SCIENTIFIC ARTICLE

Isotopic Tracing for Calculating the Surface Density of Arginine–glycine–aspartic acid-containing Peptide on Allogeneic Bone

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Objective: To investigate the feasibility of determining the surface density of arginine–glycine–aspartic acid (RGD) peptides grafted onto allogeneic bone by an isotopic tracing method involving labeling these peptides with ^{125}I , evaluating the impact of the input concentration of RGD peptides on surface density and establishing the correlation between surface density and their input concentration.

Methods: A synthetic RGD-containing polypeptide (EPRGDNRY) was labeled with ^{125}I and its specific radioactivity calculated. Reactive solutions of RGD peptide with radioactive ^{125}I -RGD as probe with input concentrations of 0.01 mg/mL, 0.10 mg/mL, 0.50 mg/mL, 1.00 mg/mL, 2.00 mg/mL and 4.00 mg/mL were prepared. Using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a cross-linking agent, reactions were induced by placing allogeneic bone fragments into reactive solutions of RGD peptide of different input concentrations. On completion of the reactions, the surface densities of RGD peptides grafted onto the allogeneic bone fragments were calculated by evaluating the radioactivity and surface areas of the bone fragments. The impact of input concentration of RGD peptides on surface density was measured and a curve constructed.

Results: Measurements by a radiodensity γ -counter showed that the RGD peptides had been labeled successfully with ^{125}I . The allogeneic bone fragments were radioactive after the reaction, demonstrating that the RGD peptides had been successfully grafted onto their surfaces. It was also found that with increasing input concentration, the surface density increased.

Conclusion: It was concluded that the surface density of RGD peptides is quantitatively related to their input concentration. With increasing input concentration, the surface density gradually increases to saturation value.

Key words: Allogeneic bone; Isotopic tracing; Arginine–glycine–aspartic acid (RGD); Surface density

Introduction

The tripeptide sequence of Arg-Gly-Asp (RGD) reported by Pierschbacher and Ruoslahti in 1984 is present in many kinds of protein¹. It can be recognized by, and combined with, integrin on the cell membrane, thus inducing adhesion between the cell and extracellular matrix and initiating signal transduction across the membrane, thus influencing cellular activity. Because it has been accepted that this peptide sequence promotes adhesion efficiently² and acts on long term

cellular behavior^{3–5}, surface modification with RGD peptides has attracted considerable interest from many biomaterials researchers.

Surface density is a key factor in the biologic activity of RGD. The topography of these peptides determines both primary adhesion and long-term mineralization. When surface density is very low, cell adhesion is impaired, yet crowding can limit a cell's behavior. Much research has focused on determining the optimal surface density of RGD peptides for biological

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activity; this is difficult because there are striking differences attributable to various scaffold materials, molecular weights of peptides and varying research methods^{6–10}.

In the present study, we grafted RGD-containing peptides labeled with ¹²⁵I onto allogeneic bone. Our aims were to: (i) investigate the feasibility of determining the surface density of RGD peptides by an isotopic tracing method; (ii) evaluate the impact of input concentration of RGD peptides on surface density; and (iii) establish the correlation between surface density and input concentration of RGDs.

Materials and Methods

Sample Preparation

Allogeneic bone (Ruiao Bio-material, Shanxi, China) was machined to 10.0 mm × 5.0 mm with a thickness of 150 ± 50 μm. The fragments were subjected to repeated saline lavage and then frozen to –80 °C for 1 month, followed by ultrasonic cleaning and freeze-drying for a week. After vacuum packing the pieces were sterilized by γ-radiation (25 kGy).

