Biosensors and Bioelectronics 56 (2014) 159–166

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

The effect of acidic pH on the inhibitory efficacy of peptides against the interaction ICAM-1/LFA-1 studied by surface plasmon resonance (SPR)

Shu-Han Wu [a,b], David Núnez [c,d,e], Shih-Yang Hu [a], María Pilar Domingo [c,d], Yi-Chun Chen [f], Pei-Kuen Wei [a,b], Julián Pardo [c,e,g,h,**], Eva M Gálvez [c,d,***], Arthur Chiou [a,i,*]

[a] Institute of Biophotonics, National Yang-Ming University, No. 155, Section 2, Linong Street, Taipei 11221, Taiwan, ROC
[b] Research Center for Applied Sciences, Academia Sinica, Taipei 11529, Taiwan, ROC
[c] Immune Effector Cells Group, Aragón Health Research Institute (IIS Aragón), Biomedical Research Centre of Aragón (CIBA), Zaragoza 50009, Spain
[d] Instituto de Carboquímica ICB-CSIC, Zaragoza 50018, Spain
[e] Department of Biochemistry and Molecular and Cell Biology, Fac. Ciencias, University of Zaragoza, Zaragoza 50009, Spain
[f] Institute of Imaging and Biomedical Photonics, National Chiao Tung University, Tainan 71150, Taiwan, ROC
[g] Aragón I+D Foundation (ARAID), Government of Aragon, Zaragoza 50004, Spain
[h] Nanoscience Institute of Aragon (INA), Aragón I+D Foundation (ARAID), University of Zaragoza, Zaragoza 50009, Spain
[i] Biophotonics & Molecular Imaging Research Center (BMIRC), National Yang-Ming University, No. 155, Section 2, Linong Street, Taipei 11221, Taiwan, ROC

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form
2 January 2014
Accepted 3 January 2014
Available online 16 January 2014

Keywords:
Surface plasmon resonance (SPR)
ICAM-1
LFA-1
Acid pH
Peptide drug

ABSTRACT

Synthetic peptides have been developed for therapeutic applications for decades. The therapeutic efficacy often depends not only on the stabilization of the peptides but also on their binding specificity and affinity to the target molecules to interfere with designated molecular interaction. In this study, the binding affinity of human intercellular adhesion molecule 1 (ICAM-1) chimera and leukocyte function-associated antigen-1 (LFA-1) derived peptides was measured by surface plasmon resonance (SPR) detection, and the results were compared with that of the interaction (of ICAM-1) with the LFA-1 whole protein. To mimic diverse pathological situations *in vivo* where a low pH has been reported, we studied pH regulated binding affinity of ICAM-1/LFA-1 at pH 7.4, 6.5, and 4.0 without and with magnesium ion. We have found that the binding affinity of LFA-1 whole protein and ICAM-1 increases significantly as the environmental pH decreases, regardless of the absence or the presence of magnesium ion. The affinity of different (LFA-1) derived peptides also depends on the pH, although in all cases the peptides retain its ability to inhibit ICAM-1/LFA-1 interaction. The biomedical relevance of these data has been confirmed using a cell aggregation assay, suggesting that LFA-1 derived peptides show great potential for peptide drug development with a wide functional window of pH range for potential applications in LFA-1 related tumor therapy and autoimmune disease treatment.

1. Introduction

In the research and development of novel therapeutic methods, peptide derived drugs have emerged as an attractive option due to their desirable properties including biocompatibility, low toxicity, and low side effect to normal cell (Zhou et al., 2011), which are in sharp contrast to the traditional chemical compound drug. The basic working principle of peptide drug is to interfere with specific molecular interaction to block the undesirable signal transduction. For example, the interaction between integrins and adhesion molecules plays an essential role in the immune cell activation and migration (Palecek et al., 1997; Hayflick et al., 1998; Millan et al., 2006). Quite often, these molecular interactions may initiate a wide variety of signaling events, which dictate the subsequent fate of the cells through the route of normal vs. abnormal functions. Among these molecular interactions, the interaction between the integrin leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18; αLβ2) and one of its ligands, the intercellular adhesion molecule-1 (ICAM-1; CD54) is of special importance during different stages of the cellular immune response including migration, activation and effector functions.

ICAM-1 is a membrane glycoprotein formed by 5 extracellular Ig-like domains (Springer, 1990). Domain 1 is the major binding site of LFA-1, while domain 2 helps maintain a proper conformation of domain 1 (Staunton et al., 1990). LFA-1 is expressed on all kinds of leukocytes and interacts with the cell via ICAM-1(CD54) (Berendt et al., 1992), ICAM-2 (CD102) (De Fougerolles et al., 1991), and ICAM-3 (CD50) (Fawcett et al., 1992). The binding site of LFA-1 is known as metal ion dependent adhesion site (MIDAS) motif located

* Corresponding author at: National Yang-Ming University, Institute of Biophotonics, No. 155, Section 2, Linong Street, Taipei 11221, Taiwan, ROC. Tel.: +886 2 28237141; fax: +886 2 28236314.
** Corresponding author at: Immune Effector Cells Group, Aragón Health Research Institute (IIS Aragón), Biomedical Research Centre of Aragón (CIBA), Zaragoza 50009, Spain. Tel.: +34 876554338; fax: +34 976762123.
*** Corresponding author at: Instituto de Carboquímica ICB-CSIC, Zaragoza 50018, Spain. Tel.: +34 976733977; fax: +34 97673318.
E-mail addresses: pardojim@unizar.es (J. Pardo), eva@icb.csic.es (E. Gálvez), aechiou@ym.edu.tw, ejchiou@gmail.com (A. Chiou).

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http://dx.doi.org/10.1016/j.bios.2014.01.008

in the α subunit I-domain (Huang and Springer, 1995). The ICAM-1/LFA-1 interaction is facilitated by the divalent cations, magnesium and manganese ions, assorted with five amino acids of MIDAS in LFA-1 and glutamate in domain 1 of ICAM-1 (Lee et al., 1995).

