Effects of endothelin-1 on melanocyte adhesion and migration

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Abstract: Objective To investigate the effect of endothelin-1 (EP-1) on the adhesion and migration of melanocyte in vitro. Methods Human epidermal melanocytes that had been cultured and purified were treated with EP-1 and observed for adhesion to bovine serum fibronectin-coated culture dishes. Stem cell factor (SCF) and EP-1 treated cells were also examined for migration into micropore filters coated with the same protein. Results Compared with the SCF group, EP-1 treated melanocytes were easier to adhere to the dishes and to be moved into the filters, especially when the concentration was 32 nmol/L. When the concentration of EP-1 was 128 nmol/L or more, it could inhibit the adhesion and migration of melanocyte. At any concentration of EP-1 except 2 nmol/L, there was a significant difference between EP-1-treated and untreated melanocytes (P < 0.01) when the adhesion test was carried out. However, even at 2 nmol/L, EP-1, there was obviously increased migration compared with those of EP-1 untreated melanocytes and SCF-treated cells (P < 0.01). Conclusion EP-1 may influence the adhesion and migration of melanocyte, which can partly explain the capacity of EP-1 to regulate the melanocyte function in vitiligo lesions.

Key words: melanocyte; endothelin-1; adhesion; migration

Today, a popular theory is that vitiligo is an autoimmune disease[1]. It is a prevalent and often disfiguring disease of acquired hypopigmentation that is due to destruction of cutaneous melanocytes. Melanocytes normally produce the pigment melanin in melanosomes, which are specialized vesicles found in the endocytic pathway. Not like other autoimmune diseases, many pre-melanocytes situated in hair follicles are not affected by autoantibod-
ies\(^2\). So stimulating the proliferation and the migration of pre-melanocytes in hair follicles is a feasible way to treat vitiligo\(^3\). The previous studies have implicated that many cytokines can promote melanocyte adhesion and migration. For example, integrins\(\alpha_\beta\)\(^4\) can promote melanocyte adhesion to fibronectin; leukotriene C4 and TGF-Alpha can also stimulate melanocyte adhesion and migration\(^5\), etc. Among these we found that ET-1 and SCF had close relation with melanocytes\(^6\), because they can change the secretion of cell stromatin, modulate the matrix connection with cytoskeleton and influence on the level of adhesion molecule obviously, so we choose ET-1 to start our study.

1 MATERIALS AND METHODS

1.1 Chemicals and reagents

Keratinocyte growth media (K-SFM, Gibco, Grand Island, NY), phorbol-12-myristate-13-acetate (PMA), cholera toxin (CT), trypsin, bovine serum albumin and bovine serum were purchased from Sigma (St Louis, MO, USA). The 24-well microtire plates and 6-well microchemotaxis chambers were purchased from Nule-pore (Pleasanton, CA, USA). Endothelin-1 and stem cell factor were purchased from Santa Cruz.

1.2 Human melanocyte’s culture

Modified Esinger and Marko’s way\(^7\), penicillin or anti-fungal drugs were not used since they could affect the growth of melanocytes. In brief, skin comes from neonatal foreskin was co-cultured in keratinocyte growth media added with CT (2 \(\mu\)g/100 ml) and PMA (4 \(\mu\)g/100 ml) since PMA could promote the melanocyte proliferation and CT could inhibit the fibroblast growth selectively. About 11 days later, we could acquire the purified melanocytes. Cells were verified by Dopar-staining. Second generation melanocytes were chosen for our study.

1.3 Adhesive assay\(^8\)

Wells of 24-well culture dish were treated with 10 \(\mu\)g of fibronectin in 0.5 ml serum-free keratinocyte growth media over night. Bovine serum albumin (10 \(\mu\)g) served as a control. At the end of the incubation period, the wells were washed in phosphate-buffered saline to remove unbound protein. The concentrations of ET-1 and SCF were used as follows: 2, 8, 32, 128, and 512 nmol/L. A suspension of 10,000 melanocytes in 400 \(\mu\)l keratinocyte growth media was placed to each well. Control and ET-1-treated melanocytes (for 24 hours) were harvested with trypsin, terminated with melanocyte growth media (10% FCS), washed twice in keratinocyte growth media, and added to the wells in this buffer. The dishes were then incubated at 37 °C for 4 hours, and 4 wells were investigated at every concentration. After that, PBS was used to wash twice to remove the unattached cells. The attached cells were counted under the light microscope. This would be duplicated for 3 times.