Preparation of the Tracer Solution

Preparation of ¹²⁵I-RGD

Ten mg of the synthetic RGD-containing peptide (Glu-Pro-Arg-Gly-Asp-Asn-Tyr-Arg, EPRGDNYR, Bomaijie Bio-tech, Beijing, China) was dissolved in 100 μL phosphate buffer (50 μmol/L, pH 7.4). 10 μL Na¹²⁵I (radioactive 1.0 mCi, PerkinElmer, Boston, MA, USA) and 50 μL (1 mg/mL) chloramine T trihydrate were added, the mixture oscillated at room temperature for 15 min and the reaction terminated with 50 μL sodium sulfite (1 mg/mL). The reaction mixture was transferred to a Sephadex-G75 separation column and eluted with phosphate buffer (50 μmol/L, pH 7.4). 70 tubes of the eluent each containing five drops were collected. 50 μL was sampled from each tube and dissolved in 0.3 mL water. The radioactivity of the samples was subsequently measured by a radiodensity γ-counter of SN-6100 model (Hesuo-Rihuan Photoelectric Instruments, Shanghai, China).

Calculation of the Specific Radioactivity

The mixed eluents of the 16th to 20th tubes, which were regarded as the stoste of ¹²⁵I-RGD (close to pure solution with possibly negligible amounts of free ¹²⁵I or RGD), were used to calculate the specific radioactivity by radiodensity γ-counter.

Preparation of the Arg-Gly-Asp Solution

The RGD peptides were dissolved in tri-distilled water at concentrations of 0.01, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/mL in 2 mL for each sample. All samples contained ¹²⁵I-RGD 1.6 μL; and the other portion of the solute was the non-radioactive RGD-containing peptides.

Grafting Reaction

Forty-eight fragments of allogeneic bone were chosen randomly and soaked in sterile deionized water for 24 h at room

temperature. The fragments were then dried, placed 40 cm away from a 20 W Burdick lamp (Siemens, Erlangen, Germany) and irradiated on both sides for 1 h. The irradiated bone fragments (eight fragments per concentration) were soaked in RGD solution at different concentrations. The reaction solution was rocked on a PY-120 shaker (Dingguo Bio-Tech, Beijing, China) for 30 min at room temperature. Care was taken to ensure that the reaction occurred within the pH range 4.5–4.7, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added as a cross-linking agent and the mixture shaken for a further 48 h. Next, all bone fragments were successively washed in tri-distilled water and acetone. After drying, all fragments were measured in the radiodensity γ-counter and the data analyzed by SPSS software.

Results

Radioactive Tracer ¹²⁵I Acts as a Probe for Labeling Arg-Gly-Asp-containing Peptides

The results of measurement of radioactivity of all the eluant tubes after separation are shown in Figure 1. When we separated the reaction mixture of ¹²⁵I-RGD, RGD peptides and free ¹²⁵I through a Sephadex-G75 column, the elution speed was positively correlated with the molecular weight of the solute. The first crest consisted of the 16th–20th tubes and represented the components of ¹²⁵I-RGD and RGD-containing peptides of high molecular weight; the second crest represented free ¹²⁵I. The presence of the first crest confirmed that the RGD-containing peptides had successfully been labeled by the radioactive ¹²⁵I as probe.

In addition, as shown in Figure 1 we found that the radioactivity of the 15th, 21st and 22nd tubes, which were adjacent to the first crest, was higher than that of the other nearby eluants, which means they contained a small amount of ¹²⁵I-RGD and RGD-containing peptides. Therefore the percentage of RGD peptides in the 16th to 20th tubes, which were regarded as the baseline for the following reaction, was 91.38%, this figure being calculated on the basis of the proportion of radioactivity (cpm) in the RGD-containing eluent.

Another important factor is the specific radioactivity (including mass specific and volume specific radioactivity), namely the ratio of radioactivity to mass or volume. Calculations indicated that the mass specific radioactivity was 34.99 ± 0.56 mCi/mmol and the volume specific radioactivity 0.40 ± 0.01 mCi/mL when the efficiency of detection of our γ-counter to ¹²⁵I was 70%.