Apart from its function during normal immune response ICAM-1/LFA-1 interaction is a key to several pathological processes, such as cancer metastasis/elimination and inflammatory autoimmune diseases (Tominaga et al., 1998; Frank and Lisanti, 2008). The ICAM-1 expression have also been studied in the context of the drug resistance in myeloma and LFA-1 associated inflammatory diseases, ulcerative colitis (UC) rheumatoid arthritis (RA), multiple sclerosis (MS), thyroiditis, and insulin-dependent diabetes mellitus (IDDM) (Zheng et al., 2013; Anderson and Siahaan, 2003. These effects are related to the enhanced ability of different types of leukocytes to migrate and adhere to inflammation sites through ICAM-1/LFA-1 interaction. Hence, the blocking of this interaction may find applications in autoimmune disease treatment and in the inhibition of tumor metastasis (Yusuf-Makagiansar et al., 2002).

In the evaluation of the therapeutic efficacy of peptide drugs, the binding affinity to the target molecule is a critical parameter. Besides, the physiological microenvironment where the target molecules exert their function should also be taken into account. The physical and chemical characteristics of the molecules may depend critically on the microenvironment, such as type of salt and ion, osmolarity, and pH (Sahin et al., 2010). In addition, histopathological studies have revealed that the extracellular microenvironment may be significantly different in pathological vs. normal tissues (Gerweck et al., 2006). For example it is well known that the extracellular pH in solid tumors can be as low as 5 due to changes in cell metabolism, which enhances tumor malignancy including invasiveness and drug resistance (Iessi et al., 2008). In addition, the pH is often lower in inflammatory micorenvironments like arthritic synovium (Farr et al., 1985) or colon during active ulcerative colitis (Fallingborg et al., 1993).

We chose surface plasmon resonance (SPR) biosensor to measure the binding affinity of the molecular interactions because it does not require the use of external dyes and allows the analysis of the interaction dynamics. In contrast, other techniques such as electrophoresis, enzyme-linked immunosorbent spot (ELISPOT) assay or flow cytometry require either dye staining or fluorescence tagging and do not provide dynamic information (Homola et al., 1999). The resonance condition in SPR detection is highly sensitive to the refractive index change near the sensor surface resulting from the molecular adsorption and has been applied to the study of ICAM-1/LFA-1 interaction (Gopinath, 2010; Jun, et al., 2001; Song et al., 2006).

In this study, we focused on the interaction between the integrin leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18; αLβ2) and one of its ligands, the intercellular adhesion molecule-1 (ICAM-1; CD54) and analyzed the effect of lowering pH. LFA-1 derived inhibitory peptides were also studied to compare their ability to interfere with the interaction between ICAM-1 and LFA-1 whole protein, as an assessment of the candidates for peptide drug therapy (Anderson and Siahaan, 2003). In our experiments, we used a commercial SPR detection system, BIAcore 3000, based on prism-coupling excitation mechanism for the coupling of an incident optical beam into surface plasmon polaritons (SPP) at gold/medium interface. The biological relevance of these measurements was confirmed via a cell aggregation assay.

2. Material and methods

2.1. Chemicals and reagents

The chemicals and reagents used in our experiments include phosphate buffered saline 10 × (PBS 10 ×), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *n*-hydroxysuccinimide (NHS), ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), lovastatin (Sigma-Aldrich, Saint-Louis, USA), magnesium chloride (J. T. Baker, Center Valley, USA), and human antibody capture kit for ICAM-1 immobilization (BR-100839; GE Healthcare, Little Chalfont, UK). 1 mM EGTA without and with 5 mM of magnesium ion at pH 7.4, 6.5, and 4.0, were prepared in 1 × PBS for molecular interaction affinity tests. Magnesium ion concentration of 5 mM was superabundant to test the magnesium ion dependence of ICAM-1/LFA-1 interaction.

2.2. ICAM-1 and LFA-1 derived peptides

Recombinant human ICAM-1/CD54 Fc chimera (D1D5-Fc) and recombinant human integrin αLβ2 (LFA-1) were purchased from R&D systems (Minneapolis, USA). LFA-1 derived peptides, $CD11a_{237–261}$ and $CD11a_{456–465}$ were purchased from GenScript (Piscataway, USA). The sequences of $CD11a_{237–261}$and $CD11a_{456–465}$ are ITDGEATDSGNIDAAKDIIRYIIGI, and APLFYGEQRG, respectively (Tibbetts et al., 2000).

The synthetic first two domains of ICAM-1 linked with human IgG1 Fc region (D1D2-Fc) was expressed in *Escherichia coli* (*E. coli*) and purified by single step column refolding as described (Núñez et al., 2013).

2.3. Surface plasmon resonance detection

The interactions of LFA-1 and derived peptides with immobilized ICAM-1 were studied by BIAcore 3000 biosensor instrument (Biacore International SA, Neuchâtel, Switzerland). The BIAcore 3000, CM5 sensor series chips with carboxymethylated dextran covalently attached on the gold surface (thickness = 50 nm) were used throughout the experiments. The sensor surface was activated by 400 mM EDC/100 mM NHS for 7 min. In order to control the orientation of ICAM-1 proteins (either the D1D5-Fc: commercial proteins or the D1D2-Fc proteins synthesized via single step column refolding and purification method) on the sensor surface, 25 μg/ml anti-human IgG (Fc) was flowed into the channel and immobilized on the sensor chip by amine coupling before the ICAM-1 injection. 40 μg/ml of ICAM-1 was then flowed into the channel and immobilized on the sensor surface for subsequent capturing of LFA-1 and LFA-1 derived peptides. In the binding affinity analysis, LFA-1 (50, 100, 200, 250, and 300 nM) and LFA-1 derived peptides ($CD11a_{237–261}$ and $CD11a_{456–465}$), in the concentration of 250, 300, 350, 400, 450, and 500 nM were evaluated for their interactions with ICAM-1 immobilized on the sensor surface. The sensor surface was regenerated by the regeneration buffer (from the human antibody capture kit: 3 M magnesium chloride). All the experiments were repeated 3 times and the affinity parameter (i.e., the dissociation constant K_D) was deduced by BIAcore analysis software (BIAevaluation 3.2 RC1) based on the repeated experimental curves at each concentration.

