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Thymosin \( \alpha_1 \) Stimulates Endothelial Cell Migration, Angiogenesis, and Wound Healing

Katherine M. Malinda,* Gurmel S. Sidhu,† Krishna K. Banaudha,‡ Jaya P. Gaddipati,† Radha K. Maheshwari,† Allan L. Goldstein,‡ and Hynda K. Kleinman*1

In wound healing, lymphoid cells release soluble factors that attract fibroblasts and macrophages, initiating repair, endothelial cell migration, angiogenesis, and matrix production. We analyzed the effect of thymosin \( \alpha_1 \) (Th1) on endothelial cell migration, angiogenesis, and wound healing. Th1, a 28 amino acid peptide initially isolated from the thymus, enhanced the morphologic differentiation of endothelial cells and was a potent chemoattractant for endothelial cells and monocytes in vitro. In vivo, Th1 stimulated angiogenesis in a subcutaneous model. When given either topically or i.p., it accelerated wound healing in a punch model, demonstrating that Th1 promotes angiogenesis and wound healing. The Journal of Immunology, 1998, 160: 1001–1006.

Angiogenesis involves endothelial cell attachment; basement membrane degradation; and synthesis, migration, and proliferation. Because of the importance of angiogenesis in tissue formation, wound healing, and in pathologic conditions such as cancer, Wegener’s granuloma, Takayasu’s arteritis, systemic lupus erythematosus, and other autoimmune diseases, identification of factors influencing blood vessel formation is of major importance. We previously found that a thymic-derived protein, thymosin \( \beta_\text{II} \), is angiogenic (1, 2). Here we investigated the role of another thymic peptide, thymosin \( \alpha_1 \) (Th1)2, in angiogenesis and wound repair.

Th1 is a highly conserved 28 amino acid peptide that has been extensively studied as an immunomodulatory factor. It affects the maturation, differentiation, and function of T-cells in vitro and in vivo (3, 4). Th1 is in late stage clinical testing as a treatment for a number of infectious diseases including hepatitis B, C, and HIV, either as a sole therapy or in combination with IFN-\( \alpha \) (5). Studies indicate that Th1 is better tolerated and is as effective as IFN-\( \alpha \) as a monotherapy in the treatment of hepatitis B (6) and can rescue IFN failures with clinical hepatitis B patients (7). It has been reported that in patients with chronic hepatitis C a combination of Th1 and IFN-\( \alpha \) was more effective than IFN alone (8).

Th1 normally circulates in the blood and has several immune functions (9). Th1 has a number of activities as a biologic response modifier in the immune system (10). It can enhance the production of IL-2 and IFN-\( \alpha \) and up-regulate the expression of IL-2 receptors on mitogen-stimulated T-cells (11). Additionally, Th1 down-regulates the activity of terminal deoxynucleotide transferase (TdT) in TdT+ thymocytes, suggesting a role for Th1 in thymocyte maturation (12). Th1 has also been found to antagonize both activation-induced (anti-CD3) and glucocorticoid-induced thymocyte apoptosis (13).

In addition, Th1 has important actions outside the immune system, related to a role for this peptide and its 113 amino acid parent molecule, prothymosin \( \alpha \), in regulating cell proliferation and apoptosis (14). Th1, the N-terminal 28 amino acids of prothymosin \( \alpha \), regulates the proliferation of fibroblasts and human non-small cell lung cancer cells (15, 16). These effects on non-lymphoid cells and the action of Th1 on T-cell development strongly suggest that it is important in other cellular processes.

In this study we investigated the effect of Th1 on HUVEC differentiation in vitro and on migration in vitro and in vivo. Our in vitro results demonstrated that Th1 enhances the formation of tube-like structures by HUVECs cultured on Matrigel. Additional experiments demonstrate that Th1 acted as a chemoattractant stimulating directional HUVEC migration. Boyden chamber assays with a variety of cell types demonstrated that migratory stimulation occurred specifically with endothelial cells and monocytes. Th1 stimulated cell migration in vivo using s.c. implanted Matrigel and stimulated wound healing when applied topically or injected i.p. in a rat wound model. We conclude that Th1 has potent effects on endothelial cell migration, angiogenesis, and wound repair.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly delivered cords as reported previously (17) and grown on Nunc dishes (Nunc, Denmark) in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 20% bovine calf serum (HyClone Laboratories, Logan UT), 100 U/ml penicillin/streptomycin (Life Technologies, Gaithersburg, MD), 50 \( \mu \)g/ml gentamicin (Life Technologies, Gaithersburg, MD), 2.7 \( \mu \)g/ml amphotericin B (fungizone) (Life Technologies, Gaithersburg, MD), 5 \( \mu \)g/ml sodium heparin (Fisher Scientific, Pittsburgh, PA) and...
Tube assay

