Published in final edited form as:

J Tissue Eng Regen Med. 2013 June ; 7(6): 421–433. doi:10.1002/term.528.

Omental grafting: a cell-based therapy for blood vessel repair

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Abstract

Clinicians regularly transplant omental pedicles to repair a wide variety of injured tissues but the basic mechanism underlying this efficacious procedure is not understood. One possibility that has not been addressed is the ability of omentum to directly contribute regenerative cells to injured tissues. We hypothesized that if omental progenitor cells could be mobilized to incorporate into damaged tissue, the power of this therapy would be greatly expanded. Labeled omental grafts were transplanted into a murine carotid artery injury model. Selected grafts were treated with Thymosin β 4 prior to transplantation to investigate the effects of chemical potentiation on healing. We found treatment of grafts with Thymosin β 4 induced progenitor cells to fully integrate into the wall of injured vessels and differentiate into vascular smooth muscle. Myographic studies determined that arteries receiving Thymosin β 4-stimulated grafts were functionally indistinguishable from uninjured controls. Concurrent in vitro analyses showed that Thymosin β 4 promoted proliferation, migration, and trans-differentiation of cells via AKT signaling. This study is the first to demonstrate that omentum can provide progenitor cells for repair, thus revealing a novel and naturally occurring source of vascular smooth muscle for use in cell-based therapies. Furthermore, our data show this system can be optimized with inducing factors, highlighting a more powerful therapeutic potential than that of its current clinical application. This is a paradigm-setting concept that lays the foundation for the use of chemical genetics to enhance therapeutic outcomes in a myriad of fields.

Keywords

Omentum; Thymosin β 4; Vascular Smooth Muscle; Tissue Grafting; Mesothelium; Wound Healing; Blood Vessel

1. Introduction

Clinicians commonly transplant omental pedicles onto injured tissues including the hepatic artery of patients receiving liver transplants (Ishikawa et al., 2001) and perforated gastric ulcers (Matoba et al., 1996) to promote healing. Additionally, omentum has been experimentally grafted onto the wrists of patients afflicted with carpal tunnel syndrome (Goitz and Steichen, 2005), the brains of Alzheimer patients (Goldsmith, 2007), and

ischemic hearts (Taheri et al., 2005) to promote revascularization. While these treatments are efficacious in promoting healing, the mechanisms underlying omentum-based repair are not understood. Previous studies suggested that the omentum secretes pro-angiogenic factors and modulates the inflammatory response (Litbarg et al., 2007) through a paracrine mechanism, but this explanation does not explain the broad reparative properties that omentum brings to the healing process.

Our previous work demonstrates that serosal mesothelium, the main component of omentum, is a rich source of diverse progenitor cell types that form the gut vasculature during development (Wilm et al., 2005). With this in mind, we explored whether grafted adult omentum could likewise produce such progenitors for healing injured vessels. We hypothesized that if omental progenitor cells could be mobilized to incorporate into injured tissues, the efficacy of this therapy would be maximized.

Until now, the ability of omentum to directly contribute reparative cells to injured tissues has not been studied. Furthermore, molecular manipulation of grafts in order to potentiate cellular contribution has been completely unexplored. Here, we reveal a novel cellular mechanism in which the omentum accelerates tissue repair by providing regenerative progenitor cells upon treatment with Thymosin β 4 (T β 4), a ubiquitously expressed protein with broad biological activities (Sosne et al., 2010). Initially characterized as a G-actin monomer binding protein (Safer et al., 1991), T β 4 is now known to play a role in cell migration (Fan et al., 2009; Malinda et al., 1997; Smart et al., 2007), angiogenesis (Cha et al., 2003; Philp et al., 2003b), inflammation (Badamchian et al., 2003; Sosne et al., 2001), and wound healing (Li et al., 2007; Malinda et al., 1999; Sosne et al. 2001). T β 4 has also shown clinical promise in trials regarding dermal and corneal wound healing and may be cardio-protective in cases of myocardial infarction (Marx, 2007). Because of its ability to mobilize cells and promote healing, we explored whether T β 4 could promote incorporation of omental cells into the walls of damaged blood vessels.

Our results show addition of T β 4 to cultured omentum promotes increased cell proliferation, migration, and differentiation into vasculogenic lineages. Importantly, T β 4-treated grafts transplanted onto an injured artery provide new smooth muscle cells that integrate into the vessel wall, accelerating repair. Additionally, smooth muscle cells derived from omental precursors are functionally identical to native vascular smooth muscle cells. This study highlights a novel cell-based therapy for vascular healing and provides a paradigm-setting mechanism underlying the ability of omentum to facilitate healing by directly contributing reparative progenitor cells to injured tissue. Finally, our data reveal that treatment of omental grafts with factors like T β 4 potentiates cellular outcomes and maximizes therapeutic benefits.