In the inhibition test of the interaction between ICAM-1 and LFA-1, the sensor surface was prepared by the same procedure as was described above (for the affinity test). Lovastatin in the concentrations of 10, and 100 μM, and $CD11a_{237–261}$ in the concentrations of 0.1, 1, and 6 μM, were each separately mixed with 500 nM of LFA-1, and injected into the channel to interact with ICAM-1 immobilized on the sensor surface.

2.4. Aggregation assay

The ability of the peptides from LFA-1 to inhibit cell homotypic aggregation of non-adherent cells at different pH was analyzed by using the Epstein–Barr Virus (EBV) transformed human B lymphoblastoid cell line R69, a generous gift from José A. López de Castro (Paradela et al., 1998). R69 human cells were washed twice in

DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and resuspended at a final concentration of 10^6 cells/ml. Then, LFA-1 expression was induced by incubating cells with 10 ng/ml PMA (phorbol myristate acetate) for 2 h at 37 °C. Cells were pipetted up and down several times to break up any pre-existing aggregates. Subsequently, cells were incubated at 37 °C for 6 h in flat bottom 96-well plates in which 1×10^5 cells were added to each well in the presence of 250 μM of peptides derived from LFA-1. Identical tests were conducted with other specific inhibitors (of the interaction of LFA-1 and its counter-receptor ICAM-1), such as D1D2-Fc (10 μg) and EGTA (1 mM). A qualitative aggregation assay was carried out as described (Tibbetts et al., 1999, 2000) with a slight modification. Briefly, PMA-stimulated cell samples were arbitrarily assigned a clumping index of 10 which represents the percentage of aggregation between 90 and 100, and test samples were ranked from 1 to 10, based upon their degree of clumping relative to PMA-treated samples. Results were verified by blind, independent ratings performed by a second observer. Clumping index for each peptide was roughly quantified by determining the mean clumping index per experiment ± SEM. All experiments were performed at least twice with similar results.

3. Results and discussion

3.1. The orientation control of immobilized molecules

In the applications of biosensors for the analysis of molecular interactions, a conventional protocol is to immobilize one of the target molecules on the sensor chip and to flow an analyte (containing the other target molecules) into the sensing chamber to enable the two types of molecules to interact with each other. The binding affinity between the two types of molecules could be analyzed and quantified from the sensor response by taking into account the concentration, the molecule weight, and the number of binding sites of the molecules. In this approach, proper orientation of the immobilized molecules such that the binding sites are readily accessible is crucial for the interaction to take place, and for accurate determination of the binding affinity. In this work, we apply the anti-human IgG (Fc) to control the orientation of ICAM-1. The sensor surface preparation procedure is outlined below and the corresponding SPR sensor response in each step is shown in Fig. 1. The sensor surface with carboxymethylated dextran covalently attached was first activated by EDC/NHS chemical solution. Anti-human IgG (Fc) was then injected into the channel to form the first layer immobilized on the sensor surface. Afterward, the ICAM-1 molecules linked with Fc region was flowed and captured by the anti-human IgG (Fc). PBS buffer (1 ×) was used to refresh the channel at each wash step. In Fig. 1, the response (the *y*-axis) represents the quantitative information in sensor detection at each step, where 1000 RU corresponds to the surface concentration of approximately 1 ng/mm^2, for typical protein molecules. From the figure, we could estimate the efficiency of the immobilization of the molecules and the optimum molecular concentrations to ensure good sensor performance in subsequent experiments. For example, 9000–10,000 of sensor response in anti-human IgG immobilization and 3000–4000 for the immobilization of ICAM-1.

The procedure outlined above (along with the sensor response curve shown in Fig. 1) was used to compare the binding affinity of the synthetic vs. the commercial ICAM-1 with LFA-1 whole protein and LFA-1 derived peptides, without vs. with magnesium ion in the buffer at pH 7.4. In our previous study (Núñez et al., 2013), the characteristics of the synthetic ICAM-1 were determined as follows. The molecular weight was determined by electrophoresis and western blot. The secondary and tertiary structures were determined by circular dichroism, and fluorescence spectroscopy.

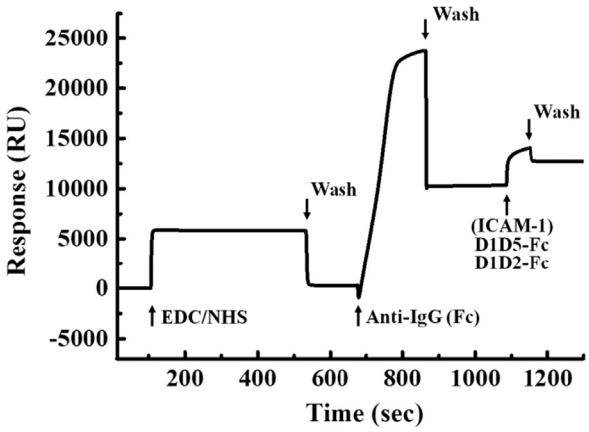

Fig. 1. The SPR response curve during the course of ICAM-1 immobilization on the sensor chip.

