Journal of Southeast University (English Edition) Dec. 2001 Vol. 17 No. 2 ISSN 1003—7985

Cell Adhesion Inhibition by RGD Peptides Linked with a Photoisomerizable Nonnatural Amino Acid

Zhang Haiqian[1,2*] Shinohara Hiroaki[2] Gu Ning[1] Sasaki Hiroshi[2] Sisido Masahiko[2]

([1]Laboratory of Wu Chien-shiung, Southeast University, Nanjing 210096, China)

([2]Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Okayama 700-8530, Japan)

Abstract: RGD peptides linked with a nonnatural amino acid, phenylazophenyl alaninine (azoAla), were synthesized and applied to cell adhesion inhibitors. The RGD peptides linked with azoAla at C-terminal showed potent binding to the integrin on the surface of HeLa cells. Photoisomerization effect of the azobenzene side chain of synthesized peptides on the cell adhesion inhibition was further investigated. It was demonstrated that the cis-form of azoAla-linked RGD peptides revealed a little weak cell adhesion inhibition effect as compared with trans-form of azoAla-linked RGD peptide.

Key words: nonnatural amino acid, cell adhesion, photoisomerization, integrin, peptide

A family of cell surface adhesion receptors, referred to integrins[1], has been extensively studied recently[2-4], and is considered to be responsible for the adhesion of cell to cell or cell to extracellular matrices (ECMs)[1,5]. The integrin is structurally clarified as an integral plasma membrane heterodimeric glycoprotein consisting of an α-subunit and a smaller β-subunit[5,6]. It is involved in specific adhesive interaction during tumor metastasis and establishment of cell-cell tight contact during tissue organization. It possesses specificity for binding of adhesion proteins which contain short hydrophilic amino acid sequence arginine-glycine-aspartic acid (RGD) which has been experimentally confirmed to play a central role in cell adhesion.

The specific recognition and binding among the integrin and the cell adhesion protein such as fibronectin is of great interest concerning application of RGD peptides as cell adhesion inhibitors[1,5,7] to some integrin-related pathological process, such as thrombosis[8], osteoporosis[9,10], and tumor metastasis[11]. Consequently, the synthetic RGD peptides acting as the ligands for integrins have been extensively studied. So far, diverse RGD peptides with various length of residuals or followed by different residual sequences have been synthesized and , in particular, compared concerning their abilities in binding to the integrin. Furthermore, conformation of RGD-containing peptides was speculated in the specific interaction. A qualitative model was proposed by R. Haubner et. al.[12,13] to describe the binding in which steric restriction, ionic interaction, hydrogen bond and hydrophobic interaction were concerned. Of which the hydrophobic interaction between the forth residual, X, in RGDX-peptides and the integrin is the most interesting and confirmed to be capable of enhancement of the binding. Accordingly, variation in the hydrophobicity and/or hydrophilicity of the forth residual X can result in change[12,13] of the binding and be potentially applied to control cell adhesion on adhesion protein-coated substrates.

On the other hand, in our laboratory functionalization of peptides and proteins have been performed by chemical or biochemical incorporation of a nonnatural amino acid which contains a group being sensible to external signals, such as photoirradiation. Previously a nonnatural amino acid carrying an azobenzene group, L-p-(phenylazo)phenylalanine (abbreviated to azoAla)[14], had been synthesized and linked to N^6-carboxymethyl-substituted NAD^+ to perform control of the azoAla-NAD^+ mediated enzyme reaction with antibody against the trans-azobenzene group[15]. These experimental data suggested that conformation change of azoAla by UV irradiation might be available to achieve biofunctional control.

In this study, an azobenzene side chain-carrying nonnatural amino acid, L-p-(phenylazo)phenylalanine was incorporated into C-terminal or N-terminal of RGD-containing peptides by solid phase peptide synthesis (SPPS). The synthesized peptides might be of both light-sensible characteristic due to azobenzene side chain and specific binding characteristic due to the RGD motif. Therefore, we had tried to use the synthesized peptide to control adhesion of human

Received 2001-06-27.