The tube formation assay was performed (19) with the following modifications. Twenty-four-well Nunclon plates were coated with 320 μl of Matrigel (10 mg/ml) and incubated at 37°C for 30 min to promote jelling. 30,000 HUVECs were resuspended in growth medium and added to each well with 1, 10, 100, and 1000 ng of synthetic Tα1, 20 and 200 μg/ml ECGS (a bovine brain extract that contains αFGF and bFGF and acts as a positive control), or medium alone. The final serum concentrations were 10% or 5%. After 18 h, plates were fixed with Diff-Quik (Baxter Healthcare Corporation, McGraw Park, IL) and the length of the tubes was measured using an Olympus CK2 microscope with a ×2 objective connected to a Javelin CCTV camera with a ×3.3 coupler and National Institutes of Health Image 1.57 software. Three random measurements of each of four wells at each culture condition were measured. Each experiment was repeated at least three times.

Migration assays

HUVEC migration assays were conducted in Boyden chambers using 12-μm pore PVP free filters coated with either a 0.1 ng/ml or 0.05 ng/ml solution of collagen IV as previously reported (2). Each condition was assayed in triplicate wells at least three times unless indicated. HCAECs, AOSMCs, foreskin fibroblasts, HT1080 cells, and neutrophils were cultured or isolated and assayed for migration as previously reported (2).

“Scratch” wound closure assay

Confluent monolayers were “scratch” wounded using the tip of a universal blue pipette tip and rinsed with PBS. Tα1 (1, 10, 100, and 1000 ng/ml) or ECGS (200 μg/ml) was added to the wells in fresh medium lacking ECGS but containing 10 mM thymidine to inhibit cell proliferation (2). Ab ini- tiation experiments were conducted as described above by wounding the cell monolayer and then adding either anti-Tα1 polyclonal Ab (1/25, 1/50 dilution) or pre-immune serum (1/25 dilution), or fresh medium to the plate. A total of three random measurements for each of three wounds at each time point was measured for each test condition. The experiment was repeated four times.

In vivo plug assay

Matrigel was mixed with Tα1 (5, 50, and 500 μg/ml) or ECGS (10 and 100 ng/ml) and injected s.c. into three or four C57Bl/6N female mice for each condition (2). Three random fields were collected and analyzed per plug in each of three experiments.

In vivo wound assay

Six full-thickness 8 mm punch biopsy wounds were made on the dorsal surface of rats as previously reported (20, 21) and Tα1 was given topically at the time of wounding (5 μg in 50 μl) and again after 48 h. Controls for the topical treatment received identical amounts of saline at the time of wounding and at 48 h. Three rats in each group also received i.p. injections at the time of wounding (60 μg in 300 μl) and again every other day. Controls for these animals received identical amounts of saline i.p. on the same injection schedule. At days 8 and 9 postwounding, tissue was collected and fixed in 10% buffered formalin (i.p. n = 18 samples; topical n = 9 samples). The samples were sectioned and stained with H&E and Masson’s Trichrome (American Histolabs, Gaithersburg, MD). This experiment was performed twice.

Results

Tube formation is enhanced in the presence of Tα1

Certain steps in angiogenesis, such as migration and differentiation, are studied in vitro using Matrigel, a basement membrane matrix on which HUVECs undergo capillary-like tube formation (19, 22). When 10% serum was used, cells incubated with 100 or 1000 ng/ml Tα1. Data points represent the average length of tubes in pixels of three random fields of quadruplicate wells ± SEM. *p < 0.0001, significantly different from medium alone (Welch’s t-test). B, Examples of tubules formed in the presence and absence of Tα1. Representative pictures of tubes that formed with medium alone containing 10% (A) or 5% (C) serum. Tubes that formed in the presence of 1000 ng/ml Tα1 in either 10% serum containing medium (B) or 5% serum containing medium (D). Note that tubes that form without Tα1 (A) contain large areas of flattened cells (arrow). Bar = 100 μm.

Tα1 alters HUVEC migration

Since tube formation involves migration, the chemotactic response of HUVECs to Tα1 was tested using Boyden chambers. After 4 h, cell migration on collagen IV-coated filters in response to Tα1 was enhanced three- to sixfold (p < 0.0001) over migration in control
of medium alone (Fig. 2A). The effects were dose-dependent with HUVECs exposed to the highest dose, 1000 ng/ml, showing the greatest response to TA1. On uncoated polycarbonate filters, migration was stimulated as much as threefold over the control (p < 0.0001) when cells are treated with 100 and 1000 ng/ml of TA1 (108 ± 7 and 146 ± 2 cells, respectively, vs 37 ± 3 cells for medium alone). A dose-response was also observed on the uncoated filters and migration at the highest dose exceeded that observed for the positive control.