3.2. The binding affinity comparison of synthetic and commercial recombinant human ICAM-1

In this study, we further measured and compared the binding affinity between the synthetic ICAM-1 (D1D2-Fc) vs. the commercial recombinant human ICAM-1/CD54 Fc chimera containing all 5 domains (D1D5-Fc) with LFA-1 whole protein vs. LFA-1 derived peptides, CD11a$_{237–261}$, and CD11a$_{456–465}$, without vs. with magnesium ion in the buffer at pH 7.4; the results are summarized in Fig. 2(a)–(c), respectively. The rational for this study and related earlier works is explained as follows: It has been reported earlier (Tibbetts et al., 2000) that the LFA-1 derived peptides, CD11a$_{237–261}$ and CD11a$_{456–465}$, both interact with ICAM-1, but with relatively high and low binding affinity, respectively. In addition, another earlier study (Huang and Springer, 1995) has indicated that the binding of LFA-1 whole protein and ICAM-1 protein is magnesium ion-dependent. However, whether this magnesium ion-dependence is a general property that is applicable to the binding of LFA-1 derived peptides with either the commercial or the synthetic ICAM-1 remains an open question.

From our experiments, the similarity (or the equivalence) of the chemical activities of the synthetic ICAM-1 and that of the commercial one was verified experimentally. These results indicate that the first two domains of ICAM-1 are adequate to bind LFA-1 with high affinity as previously indicated (Staunton et al., 1990). Our results indicated that in the case of LFA-1 and CD11a$_{456–465}$, which exhibited relatively low binding affinity in comparison with CD11a$_{237–261}$, the dissociation constant (K_D) decreased in the presence of magnesium ion; i.e., the interaction affinity is magnesium ion-dependent and increases in the presence of magnesium. In contrast, for the case of CD11a$_{237–261}$, which exhibited higher binding affinity with LFA-1, the dissociation constant (K_D) increased in the presence of magnesium ion. These results confirm that the magnesium ion plays a critical role in the conformation of not only the whole protein but also the peptides, and may thereby change the binding affinity of the molecular interactions.

3.3. The effect of pH in the interaction of human ICAM-1 chimera and LFA-1

After we experimentally verified that the synthetic ICAM-1 (D1D2-Fc) and the commercial one (D1D5-Fc) show comparable binding affinity with LFA-1 and peptides derived from LFA-1,

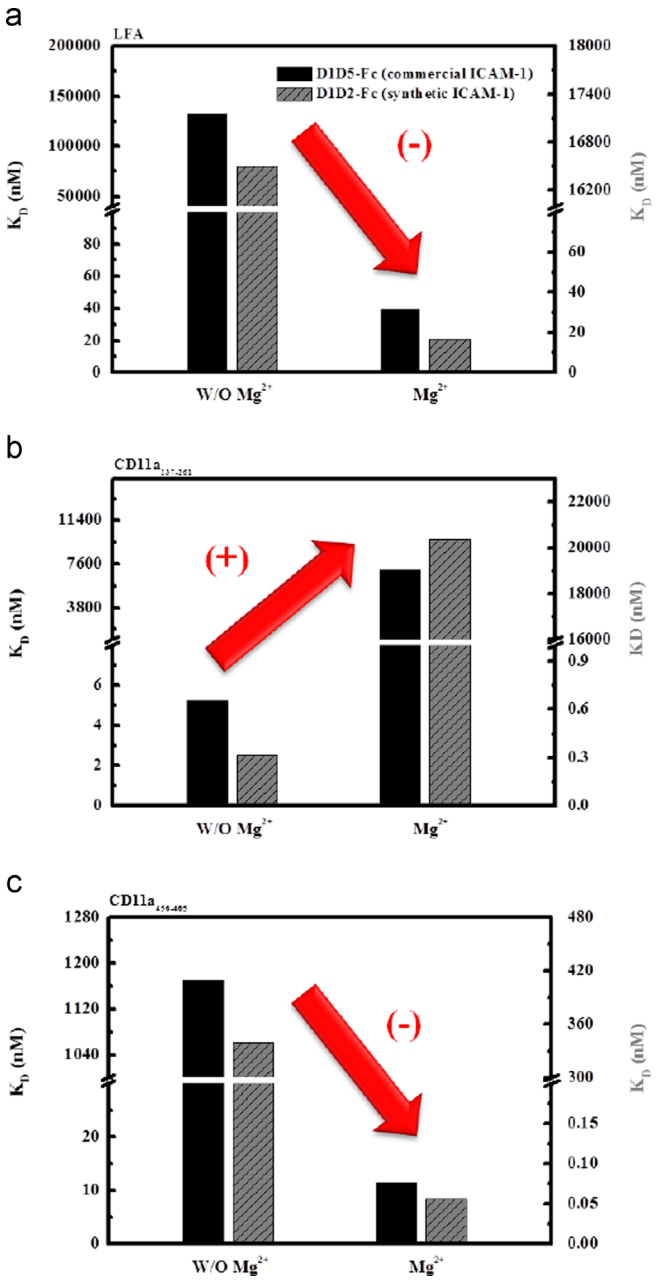

Fig. 2. The binding affinity of commercial ICAM-1 (D1D5-Fc) and synthetic ICAM-1 (D1D2-Fc) with (a) whole LFA-1 protein, (b) $CD11a_{237–261}$ peptide derived from LFA-1, and (c) $CD11a_{456–465}$ peptide derived from LFA-1, in the absence of or presence of magnesium ion at pH 7.4. The vertical scale on the left is associated with the left (dark) bars, and that on the right with the right (gray) bars.