* Born in 1963, male, associate professor, doctor.

cervical carcinoma (HeLa) cells on the fibronectin-coated substrates and considered whether the isomerization of the azobenzene side chain, trans to cis, could result in distinguishable effects for cell adhesion inhibition by conformational transformation and hydrophobicity change. It was expected that the trans-cis conformation change by UV irradiation induced the decrease of steric hindrance for the binding and the reduction of hydrophobic interaction, decreasing likewise the binding. Our results showed that the RGD peptides linked with azoAla at C-terminal had potent cell adhesion inhibition effect as compared to the other commercial available RGD peptides, whereas the difference of inhibition effect was a little between trans- and cis-form. This research is the first step to develop the intelligent peptide drugs which are responsive for external signal.

1 Experiment

1.1 Syntheses of RGD peptides

The RGD peptides linked with azoAla were synthesized by solid phase peptide synthesis (SPPS). A support, Fmoc-NH-SAL Resin (0.05 g, 0.025 mmol NH_2 group) (WataNabe chemical industries, LTD), was used in peptide syntheses. The normal procedure for peptide synthesis is as follows. Deprotection of Fmoc group was carried out with 20 % piperiding in N, N-dimethylformamide (DMF) for 10 min; Ninhydrin test was used to indicate that the protecting group was removed from amino group which was now ready for coupling of the next Fmoc-amino acid. The Fmoc-amino acid was coupled to the amino group for 1 h in DMF solution containing coupling reagents: 1-hydroxybenztriazole (HOBt), N,N-diisopropylethylamine (DIEA) and benzotriazol N-oxytrisdimethylaminophosphonium hexaflurorophosphate (BOP). Ninhydrin test assured if the coupling reaction was complete or not. Repetition of the above steps could result in peptide with sequence as designed; finally, cleavage of peptide was executed with trifluoroacetic acid (TFA) containing scavengers, m-cresol and thioanisol (m-cresol : thioanisol : TFA was 2 : 12 : 86).

In this study, we manually synthesized four RGD peptides: Gly-Arg-Gly-Asp-NH_2 (GRGD), Gly-Arg-Gly-Asp-azoAla-NH_2 (GRGDazoAla), azoAla-Gly-Arg-Gly-Asp-NH_2 (azoAlaRGD) and Gly-Arg-Gly-Asp-Phe-azoAla-NH_2 (GRGDFazoAla). These peptides were purified by HPLC with ODS column and identified by molecular weight with MALDI-TOF-MASS (PE Biosystems, Voyger DEPro). Their structures are schematically represented in Fig. 1.

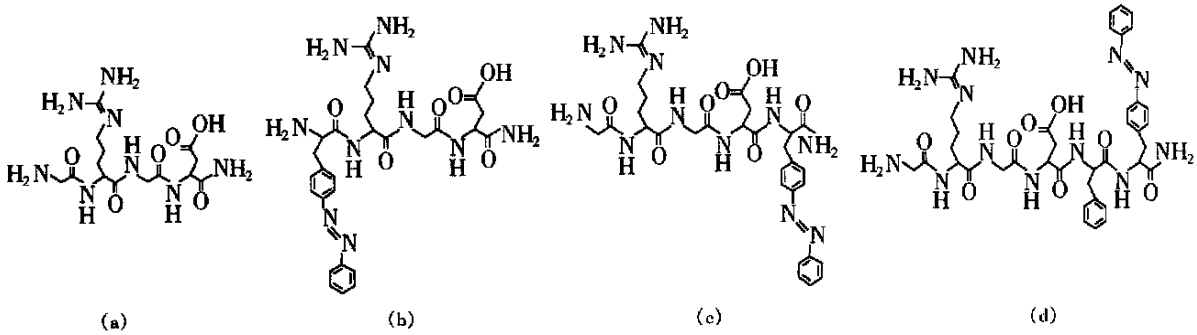

Fig. 1 Chemical structures of GRGD peptide and the synthesized RGD peptides linked with azoAla.
(a) GRGD; (b) azoAlaRGD; (c) GRGDazoAla; (d) GRGDFazoAla

1.2 Cell culture

The human cervical carcinoma (HeLa-S3) cells were obtained from RIKEN Cell Bank (Wako, Saitama), and were cultured in dulbecco's modified eagle's Medium (DMEM) (Gibco BRL) containing 10 % FCS. Cells were harvested for experiment during the logarithmic growth phase.