1.4 Migration assay

Cell migration was assessed by using the micropore filter method\(^9\). The 8 \(\mu\)m (pore diameter) nitrocellulose filters were coated with 10 \(\mu\)g bovine serum fibronectin in 0.5 ml keratinocyte growth media for 2 hours, then were placed in the holders, which was separated to 2 compartments by the filters. A suspension of 20,000 melanocytes in 1 ml keratinocyte growth media was placed in the upper compartment. The lower compartment was filled with 200 \(\mu\)l of the chemoattractant (ET-1, SCF at varying concentrations). After 4 hours incubation at 37 °C, the cells in the upper surfaces of the filters were mechanically cleaned, and then were fixed and stained to analyze the number of cells that migrated through the filters. Counting of migrated melanocytes was performed by using an image analysis system (100 \(\times\), 6 fields of vision by 2 independent investigators). The cells in 4 wells at every concentration were observed. To each well, it must be duplicated for 3 times.

1.5 Statistical analysis

To detect statistically significant differences among the adhesion of ET-1-treated \(\Delta\) SCF-treated and untreated melanocytes to fibronectin, we used the Student’s \(t\) tests. All are expressed as means ± standard deviation in the text. Significant differences among the chemotactic behaviors of ET-1-treated, SCF-treated, and untreated melanocytes were also evaluated by using the Student’s \(t\) tests, with a two-sided \(p\) value of less than 0.05 considered to indicate statistical significance.
2 RESULTS

2.1 Cultured cells verified by Dopa-staining

Epidermis constituted of keratinocytes, fibroblast, and melanocyte. In process of pure melanocyte culture, keratinocyte and/or fibroblast contamination occurred very commonly; meanwhile cultured melanocyte was similar to cultured fibroblast in shape under light microscope; so it was necessary to identify cultured cells and the common way was Dopa-staining (Fig. 1).

2.2 Effects of ET-1, SCF on melanocyte adhesion to bovine serum fibronectin

We performed 12 (4 wells ×3) times assay for each concentration, but there was no significant differences among 2 nmol/L ET-1 group, 2 nmol/L SCF group and untreated group (Fig. 2) (P > 0.05). One thing should be mentioned that if the concentration of ET-1 was higher than 128 nmol/L, the attached cells could not increase but show decrease. If the concentrations were at 8 32 nmol/L, there was a significant difference between ET-1 group and SCF group (P < 0.01). When the concentrations were higher than 32 nmol/L, ET-1 groups showed inhibition functions on cell adhesion compared with the untreated group or lower concentrations of ET-1 (<32 nmol/L) and SCF, SCF groups also could lift the adhesion cell numbers, but there was no significant difference when the concentration of SCF was higher than 32 nmol/L compared with the lower concentrations of SCF (P > 0.05) (Tab. 1).

| Tab. 1 Effects of ET-1 and SCF on melanocyte adhesion (€ ± s) |
|-----------------|----------------|----------------|----------------|----------------|----------------|
| Groups          | 0              | 2              | 8              | 32             | 128            | 512            |
| ET-1            | 224 ±10        | 226 ±9         | 284 ±13        | 369 ±15        | 178 ±7         | 156 ±6         |
| SCF             | 216 ±10        | 226 ±9         | 223 ±8         | 290 ±12        | 288 ±5         | 291 ±11        |
| P               | > 0.05         | < 0.01         | < 0.01         | < 0.01         | < 0.01         |< 0.01          |

Compared with untreated group , * * P < 0.01

Fig. 1 Outcome of Dopa-staining verifying cultured cells. Nuclears are sky-blue, areas of Dopa oxidase are brown (10 ×10).

Fig. 2 Untreated melanocytes (adhesion assay) (10 ×10). Photograph shows untreated melanocytes adhered to fibronectin.

Fig. 3 ET-1-treated melanocytes (migration assay at 512 nmol/L) (10 ×10). Photograph shows great number of migration cells. Blue dots are pores of the filter.

