of the fluid for the microchannel. The inlet was connected to a 30 cm^3 syringe of a servo-controlled infusion pump (Masterflex A-74900-05, Cole Parmer Instrument) having a variable speed control to induce flow of PBS-HSA into the microchannel. Flow rates were increased in steps from 30 µl/min up to 800 µl/min.

Figure 1 show the schematic drawing of the flow chamber. The chamber was installed on the stage of an optical inverted microscope (Union Optical, magnification 60x) in such a way that the cover glass was the floor of the microchannel. A CCD (charge coupled device) camera was placed in the ocular tube of the microscope and connected to a Digital Image Processor (IPPLUS System) to digitize record and analyze microscopic images.

Prior to the assembly of the flow chamber, the cover glass was covered with a layer of sodium hyaluronate and dried during 30 min. at 37ºC. After sealing, the microchannel was filled with PBS-HSA avoiding the formation of air bubbles, which can disturb the laminar flow in the chamber. The inlet of the flow channel was connected to a 20 cm^3 syringe containing the sample. Immediately, the cell suspension was poured into the channel by mean of the servo-controlled infusion pump see figure 1 (Schematic drawing of the Flow Chamber, where).

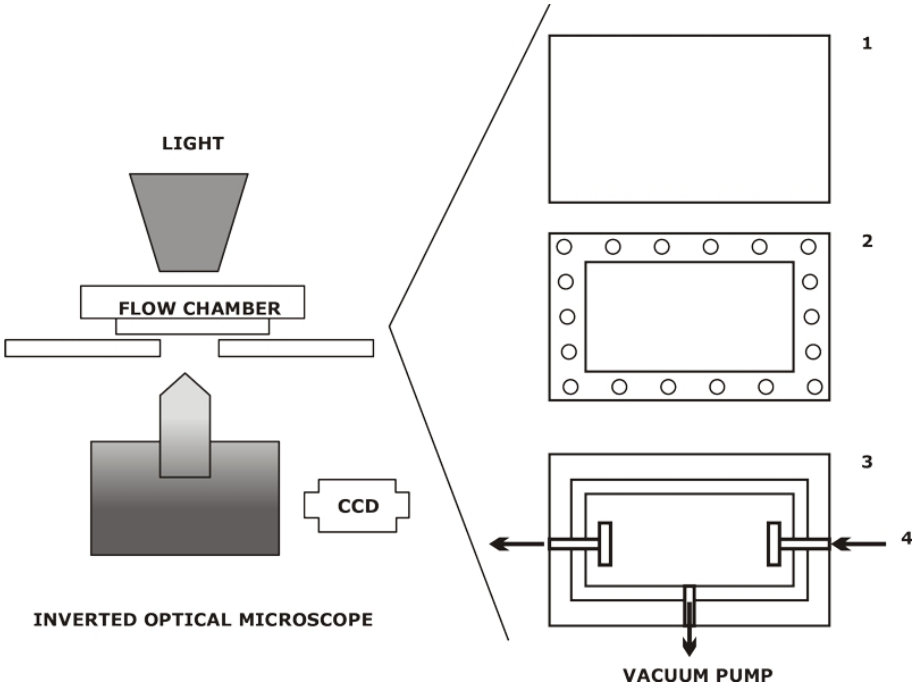

Figure 1:
Schematic drawing of the Flow Chamber, where

1. Cover Glass
2. Teflon Film
3. Thick Acrylic Base
4. Servo-Controlled Infusion Pump
5. **CCD:** Charge Coupled Device connected to a Digital Image Processor (IPPLUS System).

Then, a stationary state of 10 minutes allows the RBCs adhesion on the channel floor. After this, a cell-free PBS-HSA medium was pumped through the channel for produce the desired shear stress. The flow rate (Q) was increased in a stepwise manner to achieve a shear stress range from 2 to 80 mPa. After the first step (2.90 mPa) the channel floor was scanned (while the suspension was at rest) in order to choose a field where two or more cells were adhered. At each level of shear stress the chosen field was observed, digitized, recorded and analyzed. All the experiments were performed at room temperature.