Steerable Surface Density of Arg-Gly-Asp-containing Peptides Grafted onto Allogeneic Bone Related to Different Input Concentrations

By γ-counter measurements, we found that after the graft reaction the allogeneic bone had a certain amount of radioactivity, proving that the ¹²⁵I-RGD peptides had successfully bound to the bone fragments. The RGD-peptides surface densities were then calculated and are shown in Table 1. Statistical analysis showed heterogeneity of variance between samples of different

Fig. 1 The spectrum of ^{125}I -RGD sephadex-G75.

TABLE 1 Surface density of RGD peptides with different input concentrations

Input concentration (mg/mL)	Surface density (mean $\pm$ SD, nmol/cm ²)
0.01	0.96 $\pm$ 0.22
0.10	7.74 $\pm$ 1.02
0.50	14.26 $\pm$ 1.27
1.00	27.43 $\pm$ 3.83
2.00	34.14 $\pm$ 4.58
4.00	40.20 $\pm$ 4.82

input concentrations. Therefore we used the Tamhane comparison method to confirm that there were no significant differences between the surface density with input concentrations of 1.00 mg/mL and 2.00 mg/mL and that with concentrations of 2.00 mg/mL and 4.00 mg/mL. The differences between samples with input concentrations of 0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1.0 mg/mL differed to a statistically significant degree ($P < 0.05$). On the other hand, we found that RGD surface density was positively correlated with the input concentration ($r = 0.899$, $P = 0.007$). Correlation analysis indicated that the RGD surface density increased in parallel with input peptide concentration between 0.01 mg/mL and 4.00 mg/mL. Through regression analysis of the saturation curve shown in Figure 2, we derived the following formula for

Fig. 2 Curve showing the relationship between surface density and input concentration of RGD peptides.

the relationship between RGD surface density (ρ , nmol/cm²) and peptide input concentration (C, mg/mL):

$$\rho = -(0.0241C + 0.0168)^{-1} + 59.52$$

Discussion

Feasibility and Design Features of Determination of Surface Density of Arg-Gly-Asp-containing Peptides by Isotopic Tracing

Up to now, researchers have used several methods to determine peptide surface density, including isotopic tracing, ellipsometry, total internal reflection fluorescence and surface plasma resonance. Early on, Massia and Hubbell explored determination of peptide surface density by means of a radioactive probe¹¹. In their experiment, 20 μ g GRGDY peptides was labeled with 5.0 mCi Na¹²⁵I, after which the reaction mixture was purified through a Sep Pak C18 separator column. They then measured the absorbance at 220 nm of the eluent samples to determine the content of RGD peptides. The specific radioactivity of the RGD reaction solution was 44.0 ± 2.0 mCi/mmol. Different input concentrations of RGD-containing peptides labeled with ¹²⁵I were grafted onto a glass surface, following which their radioactivity was measured to determine the surface density of the peptides. Subsequently, there have been several studies of RGD peptides surface density that used ¹²⁵I¹², ³⁵S¹³ or ¹⁴C¹³ as probes.

We thought that the radioactive tracer we used was appropriate to our experiment in light of the following allogeneic bone characteristics: non-conducting, light-proof and complex surface ultrastructure. In the labeling reaction, the free ¹²⁵I bound to the peptides by substituting the -H of the benzene ring in tyrosine in a proportion of 2:1 or 1:1. This method has the advantages of less requirement for carrier material and broad scope of application but the disadvantage of being less accurate. Nevertheless, we improved the accuracy by increasing the number of samples and labeling rate, which means the ratio of radioactive production in all the reactants in the labeling reaction. In addition, to improve the specific radioactivity, we chose the most highly radioactive eluent with a high content of ¹²⁵I-RGD for the grafting reaction.