2. Materials and Methods

2.1 Immunofluorescence and Histology

Vessels were fixed in 4% paraformaldehyde for 2 hours prior to sucrose infiltration and cryo-sectioning. Cultured cells and whole mount omental preparations were fixed in 4% paraformaldehyde for 30 minutes prior to staining. Blocking solution (10% goat serum, 0.25% Triton X-100 in PBS) was applied for 1 hour at room temperature. Primary and secondary antibodies are listed in supporting information Table S1. Samples containing primary antibodies were incubated overnight at 4°C. Samples containing secondary antibodies and DAPI were incubated 3 hours at room temperature. For hematoxylin and eosin staining, vessels were fixed in 10% formalin prior to dehydration and paraffin embedding. 7mm sections were cut and mounted on slides for routine hematoxylin and eosin (Newcomer Supply) staining.

2.2 Omental Cultures

Omentum was isolated from adult outbred ICR mice as previously reported (Kawaguchi et al., 2007). Explants were cultured on glass Lab-Tek chamber slides in supplemented media (10% fetal bovine serum, 1% penicillin/streptomycin, DMEM). After 48 hours, some cultures received 10 ng/mL T β 4 (Abcam) and/or 100nM Wortmannin (Sigma) and were cultured for an additional 48 hours.

2.3 Quantitative RT-PCR Analysis

RNA was isolated using Trizol. 100ng of cDNA template was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative levels of gene expression were determined using TaqMan-based quantitative RT-PCR on a 7900HT platform. TaqMan Gene Expression Assays (Applied Biosystems) are listed in supporting information Table S2. The housekeeping gene 18S was used as an internal control. Triplicate DCT values were generated for each assay. The fold change in expression was determined by dividing experimental values by the control value, which was then set to 1.

2.4 Migration Assay

Cell migration was assessed as previously reported (Shelton and Yutzey, 2008). Cells were detached using 1 $\times$ Trypsin-EDTA (Invitrogen) after 48 hours in culture. Cell suspensions were collected, passed through a 25G1 $\frac{1}{2}$ needle, and resuspended in supplemented media at 1 $\times$ 10⁵ cells/mL. Each cell suspension was added to the top chamber of a Boyden chamber culture plate insert (8 μ m pores; Millicell) and placed in a 24 well culture plate (Becton Dickinson) containing supplemented media. Cells were incubated for 6 hours. Cells that migrated through the pores and were adherent to the bottom of the insert were fixed in 100% MeOH and stained with Giemsa (Sigma-Aldrich). The fold change in migration was calculated by dividing the number of migrated cells in the experimental groups by the number of migrated cells in the control group. The control value was set to 1.

2.5 Proliferation Assay

Omental cells were cultured for 48 hours. Immunohistochemistry for phosphorylated histone H3 (pHH3) was performed. The percent of proliferative cells was calculated by dividing the number of pHH3 labeled nuclei by the total number of DAPI labeled nuclei per microscopic field.

2.6 Carotid Artery Injury

Adult outbred ICR mice were anesthetized by intraperitoneal injection of Ketamine (Hospira) and Xylazine (Lloyd Laboratories). The right carotid artery was exposed and injured with liquid nitrogen delivered by a cryo-spray device (Brymill) for 5 seconds. Immediately following injury, omentum was applied to the vessel. Omental mesothelium isolated from a littermate was labeled with CFDA (Invitrogen) as per the manufacture's recommendations. Mice recovered and were sacrificed 2,7,10,14, or 18 days later. For some experiments, agarose beads (Sigma) were soaked in soluble T β 4 for 2 hours and transplanted with or without omental preparations. For membrane experiments, vessels were wrapped in a porous (0.4mm) polycarbonate membrane (Millipore) prior to graft transplantation.