The wall shear stress (τ) acting on the upper surface area of the settled and adhered cells was calculated from the channel thickness (h), the channel width (a), the flow rate (Q) and the viscosity of the suspension medium (η_o) as follows:

$$\tau = \frac{6 \cdot Q \cdot \eta_{0.}}{h^2 \cdot a} \qquad (1)$$

Measured values were expressed as mean ± standard deviation (SD) (the number of experimental measurements was n = 15)

Theoretical Analysis

Skalak et al. [5] proposed a differential equation of conservation of energy, which can be applied to our studies in a simpler form:

$$\frac{\partial W}{\partial t} = -\gamma \cdot \frac{\partial A}{\partial t} \qquad (2)$$

where γ is the specific adhesion energy (per unit area) and A is the adhered area of the cell membrane. In this work, the specific adhesion energy is the energy required to disrupt a unit area of interaction between the adhered area of the erythrocyte membrane and the solid surface covered with hyaluronate molecules.

The specific energy γ required to detach the cell surface adhered to the channel floor can be derived from the mean work (<W>) done by the wall shear stress:

$$\gamma = \frac{< W >}{A_i}$$

where A_i is the detached cell area.

Work was measured as the product of the force acting on the upper surface of the adhered cell and the distance through which the cell moves from its initial position (6). The total work done was estimated by integrating the area under the curve of the applied force (*F*) versus the displaced distance (D_i). One could assume that there is a linear relationship between force and distance (at least up to 50% separation). Hence, the integral could be close to one half of that product *F*. D_i and in consequence results:

$$\gamma = \frac{F \cdot D_i}{2 \cdot A_i}$$

where D_i is the length of the cell displacement.

Considering that $F = \tau \cdot A_o$, where A_o is the projected area of the upper cell surface on which the shear stress is applied, we obtain:

$$\gamma = \frac{\tau \cdot A_0 \cdot D_i}{2 \cdot A_i} = \frac{\tau \cdot D_i}{2 \cdot \Phi} \qquad (3)$$

where ϕ defined as A_i/A_o represents the fraction of the detached cell surface.

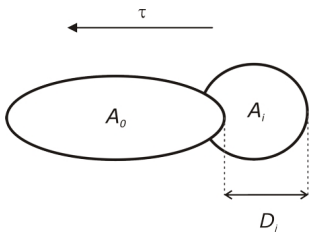

Figure 2:
Schematic representation of the RBC displaced in the flow direction, where D_i is the cell displacement length, A_o represents the area of the cell surface on which the shear stress acts and Ai is the area of the detached cell surface.

The degree of separation of adhered cells in response to shear flow was correlated with the specific energy required to attain this separation. Figure 2 shows the displacement of RBC in the flow direction.

Results

Experiments were performed on 15 red blood cell samples. Shear stress was calculated from the dimensions of the microchannel and the flow rate indicated by the infusion pump. The channel floor was microscopically scanned while the suspension was in the stationary state. In each experiment, a field was selected to be observed, recorded and analyzed.

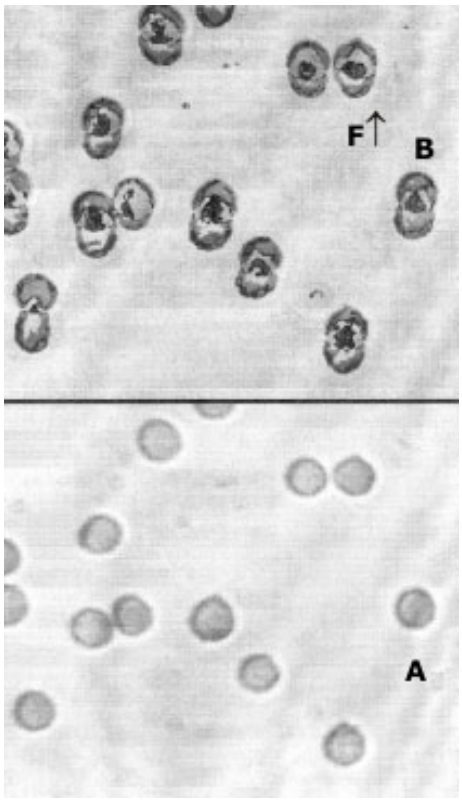

Figure 3:
Register of digitized images corresponding to adhesion of RBC to hyaluronate.
A: Image to Q = 0 mm^3/min.
B: Superposition of image **A** with the image that corresponds to Q = 400 mm^3/min showing the RBC displacement in the flow direction (τ).