We also tested the N-terminal (SDAAVDTSSIEITKDLK) and C-terminal (TTKDLKEKKEVVENAEEN) halves of TA1. When either half was tested for activity, no significant difference was observed between cells in the presence of the halves or medium alone. Thus, migration-promoting activity may require the intact peptide due to conformational requirements and/or the active site encompassing a sequence greater than the six amino acid overlap.

TA1 stimulates directional migration

We next determined if TA1 stimulated migration by chemotraction (directional migration) or by chemokinesis (random motility). Checkerboard assays were performed with various concentrations of TA1 in the top, bottom or both chambers (Fig. 2B). The greatest number of cells migrated along the chemotactic gradient i.e., increasing concentrations of TA1 in the bottom chamber, (Fig. 2B dark shading) and fewer cells migrated to the increasing chemokinetic gradient, i.e., when the concentration of TA1 was highest in either the top or both chambers (Fig. 2R, light shading). These results indicate that TA1 stimulates directional HUVEC migration.

Migration effects are cell type specific

Several cell types were tested to determine whether the migratory stimulation observed with TA1 was specific. Foreskin fibroblasts, neutrophils, HT1080 fibrosarcoma cells, and human aortic smooth muscle cells did not demonstrate any significant migration toward TA1 (Table I). The cells did migrate toward their respective positive controls. In contrast, human coronary artery endothelial cells migrated to TA1 with a twofold increase (p < 0.0001) in migration over medium alone in response to 1000 ng of TA1 (Table I). Significant migration was also observed with 1 to 100 ng doses (p < 0.0001), showing that the response to TA1 could occur in other endothelial cell types. Monocyte migration was stimulated by TA1 similarly to the positive control. Monocyte migration increased in response to 1000 ng/ml of TA1 (p = 0.003), while migration appeared to be inhibited by 100 ng/ml. Higher levels of TA1 may trigger migration similarly to an inflammatory response when the cells invade surrounding tissues and the inhibitory response to lower levels of TA1 could be due to cell activation. Thus, TA1 specifically stimulates endothelial cell and monocyte migration.

In vitro “scratch” wound closure is more rapid in the presence of TA1

During angiogenesis the cells may be presented with a constant level of TA1. The “scratch” wound closure assay was used to assess the potential effects of constant doses of TA1 on cell migration. Migration of cells into the wounded area was significantly increased in the presence of 1000 ng/ml of TA1 (p ≤ 0.0001) over migration in the presence of medium alone within 2 h after wounding (14% closure vs 1%). Wound closure was similar to that in the presence of the positive control, ECGS, and remained elevated throughout the 10 h experiment. Acceleration of wound closure was also observed in response to 100 ng/ml TA1 as early as 2 h (7% vs 1%, p ≤ 0.0001). When wounded monolayers were incubated in the presence of TA1 Ab, wound closure was inhibited 1.6-fold compared with that observed in the presence of medium alone (p ≤ 0.0001). Pre-immune sera had no effect on cell migration and there was no indication of altered cell morphology or death of the anti-TA1-treated cells. Ab reduction of the levels of TA1, therefore, altered HUVEC migration.