2.7 Myography Experiments

Experiments were performed as previously reported (Reese et al., 2009a; Reese et al., 2009b) with the following modifications. Carotid arteries were mounted in microvessel perfusion chambers filled with aerated (5% CO₂, 95% O₂) Krebs buffer. Chambers were placed on the stage of an inverted microscope equipped with a digital image capture system

(IonOptix) to record intra-luminal diameters. Vessels were pressurized to 40 mm Hg by a column of Krebs buffer and allowed to equilibrate. The pressure was increased in a stepwise manner to achieve a pressure of 100 mm Hg. Vessels were challenged with 50mM KCl in Krebs buffer until diameter measurements reached a plateau. Vessels that failed to constrict to KCl were excluded from further study. After wash out with buffer, vessels were challenged with 10^{-7} M U46619 (Cayman) diluted in Krebs buffer until diameter measurements reached a plateau. Then, 10^{-5} M acetylcholine (Sigma) was added to the U46619/Krebs solution. After wash out with buffer, vessels were challenged with 10^{-5} M phenylephrine (Sigma) until diameter measurements reached a plateau. For each vessel, the diameter when pressurized to 100 mm Hg was considered to be 100% intra-luminal diameter. Changes in diameter were calculated by dividing the challenged vessel diameter by the diameter at 100 mm Hg.

2.8 Animal Care and Use

This study was conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Procedures were performed with the approval of Vanderbilt University's Animal Care and Use Committee under the supervision of the Institutional Animal Care and Use Committee.

2.9 Statistical Analyses

Error bars represent standard error of the mean. A Student's t-test was calculated to determine statistical significance.

3. Results

3.1 T β 4 potentiates smooth muscle and endothelial lineages in omental progenitor cells

Murine omentum consists of an avascular double membrane of serosal mesothelium bordered by a ribbon of adipose tissue and vasculature (Figure 1A). This discrete separation of mesothelial tissue from adipose and vascular tissue makes this an ideal model for studying the reparative potential of mesothelium as it minimizes the confounding effects that the presence of vasculature and adipose tissue may cause. The membranous portion of native omentum robustly expresses the mesothelial markers Wilms Tumor 1 (Wt1) (Figure 1B) and cytokeratin (Figure 1D). While it is known that the progeny of embryonic serosal mesothelial cells are the main source of vasculogenic cells for the developing gut vasculature (Wilm et al., 2005), adult serosal mesothelium does not express smooth muscle (Figure 1E) or endothelial markers (Figure 1C). In our study, only the membranous mesothelial portion of this structure was used for culture and transplantation experiments.

As T β 4 restores pluripotency to adult epicardial explants (Smart et al., 2007), we sought to determine whether T β 4 directs adult gut mesothelial cells into vasculogenic lineages. After 48 hours of culture, control explants formed sheets of cells that grew out from the initial cell mass. Eighty percent of these cells expressed Wt1 (Figure 1F), while T β 4 treatment virtually extinguished Wt1 expression, suggesting a down regulation of mesothelial characteristics (Figure 1G). Regarding smooth muscle markers, 33% of control cells expressed smooth muscle α -actin (α sma), some of which also expressed Wt1 (Figure 1F, H). This expression profile was dramatically altered with T β 4 treatment. A significantly greater number of T β 4-treated cells expressed α sma (70%; see supporting information Figure S1 for quantification), none of which expressed Wt1 (Figure 1G, I). Similarly, no control cells expressed smooth muscle myosin heavy chain (smMHC), while 29% of T β 4-treated cells had smMHC expression. Furthermore, control cultures were devoid of endothelial cells, whereas PECAM-positive cells were readily identified in T β 4-treated cultures (16%; Figure 1M).

Quantitative real time RT-PCR was used to corroborate these data and demonstrated that T β 4-treated cultures expressed early and late smooth muscle markers at a significantly higher level than native omentum and control untreated cultures (Figure 2A). The expression of the smooth muscle related transcription factors serum response factor (SRF) and myocardin were also increased in T β 4-treated cultures (Figure 2A). Similarly, the expression of endothelial markers, PECAM, VEGF, and the VEGF receptor Flk-1, was increased in T β 4-treated cultures compared to controls (Figure 2B). Taken together, these data demonstrate that omental mesothelium can be stimulated to produce vasculogenic phenotypes.

3.2 T β 4 promotes migration and proliferation in cultured omental cells

A critical component to enhance cell-based repair from grafts is identifying an agent capable of mobilizing resident progenitor cells. T β 4 was an obvious candidate for this function as it promotes migration in endothelial, epicardial, and myocardial cells (Bock-Marquette et al., 2004; Malinda et al., 1997; Smart et al., 2007). Therefore cultured explants were assayed for stimulation of cell mobility after T β 4 treatment. As seen in Figure 3, T β 4 treatment of omental isolates induced cell migration significantly above control levels (Figure 3A, B). Activation of integrin-linked kinase (ILK) and AKT is the molecular underpinning of T β 4 stimulation of cell migration (Bock-Marquette et al., 2004) and, as expected, T β 4 increased expression of ILK and phosphorylated AKT in omental mesothelium demonstrating the conservation of regulatory components (Figure 4B, E). Furthermore, this T β 4-induced ILK and phosphorylated AKT was ablated by treatment of cells with Wortmannin, an inhibitor of PI(3)K (Ui et al., 1995), the upstream regulator of ILK activity (Figure 4C, F). Importantly, Wortmannin treatment produced a concomitant inhibition of T β 4-induced omental cell movement (Figure 3C, G).