1.3 Cell adhesion and the inhibition measurements

Fibronectin was coated on the bottom of a 96-well microtiter plate for the purpose of evaluating the inhibition effect of the peptides for HeLa cell adhesion. The optimal concentration of fibronectin for cell adhesion was experimentally shown to be about 10 mg/L (data was not shown). Thus, fibronectin solution of 10 mg/L was always used to coat the bottom of 96-well plate in relevant cell adhesion experiments. The fibronectin-coated plates were incubated overnight at 37 ℃; the coated surfaces were washed with phosphate buffer saline (PBS, pH7.4) twice and then blocked with 1 % bovine serum albumin (BSA) for 2 h at 37 ℃. HeLa cells were added to each fibronectin-coated well (5×10^4 cells/100 μL, 100 μL/well). The cells were incubated for 2 h at 37 ℃.

Twice washing with PBS and aspiration removed those unattached cells. Immediately after that, fresh DMEM medium of 100 μL was added to the cell-attached wells, and at the same time 10 μL of WST-1 cell counting kit solution (Dojindo, Kumamoto) was pipetted into the wells. After 4 h incubation at 37 ℃, absorbance of each well was monitored with Model 450 Microplate Reader (Bio-Rad) to measure the number of cells attached on the coated bottom. The synthesized RGD peptides, as antagonists to ECMs, were dissolved in a PBS solution (pH7.4) and injected into wells before HeLa cell plating when the adhesion inhibition effect of the peptides was investigated. The inhibitory ratio could be used to evaluate the specific binding of the peptides to cells.

2 Results and Discussion

2.1 Photoisomerization of GRGDazoAla by UV/vis light irradiation

Fig. 2(a) shows the absorption spectra of the GRGDazoAla. Large absorption at around 340 nm of spectrum t_1 attributes to trans-form of azobenzene side chain of azoAla. And the absorption decreased at 240 nm and absorption increased at around 425 nm (spectrum c_1) after the irradiation of light with wavelength of 340 nm indicated the conformation change from trans-form to cis-form in azobenzene part. Furthermore, it was demonstrated that photoirradiation at 425 nm induced the reversible conformation change to trans-form (t_2). From these results, it was confirmed that the azobenzene side chain of the synthesized peptides maintained the reversible photoisomerization function.

2.2 Stability of the cis-form of GRGDazoAla

The cis-form of GRGDazoAla can be preserved for over 2 h under dark at room temperature. Furthermore, we also examined stability of cis-form by putting the peptide solution in an incubator at 37 ℃ for 2 h and found little change in absorption spectra for the peptide in cis-form, as shown in Fig. 2(b). Therefore it was considered that the cis-form was stable for at least 2 h under dark even at 37 ℃.

Synthesized peptides and some commercial available ones were compared for their inhibition effect on adhesion of HeLa cells to fibronectin in dose-dependent manner, shown in Fig. 3. From the figure, it was shown that GRGDFazoAla and GRGDazoAla peptides have quite strong inhibition effect on adhesion of HeLa cells to fibronectin as compared with the other RGD peptides. Upon preparing the peptide solution, it was found that the water solubility of the peptides was decreased in the order of GRGD, GRGDazoAla and GRGDFazoAla. This result is in good agreement with the hydrophobicity of the side chain of the forth residual that can enhance the binding of the peptide to the integrin. On the other hand, azoAlaRGD in which azoAla was linked at *N*-terminal of RGD peptide, showed low inhibition in spite of hydrophobicity of azoAla.