When compounding the RGD reaction solutions, we added the same amount of ¹²⁵I-RGD to every concentration to ensure the same radioactivity in each sample; non-radioactive RGD was used as the remainder of the solute. To improve the accuracy, the sample with the lowest concentration (0.01 mg/mL) consisted completely of ¹²⁵I-RGD (100%, nearly 20 μ g) and the ratio of ¹²⁵I-RGD decreased with increasing concentration. This adjustment may have had a greater impact on the results of high concentration samples and a smaller effect on the results of lower concentration samples. We found that the standard deviation of the surface density was ± 0.22 in the samples of 0.01 mg/mL input concentration and that it increased gradually in parallel with increasing input concentration. For instance, ± 1.02 in samples of 0.1 mg/mL, ± 1.27 in those of 0.50 mg/mL, ± 3.83 in those of 1.00 mg/mL,

± 4.58 in those of 2.00 mg/mL and ± 4.82 in those of 4.00 mg/mL. We thought that this explained the heteroscedasticity. Because many researchers have found that RGD peptides can enhance cell adhesion and differentiation in low surface distributing concentrations, we chose the method described above to ensure accuracy and precision of the data for lower input concentrations and to create favorable techniques for future research into cellular behavior^{3,6,10,11}.

Factors Influencing the Relationship between Arg-Gly-Asp Input Concentration and Surface Density

In our experiment, we determined the relationship between RGD surface density and input concentration by grafting these peptides onto bone fragments at different reaction concentrations. We found a quantitative relationship between the two factors. The impact on surface density was obvious at lower input concentrations (0.01–1.00 mg/mL) but this gradually became less obvious at higher concentrations (1.00–4.00 mg/mL). Thus, there was a point of saturation of surface density when all the active sites on the bone fragments were occupied by RGD peptides. Our curve was similar to those reported by many early studies^{3,11,14,15}.

In our research, the theoretical saturation value of RGD surface density was 59.52 nmol/cm² based on our regression equation, which differs greatly from early reports and is far greater than a magnitude of pmol/cm²^{6,10–14}. Some factors that may explain this are described below.

Surface Chemical Properties of Allogeneic Bone

We grafted RGD peptides onto the surface of allogeneic bone with the cross-linkage agent EDC, which provides double functional groups. Presuming there is an excess of EDC, the number of active sites for binding (as -COOH) on the surface of the allogeneic bone fragments is a crucial determinant of the amount of peptides that adhere. The carriers in previous research were mostly Ti, Si, PLA and PMMA, which have far fewer active sites (such as -COOH, -OH and -NH₂) than does allogeneic bone, a bio-derived material. This factor would lead clearly to greater RGD surface density than that reported previously.

Surface Micromorphology of Allogeneic Bone

The allogeneic bone we used as the carrier for RGD peptides had been treated by degreasing, deproteinization, deep freezing, drying and radicidation. This resulted in elimination of its antigens had been but preservation of the natural network pore system consisting of the original trabeculae, intertrabecular spaces and canal systems of Sandermann. The structure of the pores in this scaffold, which have the complex stereo surface micromorphology depicted in Figure 3 would have made the actual grafting area much larger than the measurable area. That may also have affected the RGD surface density.

Washing Time after Grafting Reaction

After the grafting reaction, we repeatedly washed the bone fragments with water and acetone to remove the peptides

Fig. 3 The complex stereo surface micromorphology of allogeneic bone as shown by scanning electron microscopy ($\times 2000$).

absorbed noncovalently. However, because of the structure of the pores, some RGD peptides may have remained physically attached within them. We prolonged the washing time as much as possible to offset this factor. Chollet *et al.* suggested that the RGD surface density decreases along with the duration of washing time. In their research, the surface density tended to stabilize after 300 h of washing, indicating that washing time may be an influential factor, especially when using allogeneic bone with its unique structure¹⁶.

After our experiments using RGD labeled with ¹²⁵I and the analysis of our results, we were able to determine the RGD peptides surface density on allogeneic bone fragments by measuring the radioactive tracer. Statistical analysis showed that the surface density of RGD peptides is quantitatively related to the input concentration of the peptides. With increasing input concentration, the surface density gradually increases to the saturation value.

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