we applied the same method to study the interaction between synthetic ICAM-1 and LFA-1 at different pH without and with magnesium ion to better understand the influence of the microenvironment on the molecular interactions *in vivo* in physiological environment. The pH distribution can be different significantly in pathological vs. normal tissues. As was mentioned earlier, pH is significantly lower in the extracellular matrix of solid tumors (Gerweck et al., 2006) or in inflammation sites (Farr et al., 1985; Fallingborg et al., 1993) than in normal tissue. Notably, it has been described that in colon from patients with active ulcerative colitis pH can be as low as 3 (Fallingborg et al., 1993). Hence, an understanding of the molecular binding affinity at different pH is of interest from the medical point of view. The dynamic sensorgrams obtained from our experiments at different pH environment in the absence of vs. in the presence of magnesium ions are shown in Fig. 3, where Fig. 3(a), (c), and (e) were obtained at pH 7.4, pH 6.5, and pH 4.0, respectively, in the absence of magnesium ions, and Fig. 3(b), (d), and (f) are the corresponding results, respectively, in the presence of magnesium ions. The values of K_D at different pH are summarized in Fig. 3(g), which indicates that (i) K_D decreases (or equivalently, the binding affinity increases) in the environment with lower pH (4.0 and 6.5), independent of the presence or the absence of magnesium ions; (ii) at pH 4.0, the presence of magnesium ions increases the value of K_D (and hence, reduces the binding affinity); (iii) in contrast, in the environment with higher pH, the effect of magnesium ions is exactly opposite, and as expected magnesium ions increase the affinity of ICAM-1/LFA-1 interaction. We notice that while K_D decreases (i.e., the binding affinity increases) in the environment with lower pH (4.0 and 6.5), either with or without magnesium ions, the binding affinity is strongly enhanced in presence of magnesium ion at pH 6.5. The net-regulation by both magnesium ion and pH could be the reason that the dissociation phase does not appear in Fig. 3(d)–(f). We believe these differences provide useful information about how the interaction of ICAM-1/LFA-1 is jointly regulated by ion and pH in the environment. Altogether these data indicate that at pH lower than physiological, the interaction of ICAM-1 and LFA-1 is higher.

The sensograms show the results after the subtraction of the signal from the reference channel to remove the solvent or bulk shift during the SPR measurement. We speculate that the extra-ordinary steep slopes in the association and the dissociation phases shown in Fig. 3(c), in the case of pH 6.5 and in the absence of magnesium ion, may reflect fast binding kinetics and unstable binding, respectively. Apparently, the extra-ordinary steep slopes do not appear in all the other conditions that have been examined.

3.4. Inhibition test of peptide to the interaction of ICAM-1/LFA-1

Our data suggest that in acidic pH microenvironments, leukocytes could show a higher adhesion and activity due to a higher ICAM-1/LFA-1 interaction. As a consequence the inflammatory reactions could be enhanced and the efficacy of potential inhibitors reduced. For potential applications of LFA-1 derived peptide as the peptide drug to intervene with the interaction between ICAM-1 and LFA-1, we tested the following competition assay. 500 nM of LFA-1 and $CD11a_{237–261}$ (high affinity peptide) in the concentration of 0.1, 1.0, and 6.0 μM were mixed and applied to the sensor chip surface coated with ICAM-1, and compared with the standard drug, lovastatin, in the concentration of 10 and 100 μM. In order to evaluate the inhibition efficiency, and to take into account the low pH environment at pathological lesions, the inhibition test was conducted at pH from acidic to normal. Our results show that the amount of LFA-1 binding with ICAM-1 on sensor surface was indeed reduced by both the lovastatin and $CD11a_{237–261}$ peptide (dynamic curves not shown) under all tested conditions. Identical experiment with 500 nM of LFA-1 without any peptide drug and/or lovastatin was repeated, and the result served as the control for 0% inhibition. The inhibition efficiency (in %) is normalized to the control signal by Eq. (1) and summarized in Fig. 4.

$$\text{Inhibition efficiency}(\%) = \left(\frac{R_{\text{control}} - R_{\text{exp}}}{R_{\text{control}}}\right) \times 100\% \quad (1)$$

where R represents the ratio of the value of sensor response from the residual molecules on the sensor surface after washing and the corresponding initial value. The results are summarized in Fig. 4. Inhibition efficiency of each inhibitor at each concentration and pH is tabulated in Supplementary material. In the case of lovastatin, the inhibition efficiency, in the absence of magnesium ion,