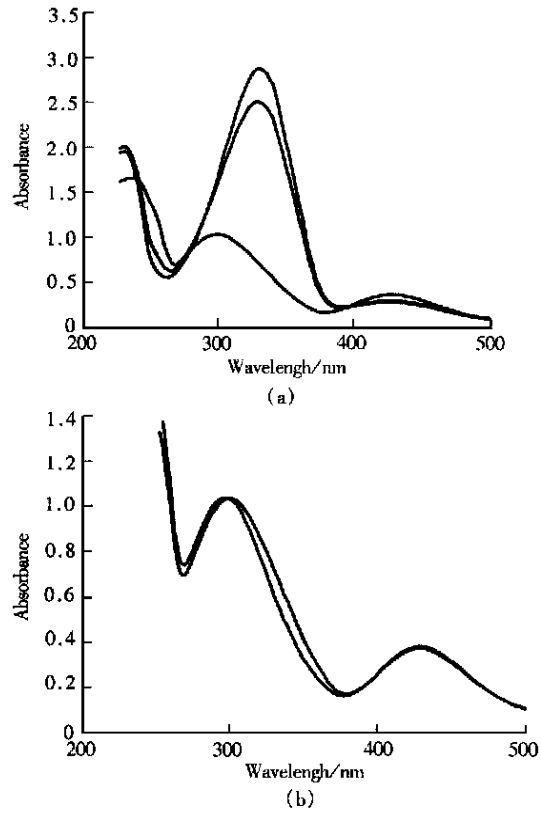

Fig. 2 Absorption spectra of the GRGDazoAla peptide. (a) The conformational change from trans-form (t_1) to cis-form (c_1) was carried out by irradiation of light of 340 nm and the reverse conformation change to trans-form, t_1, t_2 and c_1 (from top to bottom); (b) GRGDazoAla in cis-form before (lower curve) and after (upper one) being kept at 37 ℃ in dark incubator for 2 h

The effect of photoisomerization of azoAla in the peptides on the cell adhesion was furthermore investigated. Fig. 4(a) and Fig. 4(b) show the influence of trans-cis conformation change in the GRGDazoAla and the GRGDFazoAla, respectively on HeLa cell adhesion inhibition. The influence of trans-cis conformation change of azoAla was investigated at not only high inhibition on the HeLa cell adhesion inhibition concentration but also low inhibition concentration to confirm the conformation effect with the consideration for the difficulty of 100 %

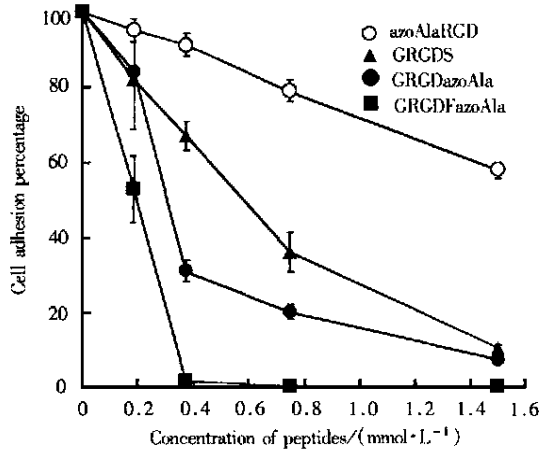

Fig. 3 Cell adhesion inhibition by the RGD-containing peptides in dose-dependent manner