Figure 3 show digitized images of RBCs, which present a displacement in the indicated flow direction. At the beginning of the detachment process, one could assume that the molecular bridges are homogeneously distributed on the contact area of the adhered membrane. This condition can be accepted until the contact area approached the 50% of the original value. After applying the shear stress, the adhered cell was peeled from the hyaluronate covered solid surface. Results are shown in the table I.

Table 1
The disaggregation specific energy (γ) for the interaction hyaluronate-CD 44.

Q: flow rate
γ: Specific adhesion energy (per unit area)
ϕ = A_i/A_o: fraction of the detached cell surface
D_i: length of the cell displacement
A_o: projected area of the cell surface on which the shear stress acts
A_i: detached cell area

Mean ± SD

Q (μl/min)	A_0* (μm²)	A_i* (μm²)	D_i* (μm)	Φ* = A_i/A_0	τ (10^{-3} Pa)	γ* (10^{-9} J/m²)
0	51.3 ± 1.3	0	0	0	0	0
30	52.6 ± 1.3	23.7 ± 0.7	2.7 ± 0.9	0.45 ± 0.02	2.93	9 ± 3
60	51.4 ± 1.3	23.6 ± 0.8	2.9 ± 0.8	0.46 ± 0.03	5.87	18 ± 6
100	51.9 ± 1.3	24.4 ± 0.9	3.0 ± 0.5	0.47 ± 0.03	9.78	31 ± 7
150	48.7 ± 1.0	23.7 ± 1.5	3.1 ± 0.5	0.49 ± 0.04	14.67	46 ± 12
200	51.8 ± 1.3	31.7 ± 1.7	3.4 ± 1.1	0.61 ± 0.05	19.56	55 ± 22
400	52.2 ± 1.9	37.5 ± 0.6	5.3 ± 0.9	0.72 ± 0.04	39.11	145 ± 27
800	51.0 ± 2.0	38.6 ± 1.2	5.2 ± 0.5	0.76 ± 0.05	78.22	271 ± 43

By estimating the forces applied by the external fluid and the work expended in detaching the cell from the solid surface, the values of specific adhesion energy γ have been calculated and shown in figure 4 as a function of the Φ.