In vivo endothelial cell migration and vessel formation are enhanced by TA1

Since TA1 enhanced HUVEC migration in vitro, experiments were performed to determine whether it promoted cell migration and vessel formation in vivo. Endothelial cells invade and form vessels in Matrigel plugs containing angiogenic factors (18, 23). Matrigel plugs containing 5 or 50 µg/ml of TA1 showed a significant 3- to 3.7-fold increase (p < 0.0001, p = 0.001 respectively) in the number of cells in the plugs compared with plugs containing Matrigel alone (Fig. 3A). At the lower and higher concentrations, 1 µg/ml and 500 µg/ml, a decrease in migration was observed. Cells migrate into the plug from the area closest to the skin forming vessels (Fig. 3B). Plugs containing TA1 (Fig. 3B, C and D) show many more cells than in the Matrigel alone (Fig. 3B, A). Cell morphology was similar to those in plugs containing 10 ng/ml of the positive control ECGS (Fig. 3B, B). These results indicate that TA1 is a potent cell migration and vessel formation factor in vivo.
Table 1. Response of different cell types to Th.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Thymosin α1 (ng/ml)</th>
<th>1000</th>
<th>100</th>
<th>10</th>
<th>1</th>
<th>0</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF*</td>
<td></td>
<td>5.2 ± 0.5</td>
<td>4.8 ± 0.7</td>
<td>5 ± 0.3</td>
<td>5 ± 0.4</td>
<td>5 ± 0.3</td>
<td>bFGF (10 ng/ml) = 12.3 ± 0.3*</td>
</tr>
<tr>
<td>AOSMC</td>
<td></td>
<td>1 ± 0.4</td>
<td>0.5 ± 0.5</td>
<td>1 ± 0.4</td>
<td>1 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>PDGF-BB (10 ng/ml) = 36 ± 4.5*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>149.3 ± 27</td>
<td>149.3 ± 13.5</td>
<td>138.7 ± 20</td>
<td>138.7 ± 12.8</td>
<td>149.3 ± 12</td>
<td></td>
<td>FMLP (10⁻⁷ M) = 1404.7 ± 58.2*</td>
</tr>
<tr>
<td>Monocytes</td>
<td>80 ± 7.6**</td>
<td>20 ± 5.4**</td>
<td>N.D.</td>
<td>N.D.</td>
<td>49 ± 8.1</td>
<td></td>
<td>FMLP (10⁻⁷ M) = 74 ± 2.1**</td>
</tr>
<tr>
<td>HCAEC</td>
<td>21 ± 0.4*</td>
<td>18 ± 0.4*</td>
<td>16 ± 0.5*</td>
<td>14 ± 0.3*</td>
<td>11 ± 0.3</td>
<td></td>
<td>ECGS (200 µg/ml) = 19 ± 2.0*</td>
</tr>
<tr>
<td>HT1080</td>
<td>0.9 ± 0.4</td>
<td>1.3 ± 0.7</td>
<td>1.8 ± 0.4</td>
<td>2.4 ± 0.8</td>
<td>2 ± 0.5</td>
<td></td>
<td>LN (1 µg/ml) = 661.2 ± 37*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LN (5 µg/ml) = 1136 ± 70.3*</td>
</tr>
</tbody>
</table>

* FF: foreskin fibroblasts, N.D.: not done, AOSMC: human aortic smooth muscle cells, HCAEC: human coronary artery endothelial cells, HT 1080: human fibrosarcoma cell line, bFGF: basic fibroblast growth factor, PDGF-BB: platelet-derived growth factor B form, ECGS: endothelial cell growth supplement, LN: laminin. Numbers represent the average number of cells that migrated ± SEM in Boyden chamber assays. *p ≤ 0.0001, **p = 0.003, significant difference from media alone (Student’s t-test).

In vivo wound healing is accelerated by Th.

Additional experiments were performed in vivo to directly determine whether wound healing was altered by Th, using a rat wound healing model. Th, whether administered topically or i.p., significantly accelerated wound healing as compared with untreated wounds (Fig. 4). Migration of cells into the granulation tissue and complete re-epithelization of the epithelium were observed (Fig. 4B and C). Th treatment by both methods resulted in considerable capillary ingrowth especially, when applied topically (Fig. 4D vs E and F). These results suggest that Th is active in vivo for the formation of granulation tissue by promoting cell proliferation, migration and vessel formation. Additionally, an increase in the accumulation/biosynthesis of collagen by Th treated wounds as compared with the untreated control (Fig. 4G vs H and I) suggests a role for Th in wound contraction (24).

Discussion

Although many of the basic biochemical steps in wound healing have been characterized, a number of key regulatory molecules have yet to be identified. We have analyzed a number of well characterized thymic peptides for their effects on angiogenesis and on endothelial cell migration to better understand the role of thymic peptides in wound healing. Initial experiments investigated whether Th acts as a stimulus of the morphologic differentiation of endothelial cells in vitro. HUVECs cultured on Matrigel showed increased tube formation in the presence of Th, demonstrating that Th significantly stimulates HUVEC migration and differentiation.

Boyd en chamber experiments demonstrated that Th is a potent chemoattractant for HUVECs in the presence and absence of a collagen IV coating on the filters. Lower but significant levels of migration were observed when no collagen IV was present. This demonstrates that migration was not stimulated by potential contaminants in the collagen IV and suggests that in cases where the basement membrane is degraded, i.e., during angiogenesis, chemotraction in response to Th could still be effective.