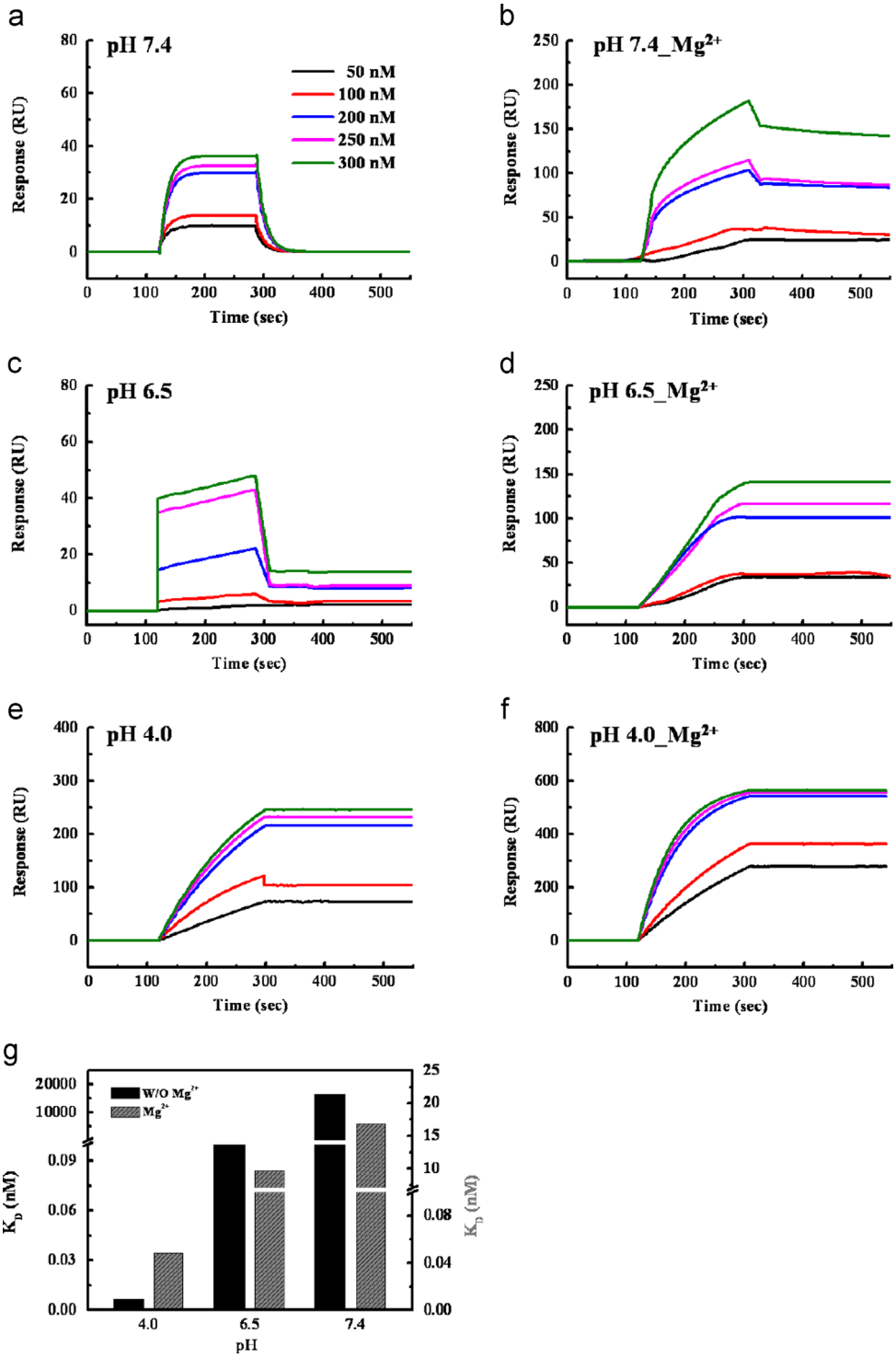

Fig. 3. The sensorgrams from the SPR sensor chip showing the dynamic interaction between synthetic ICAM-1 and LFA-1 (50, 100, 200, 250, and 300 nM, indicated by different colors as shown in the upper right of (a)) at different pH environments in the absence of ((a), (c), (e)) vs. in the presence of ((b), (d), (f)) magnesium ions. (g) A summary of (a) to (f); the vertical scale on the left is associated with the left (dark) bars, and that on the right with the right (gray) bars.

increased from approximately 10% to 47% when the pH value was reduced from 7.4 to 6.5; and the presence of magnesium ion did enhance the inhibition efficiency at both pH 7.4 and pH 6.5. At each pH, 1.0 μM of $CD11a_{237–261}$ showed inhibition efficiency comparable to 100 μM of lovastatin. We observed that the $CD11a_{237–261}$ inhibition efficiency decreased at the concentration of 1 and 6 μM in the presence of magnesium ion at pH 7.4; a plausible explanation lies in our previous experimental result, discussed earlier in association with Fig. 2, which indicates that the magnesium ion increases the binding affinity of ICAM-1/LFA-1 but decreases that of ICAM-1/$CD11a_{237–261}$.

We have compared the data for cases with vs. without magnesium ion both in lovastatin and peptide inhibitions by Student's *t*-test. (Cases where the inhibition effect is reduced in the presence of magnesium ions are not of practical interest, and are not statistically analyzed and compared). We do observe significant differences between cases with vs. without magnesium ion in both low and high concentration of lovastatin at pH 7.4 and