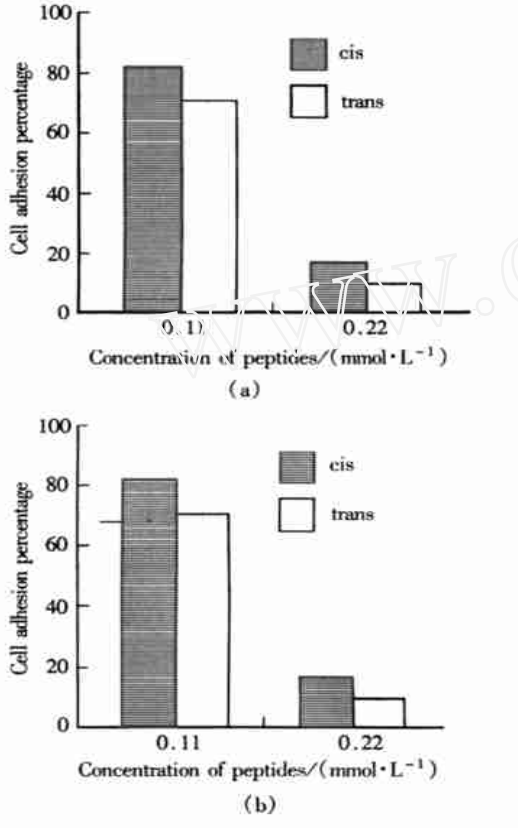

Fig. 4 Influence of trans-cis conformational change. (a) GRGDazoAla; (b) GRGDFazoAla peptides

of photoisomerization from trans- to cis-form. From Fig. 4 (a) and (b), it was indicated that trans- to cis-isomerization of azoAla in the peptides induced the decrease of cell adhesion inhibition effect, though the influence was not great as we expected. In RGD-containing peptide, RGD motif, as recognition unit by the integrin, plays a central role in cell adhesion. It is well known that substitution of any residual in the unit leads to loss of the binding. The side chains of arginine and aspartic acid are believed to be ionic interaction with the integrin[13]. It was also demonstrated that the hydrophobic interaction of the forth residual of RGDX peptides with the integrin could enhance the binding[12,13], whereas the linkage of the hydrophobic amino acid at the *N*-terminal site of the RGD motif shows little influence on the binding. This might be ascribed to the structure of the integrin. The glycine is likewise important in the binding and offers a steric restriction on orientation of the side chains of arginine and aspartic acid. Dynamic simulation reveals that RGD takes a β-type structure[15] where both the side chains of arginine and aspartic acid locate at the same flank of the peptide. A favorite structure of GRGDazoAla tends to be a hemi-circle with the side chains of arginine and aspartic acid extra-orientation and, rather, the side chain of azoAla intra-orientation. So the conformationl change of azoAla cannot form an efficient steric hindrance to the binding of the peptide to the integrin. Photoisomerization effect of GRGDF-azoAla on cell adhesion inhibition was also a little, although the side chain of azoAla was expected to be on the same flank as the side chains of arginine and aspartic acid. This result implicates that distance between the ionic interaction region and conformational change one of azoAla might be an important factor in the steric hindrance. In addition, the variation in the hydrophobicity of azoAla is not large enough to affect the binding in this situation. These results suggest that the cell adhesion undergoes influences from the conformational and hydrophobic changes of azoAla, however it can be compensated by adjustment of the peptide's structure because of its flexibility.

3 Conclusion

RGD peptides linked with the nonnatural amino acid, azoAla, were manually synthesized with SPPS. These peptides exhibit photoisomerization characteristic. Moreover, their specific binding to the integirn was greatly enhanced by linked azoAla at *C*-terminal due to the hydrophobicity of azobenzene part. However, photoisomerization has a little effect on the cell adhesion inhibition. It may be explained by high flexibility of the synthesized small peptide. In the next step, more rigid peptide structure will be designed to perform enough control of biological activity of azoAla-incorporated peptides.