When cells are attached to a dish and have synthesized extracellular matrix, removal of surrounding cells (scratch assay) permits a migrational response to a constant dose of Th. However, significant stimulation of migration was not observed in the checkerboard assay in response to Th when the cells were preincubated with Th. Preincubation with Th does not inhibit HUVEC migration a significant stimulation of migration was not observed in the check-

![FIGURE 3. Th increased cell migration and angiogenesis in vivo in the s.c. Matrigel plug assay. A. Three experiments expressed as the mean area occupied by cells (µm²) ± SEM. *p < 0.0001, **p = 0.0007, ***p = 0.03 (Welch’s t-test). B. Histologic sections of Matrigel plugs. Masson’s trichrome stains Matrigel blue and endothelial cells red. The side underlying the skin is at the top of each figure. A, Matrigel alone. B, 10 ng/ml ECGS (positive control). C and D: 5 µg/ml Th. Response to Th is variable but always significantly higher than Matrigel alone. These sections show the two extremes observed in Th containing plugs. Bar = 100 µm.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
migration in response to other chemoattractants, such as bFGF (our unpublished observations), suggesting that pretreatment saturates Tα1 receptors, activating the cells and preventing response to a Tα1 gradient. Due to the differences inherent between the checkerboard and scratch assays (attached vs freshly plated cells; cell synthesized matrix vs coated matrix, etc.), direct comparison between the two experiments is not possible. We conclude that cells can migrate in response to gradients and to constant doses of Tα1 depending on the conditions employed.

Tα1 appears to be a specific chemoattractive agent for endothelial cells and to a lesser extent for monocytes. Among the primary (and one tumor cell) lines tested, only endothelial cells and monocytes responded to Tα1 by showing increased cell migration. The migration of HCAECs and HUVECs demonstrated that the response to Tα1 was not limited to the highly specialized endothelial cells of the umbilical cord but can occur in other endothelial cells. Additional cell types tested are those located near or are likely to interact with endothelial cells in vivo. Fibroblasts, smooth muscle cells, and neutrophils showed only background levels of migration in response to Tα1, whereas monocytes showed increased migration to Tα1 at 1000 ng/ml. Tα1 is known to trigger differentiation and proliferation in certain types of T cells (25), therefore, higher levels may promote the migratory response similarly to an inflammatory response. The inhibitory response of monocytes to lower levels of Tα1 may be due a lag phase when the cells are activated.

Tα1 was active in promoting endothelial cell migration, angiogenesis, and wound healing in vivo. Previous work has shown that factor VIII-positive endothelial cells invade and form vessels in the Matrigel plugs containing angiogenic factors (18, 23). The in vivo plug results show that migration and angiogenesis were stimulated at or above the levels observed for migration and angiogenesis in response to the positive control ECGS. Additionally, we observed an acceleration of wound healing in the punch wound assay. Complete re-epithelialization was observed with both i.p. and topical applications of Tα1 and neovascularization was more extensive in treated wounds. Increased levels of collagen were also observed in treated wounds, showing that Tα1 treatment may also be accelerating wound contraction, stimulating the healing process. Injection of 60 μg/300 μl every other day yielded approximate maximal concentrations in the blood of 5 μg/ml. It should be noted that Tα1 is a very small peptide that may have a short half-life in the circulation and these estimates are probably higher than actual. It should be noted that the apparent concentrations of Tα1 used in in vitro and in vivo studies were greater than physiologic levels. In humans, the concentration of Tα1 in the blood is approximately 400 to 1000 pg/ml (26).
concentration of Tα1 varies with tissue type, age of the individual, and state of the immune system (i.e., levels of 1019 – 6, 384 pg/ml have been detected in follicular fluid of infertile females) (26, 27). The Tα1 in serum is apparently active since some migration occurred in the presence of medium containing serum in the scratch assay and was inhibited by treatment with a polyclonal Tα1 Ab.

Our data indicate that Tα1 is an important factor in angiogenesis and may play a novel role in the process of wound healing. Tα1 does not appear to resemble any known angiogenic factors. Its specificity for endothelial cells and monocytes also appears unique. The current findings strongly suggest that the effect of Tα1 on the migration of the endothelial cells in vitro and in vivo is significant. Further studies are necessary to determine the mechanism by which Tα1 acts to promote migration and wound healing in vivo. Prothymosin α has been shown to enhance HLA-DR Ag expression of monocytes from patients with multiple sclerosis (28). Our results document a direct effect of Tα1 on monocyte migration, providing a clue as to the mechanism of action of Tα1 on immune restoration following treatment observed in vivo. These results may have significant implications in developing a new family of molecules that could accelerate wound healing and regulate the process of angiogenesis.

Acknowledgments

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References