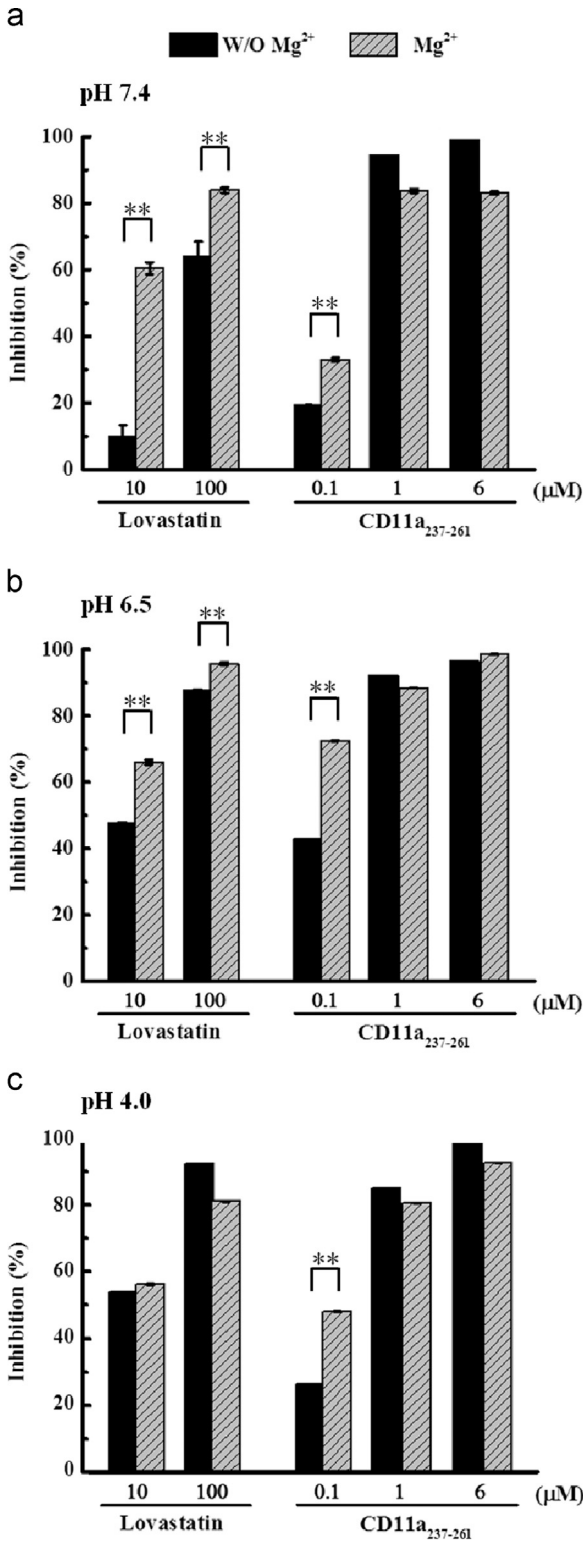

Fig. 4. A summary of the results of the inhibition efficiency of lovastatin vs. $CD11a_{237-261}$ peptide to the ICAM-1/LFA-1 interaction at (a) pH 7.4, (b) pH 6.5, and (c) pH 4.0, without vs. with magnesium ion. Significant differences in inhibition efficiency in cases with vs. without magnesium ion (Mg^{2+}) are indicated by **for $p < 0.001$.

pH 6.5. In the peptide ($CD11a_{237-261}$) inhibition, the differences are significant only in the low concentration (0.1 μM). The peptide does show smaller difference in inhibition efficiency in cases with vs. without magnesium ion; in summary, compared with lovastatin, peptide inhibition is less dependent on magnesium ion.

3.5. Inhibition test in cell based aggregation assay

The LFA-1 derived peptides as inhibitor to the interaction of ICAM-1/LFA-1, summarized in Fig. 4, shows that the inhibition efficiency is comparable to that of the standard drug, lovastatin. We further applied the peptides into the cellular system to evaluate the inhibition efficiency of cell homotypic aggregation of non-adherent cells at different pH. LFA-1 expression and activation was induced in the Epstein Barr Virus (EBV) transformed human B lymphoblastoid cell line R69 by incubation with 10 ng/ml PMA for 2 h at 37 °C. The images of cell aggregation assay at pH 7.5 are shown in Fig. 5(I, a–e) and at pH 6.5 are shown in Fig. 5(I, f–j), for cells without PMA treatment (a, f); D1D2-Fc 10 μg (b, g); EGTA 10 mM (c, h); $CD11a_{237-261}$ (LFA-1 derived peptide with high affinity to ICAM-1) 250 μM (d, i); $CD11a_{441-465}$ (LFA-1 derived peptide with intermediate affinity to ICAM-1) 250 μM (e, j). The cell aggregation at pH 7.5 and pH 6.5 were ranked by clumping index from 1 to 10 based on their relative degree of aggregation in comparison with the PMA-stimulated cells (positive control, clumping index = 10), as shown in Fig. 5(II). The clumping index represents the degree of cell aggregation which reflects the inhibition efficiency of peptides. A higher clumping index means a stronger interaction between cells due to a lower peptide inhibition efficiency. The soluble quimera D1-D2-Fc completely inhibited cell aggregation indicating that this process was dependent on ICAM-1/LFA-1 interaction. The clumping index of peptides from LFA-1, the protein D1D2-Fc and EGTA at pH 6.5 are higher than those at pH 7.5. It means at pH 6.5, the inhibition ability is lower and the interaction of ICAM-1/LFA-1 is stronger, compared with those at pH 7.5. These results are consistent with molecular interactions studied by SPR discussed in Section 3.3 (see Fig. 3), which show that the K_D of ICAM-1/LFA-1 decreases (or equivalently, the binding affinity increases) in the environment with lower pH. Importantly, the high affinity peptide still retains its ability to significantly reduce cell aggregation at low pH suggesting that this type of inhibitors would be effective even in microenvironments where the pH is more acidic.

4. Conclusion

In this work the binding affinity of synthetic ICAM-1 molecules (D1D2-Fc) with LFA-1 was compared with that of the commercial ICAM-1 molecule (D1D5-Fc) via a label-free BIAcore SPR detection system. The binding affinity was evaluated at different pH (pH 7.4, 6.5, and 4.0) to mimic the microenvironment of molecular interactions *in vivo* in normal and pathological tissues. Our results show that, in general, the binding affinity of ICAM-1/LFA-1 is higher at low pH environment, which could be related to molecular mechanisms involved in pathologies like tumor progressing and metastasis or inflammatory disorders like colitis and arthritis. Indeed, ICAM1/LFA-1 interaction has been shown to be crucial in these disorders and a plausible hypothesis is that increasing interaction affinity by low pH would enhance migration and adhesion of leukocytes, which would enhance inflammation and tissue injury.

To our knowledge, this is the first kinetic analysis study of the interaction between ICAM-1 and LFA-1 at different pH values. The reason for stronger binding in acidic pH buffer system may relate to the protein isoelectric point effect and the associated change in protein structure. The affinity between molecules could significantly decrease due to the loss of recognizer-structure (Davies et al., 1990). Acidic pH can have profound effects on the activation of the integrin β3; it has been reported that the interaction of integrin β3 with its ligand increases in environments with acidic pH (Paradise et al., 2011). Apparently, the interaction of the β2