Fig. 4 SCF-treated melanocytes (migration assay at 512 nmol/L) (10 ×10). Photograph shows fewer number of migration cells. Blue dots are pores of the filter.
2.3 Effects of ET-1 and SCF on melanocyte migration

ET-1 increased melanocyte migration at any concentration compared with untreated cells (P < 0.01) (Fig. 3) and more ET-1 in the media, more migration cell numbers. In SCF group, only when the concentrations were 128 nmol/L or more, we could find a promoting effect on the melanocyte migration (P < 0.05) (Fig. 4). There was no significant difference between 128 nmol/L and 512 nmol/L (P > 0.05). The level of 32 nmol/L ET-1 treated melanocyte was significantly higher than that of 128 nmol/L SCF-treated melanocyte (P < 0.01). All levels of ET-1 group were significantly higher than SCF group at the same level (P < 0.05) (Tab. 2).

Tab. 2 Effects of ET-1 and SCF on melanocyte migration

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentrations (nmol/L)</th>
<th>0</th>
<th>2</th>
<th>8</th>
<th>32</th>
<th>128</th>
<th>512</th>
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<tbody>
<tr>
<td>ET-1</td>
<td>250 ±15 313 ±18 330 ±14 478 ±21 455 ±24 482 ±23 * * *</td>
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<tr>
<td>SCF</td>
<td>250 ±15 248 ±13 261 ±21 246 ±16 328 ±21 Δ</td>
<td>336 ±19 Δ</td>
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<tr>
<td>P</td>
<td>&lt; 0.01 &lt; 0.01 &lt; 0.01 &lt; 0.01 &lt; 0.01</td>
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</table>

* * * Compared with the same concentration of untreated group. * * * P < 0.01. Compared with lower concentration (2, 8, and 32 nmol/L) SCF, ΔP < 0.05

3 DISCUSSION

Vitiligo is well known as an autoimmune disease, but it is unlike other autoimmune diseases which can be treated by systemic use of hydrocortisone. Because of no melanocyte in the lesion, many drugs like MOP, MOP, PUVA, and so on. have been found with the theoretical effect on vitiligo by lifting the number of adhesion or migrations melanocytes, but the real results are little. For skin or cell transplantation, the long-term effect has been found awfully recently. So till today, there are no good measure to treat vitiligo. Studies have told us there are many pre-melanocytes in hair follicle, which are not destroyed by autoantibodies. From the above, we infer that the recovery mechanism of the patients may be as follows:

After being stimulated and activated by some factors, the pre-melanocytes in hair follicle become mature, proliferate, migrate to the lesion, and adhere to fibrous tissue, then proliferate again and produce melanin, and then white lesions recover and become black. Thus, the adhesion and migration of melanocyte to lesions of vitiligo are very important. Stimulating them to proliferate and move to lesions of vitiligo may be the feasible way to solve this problem.

According to the recent reports, many factors can influence the adhesion and migration of melanocyte. Among them, ET-1 and SCF may be the most important, so we chose them firstly to study on cell adhesion and migration. The results showed that, at lower concentrations, ET-1 could promote melanocyte adhesion in a dose-dependent manner (P < 0.01); when the concentrations were beyond 32 nmol/L, melanocyte adhesion was inhibited (P > 0.05). In SCF, from the table 1 we found that only the higher concentration of SCF (more than 32 nmol/L) could promote melanocyte adhesion. Moreover, statistical results showed that there was a significant difference between the lower concentration of ET-1 (8 nmol/L) and the higher concentration of SCF (32 nmol/L) (P < 0.01). So the observation may reflect that the lower concentration of ET-1 was more effective than the higher concentration of SCF.

For cell migration, from table 2 we could find even at the low concentration, ET-1 could promote melanocyte migration, and this displayed a dose-dependent manner (P < 0.01). But in the SCF group, only when the concentration was beyond 128 nmol/L, accelerating effect on melanocyte migration could have a statistical significance. From the above we can say that ET-1 is more effective on melanocyte migration than SCF (P < 0.01), and ET-1 can promote melanocyte migration at any concentration.

Therefore, ET-1 is a good cytokine to melanocyte adhesion and migration. According to the reference about other cytokines that had been studied today, we infer that ET-1 is the most effective cytokine to the adhesion and migration of melanocyte. With the gene technical development, ET-1 may be synthesized artificially and may aid to treat vitiligo.

If ET-1 can be used to treat vitiligo, a good effect will come. So we need some further studies to fulfill this project.

Reference:

Effects of endothelin-1 on melanocyte adhesion and migration [MOU Kaai-lou et al.]