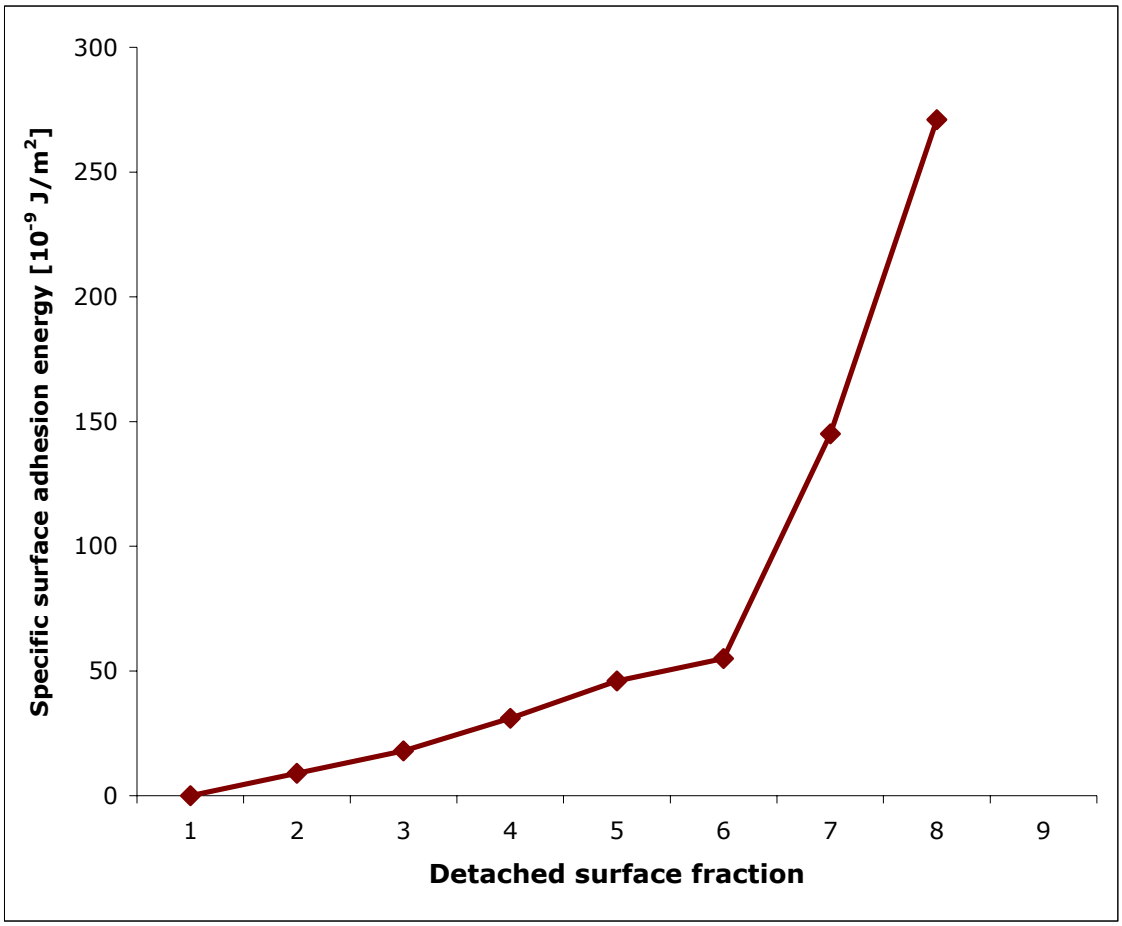

Figure 4:

The specific surface adhesion energy γ as a function of the fraction of the detached surface $\Phi = \frac{Ai}{Ao}$.

Discussion

The experiments carried out using the flow microchannel were widely used for assaying the strength of intercellular adhesion bridges [19]. The data shown in Table 1, which is quite similar to the graphic found in the reference [15], confirms the theory that the adhesion energy is not constant and that there is some redistribution of the bonds as the peeling process is in progress.

The specific energy increases as the cell becomes detached. When the adhered cells were separated from the solid surface by an external shear stress which pulled them apart and, if the specific energy is constant, one could expect that as the area of contact is reduced, a progressive failure may be observed. However, as shown by our experiments, no such progressive failure could be observed as the contact area was reduced.

These experiments showed that adhesive strength was maintained even when the contact areas were reduced to a minimum. Hence, the adhesion energy appears to increase as the contact area decreases. These results imply some rearrangement of molecular bonds, producing an increased concentration of cross-links at the remaining area of adhesion. This phenomenon has been confirmed by Skalak's studies [15], leading to the conclusion that the maximum energy value is produced when there is a total detachment of cell. Following Skalak's studies, it can be suggested that a constant equilibrium state is established during the detachment process, forming new molecular bonds to replace those that are have been destroyed. Apparently, some molecular bonds shift to the adhered area causing specific energy to increase as this area is reduced. This observation leads to the conclusion that the maximum specific energy is attained when the adhesion area tends zero value. On the other hand, it is interesting to note that as the membrane is peeled and the cell moves it seems that no new bonds are formed. There are many possible reasons for this behavior. One possible explanation is that the hyaluronate layer could be washed from the solid surface by the flowing fluid. Studies carried out by Evans et all. [1] on erythrocytes bonded by a monoclonal antibody to glycophorin A showed that the detachment was accompanied by the extraction of the membrane receptors. This observation suggests that the adhesion energy would be better estimated during the adhesion process rather than detachment.

Conclusion

The methodology proposed allows for a simple, quick, reproducible, and non-conventional way of estimating the specific adhesion energy. Calculated specific energies (table 1) are within the values (0.6×10^{-7} J/m^2 with a lectin surface density of 350 molecules/μm^2) determined for other types of cellular adhesion (agglutination by lectins and by specific antibodies) [6]. This would indicate that the method used to determine the adhesion specific energy by the analysis of digitized images in the flow chamber is convenient to be applied not only in this case but also in other types of intercellular adhesion. Studies carried out by other researches have demonstrated that cell adhesion becomes increased by cardiovascular disorders [18]. Hence, the method applied in our experiments might offer a significant help for the understanding of several physiological and pathological phenomena (vasculopathies). It can also supply a useful parameter to study adhesion as a risk factor in patients with microcirculatory disorders such as diabetic or hipertensive patients and obese people.

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