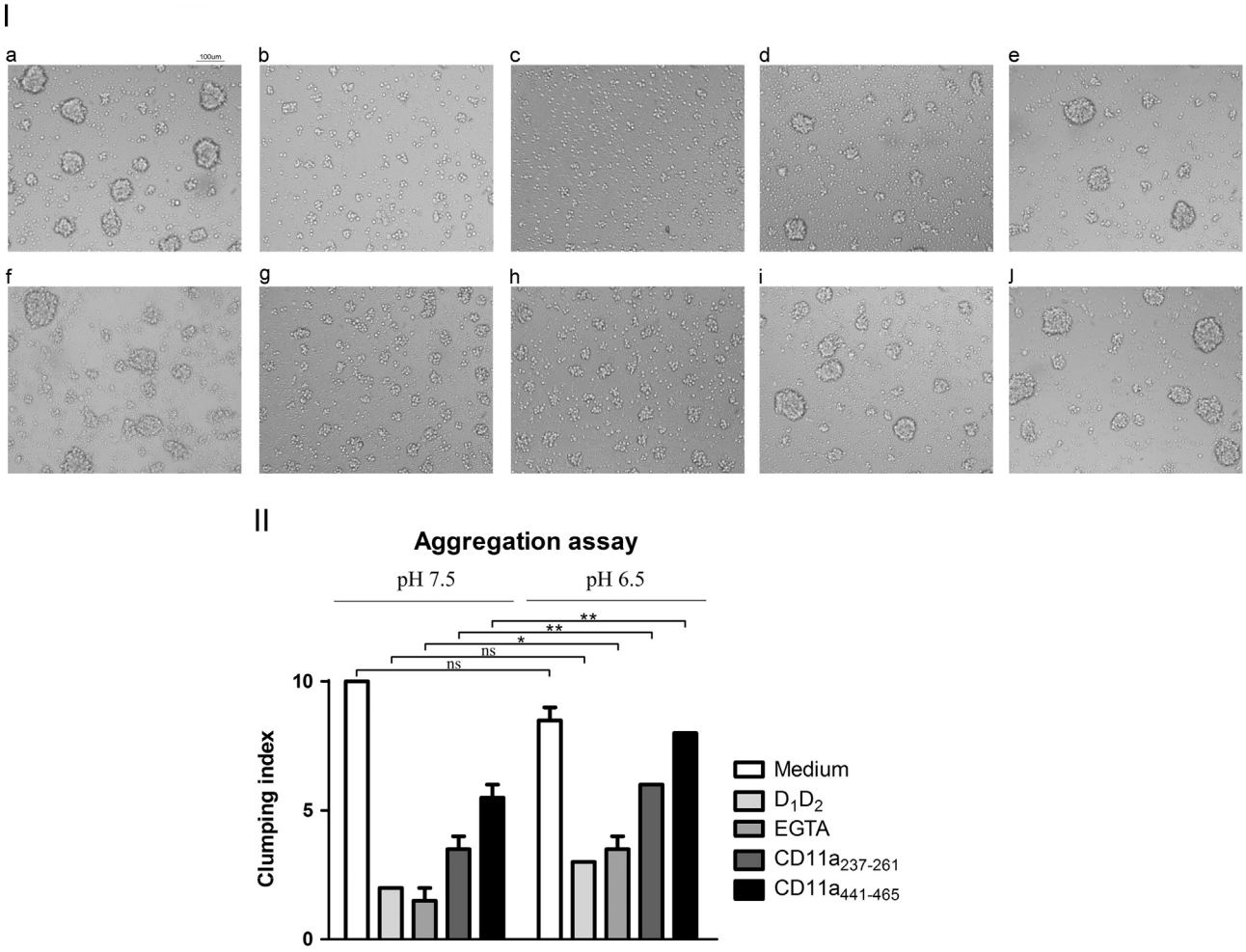

Fig. 5. Inhibition studies of ICAM-1/LFA-1-dependent homotypic aggregation of R69 cells at pH 7.5 and 6.5. R69 cells were stimulated with PMA (10 ng/ml) for 2 h. After pipetted up and down several times to break up any pre-existing aggregates, cells were incubated in flat bottom 96-well plates at pH 7.5 (a)–(e) and pH 6.5 (f)–(j) with medium (a) and (f); D1D2-Fc 10 μg (b) and (g); EGTA 10 mM (c) and (h); $CD11a_{237-261}$ 250 μM (d) and (i); $CD11a_{441-465}$ 250 μM (e) and (j). After 6 h at 37 °C from the beginning of the assay, photographs of homotypic aggregation were taken under an optical microscope (original magnification, 10 ×). A representative result of two independent experiments with similar results is shown. (II) Graphic representation of inhibition of R69 cells aggregation by peptides derived from LFA-1 at pH 7.5 and 6.5. Semiquantitative scoring of the clumping index was calculated as described in Section 2.4. PMA-stimulated cell samples, which served as positive control, were arbitrarily assigned a clumping index of 10 which represents a percentage of aggregation between 90 and 100%. The homotypic aggregation was ranked from 1 to 10 based upon degree of clumping relative to PMA-stimulated cells and results were verified by blind, independent ratings performed by a second observer. All experiments were performed at least two times with similar results. * Represents $p < 0.05$ and ** represents $p < 0.001$.

integrin LFA-1 with its ligand ICAM-1 may show the same trend. Finally, we applied the LFA-1 derived peptides to interfere with the interaction between ICAM-1 and LFA-1 whole protein at different pH (pH 7.4, 6.5, and 4.0) as a first step towards the evaluation of peptide drug efficacy. The peptides work as the inhibitor of the interaction between ICAM-1/LFA-1 not only in pure molecular analysis by SPR detection but also at the cellular level in cell aggregation assay. The results were comparable to that of the standard drug, lovastatin, over a wide range of pH; hence, we conclude that these synthetic peptides may have the potential for further development to become a peptide drug to treat disorders in which low pH may modulate ICAM-1/LFA-1 interaction.

Acknowledgement

This work is jointly supported by The National Science Council, Taiwan, ROC (NSC 99-2923-E-010-001-MY3), by The Spanish National Research Council (CSIC) (2009tw0034), SAF2011-25390 from Spanish Ministry of Economy and Competitiveness. Authors also thank Gobierno de Aragón-Fondo Social Europeo for financial support. The BIAcore 3000 instrument is provided by Research Center for Applied Sciences (RCAS), Academia Sinica, Taiwan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.01.008.

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