References

1 R. O. Hynes, Integrins: a family of cell surface receptor, *Cell*,

vol. 69 , pp. 11 - 25 , 1992
2 B. Michael ,P. Robert , and S. Dean , Characterization of the integrin $\alpha_v\beta_6$ as a fibronectin-binding protein , *J. Biol. Chem.* , vol. 267 , no. 9 , pp. 5790 - 5796 , 1992
3 W. Ann ,C. Aileen , A. Michael , and R. I. Cone , Steve nishimura , W. Elizabeth , P. Robert and S. Dean , *J. Biol. Chem.* , vol. 269 , no. 9 , pp. 6940 - 6948 , 1994
4 R. O. Hynes , and A. D. Lander , Integrins: variety , versatility , and interactions in cell adhesion , *Cell* , vol. 68 , pp. 303 - 322 , 1992
5 E. Ruoslahti , and M. D. Pierschbacher , New perspectives in cell adhesion: RGD and integrins , *Science* , vol. 238 , pp. 491 - 497 , 1987
6 M. E. Hemler , VLA proteins in the integrin family: structures , functions and their role on leukocytes , *Annu. Rev. Immunol.* , vol. 8 , pp. 330 - 3309 , 1990
7 E. Ruoslahti , Fibronectin and its receptor , *Annu. Rev. Biocheml.* , vol. 57 , pp. 1948 - 1954 , 1988
8 Z. R. Gan , R. J. Gould , J. W. Jacobs , P. A , Friedman , and M. A. Polokoff , Echistatin , a potent platelet aggregation inhibitor from the venom of viper , Echis carinaus , *J. Biol. Chem.* , vol. 106 , pp. 19827 - 19832 , 1988
9 K. Nakamura , M. Nashimoto , Y. Hori , and M. Yamamoto , Serum 25-hydroxyvitaminD concentrations and related dietary factors in peri- and postmenopausal Japanesewomen. American journal of clinical nutrition , vol. 71 , no. 5 , pp. 1161 - 1165 , 2000
10 K. Kamezawa , Inhibitory effects of combined treatment with vitamin K and D on bone loss of ovariectomized rats: a microradiographic study , *Fukuoka Igaku Zasshi* (In Japanese) , vol. 90 , no. 3 , pp. 71 - 78 , 1999
11 K. R. Gehlsen , W. S. Argraves , M. D. Pierschbacher , and E. Rouslahti , Inhibition of in vitro tumor cell invasion by Arg-Gly-Asp-conaining synthetic peptide. *J cell Biol.* , vol. 106 ,pp. 925 - 930 , 1988
12 R. Haubner , R. Gratias , B. Diefenback , S. L. Goodman , A. Jonczyk , and H. Kessler , Structural and functional aspects of RGD-containing cyclic pentapeptides ashighly potent and selective integrin $\alpha_v\beta_3$ antagonists , *J. Am. Chem. Soc.* , vol. 118 , no. 31 , pp. 7461 - 7472 , 1996
13 R. Haubner , W. Schmit , G. Holzemann , S. L. Goodman , A. Jonczyk , and H. Kessler , Cyclic RGD peptides containing β-turn mimetics. *J. Am. Chem. Soc.* , vol. 118 , no. 34 , pp. 7881 - 7891 , 1996
14 H. Shinohara , H. Q. Zhang , and Masahiko Sisido , Design and Synthesis of Photoresonsible Intelligent Bioactive Peptides , In: *Proceeding of the* 10*th Symposium on the Intelligent Materials* , Tokyo , Abstract No. B2 , pp. 62 - 63
15 T. Hohsaka , K. Kawashima , and M. Sisido , Photoswitching of NAD^+-mediate enzyme reaction through photoreversible antigen-antibody reaction , *J. Am. Chem. Soc.* , vol. 116 , no. 1 , pp. 413 - 414 , 1994
16 Y. Wang , S. Y. Goh , and K. Kuczera , Molecular dynamics study of disulfide bond influence on properties of an RGD peptide , *J. Pept. Res.* , vol. 53 , no. 2 , pp. 188 - 200 , 1999

连接光异构化非天然氨基酸的 RGD 肽抑制细胞的附着

张海黔[1,2] 篠原宽明[2] 顾 宁[1] 佐々木裕司[2] 宍户昌彦[2]

([1] 吴健雄实验室 , 东南大学 , 南京 210096)
([2] 生物科学与技术系 ,冈山大学 ,冈山 700 - 8530 , 日本)

摘 要 利用固相肽合成方法 ,将带有光敏基团偶氮苯的非天然氨基酸 ——苯基偶氮苯基苯丙氨酸与 RGD 肽连接 ,得到具有光敏功能的 RGD 肽. 非天然氨基酸连接在 C-端的 RGD 肽与 HeLa 细胞表面的整合素有很强的结合能力. RGD 侧链上的光敏基团的光致异构化对 RGD 肽与 HeLa 细胞表面的整合素的结合有一定的影响.

关键词 非天然氨基酸 ,细胞附着 ,光致异构化 ,肽
中图分类号 Q517 ; Q518. 4