While T β 4 stimulation of cell proliferation in mesothelia has been largely unexplored, simple observation of omental cultures treated with this agent suggested an increase in cell number over untreated samples. Quantification of these cultures determined that T β 4 did indeed stimulate cell proliferation in omental cells over controls (compare expression of phosphorylated histone H3 in Figure 3D and E) and that Wortmannin treatment again reversed this proliferative response (Figure 3F, H). Taken together our analyses suggest that T β 4 treatment of omental explants stimulates latent differentiative, migratory, and proliferative potentials of serosal mesothelia and thus may potentiate repair from this cell source in vivo.

3.3 Carotid Artery Injury Model

To test the reparative capacity of omental grafts with and without T β 4 intervention, we developed a carotid artery cryoinjury model. This reproducible protocol results in a complete yet transient loss of arterial endothelial, muscular and connective tissue elements where the course of vessel repair can be precisely quantified over time. This model phenocopies several clinical conditions including the loss of vascular cells associated with Hutchinson-Gilford progeria syndrome (Varga et al., 2006) as well as the hepatic artery degeneration that often accompanies liver transplantation, a case where omental transplantation is currently used to stave off such degeneration (Ishikawa et al., 2001). While the injured vessel remained patent, no nuclei were present in the vessel wall or surrounding connective tissue after 2 days, demonstrating the loss of vascular cells (Figure 5B). However, residual α sma staining is evident in necrotic myocytes in the tunica media (compare Figure 5C and D). By 7 days post injury, endothelial cells repopulated the injured area and nuclei were present in the tunica adventitia (supporting information Figure S2C). By 10 days post injury, the subendothelial, innermost smooth muscle layer was apparent, but the outer two muscle layers of the tunica media remained acellular (supporting information

Figure S2D). By 14 days post injury, two of the three layers of smooth muscle were present in the tunica media (supporting information Figure S2E) and by 18 days (supporting information Figure S2F), all three smooth muscle layers were present, rendering the injured vessel indistinguishable from an uninjured control. In all uninjured contralateral control vessels, viable nuclei were visible in the tunica intima, tunica media, and tunica adventitia (Figure 5A).

3.4 Omental grafts provide paracrine factors for healing but do not contribute cells for vessel repair

To determine the potential of omental grafts to facilitate vessel repair, grafts labeled with the fluorescent vital dye CFDA were transplanted onto injured arteries *in vivo* (Figure 5E–G). Two days following injury, graft-derived cells were visible adjacent to the vessel wall and expressed cytokeratin (Figure 6A–C) but not α sma (Figure 6D–F) implicating that the graft was still mesothelial in nature. Interestingly, 14 days following injury, omental-derived progenitors differentiated into smooth muscle (Figure 6G–O) as indicated by the colocalization of smooth muscle markers with CFDA fluorescence (Figure 6G–O).

While grafted cells did not incorporate into damaged vessel walls, it was apparent that these grafts accelerated healing. Endogenous endothelial and smooth muscle cells repopulated the injured area more rapidly than in un-grafted vessels (supporting information Figure S2H, I). By 14 days, the grafted vessel was indistinguishable from an uninjured control (supporting information Figure S2J), which is in contrast to the 18 days it took an un-grafted vessel to heal (supporting information Figure S2F). These data suggest that grafting mesothelium to an injured vessel had a mild but reproducible effect on wound healing even though omental cells did not incorporate into the vessel.

As we observed modest acceleration of repair despite the lack of cell integration, we examined the potential paracrine functions of omentum. Grafts were removed from injured and uninjured control vessels 2 days after transplantation and measured for the expression of paracrine factors known to promote vessel development. Quantitative RT-PCR revealed that grafts transplanted onto an injured vessel expressed elevated levels of VEGF, SDF1- α , and FGF-17, factors known to stimulate vessel development (supporting information Figure S3). Interestingly, expression of these factors was not stimulated in grafts placed on uninjured vessels suggesting an important interaction between the injured tissue and graft that causes the expression of these paracrine factors. Stimulation of angiogenic factors was not unrestricted as Flk-1, a known VEGF receptor, expression was not affected by contact with injured tissue (supporting information Figure S3).

To determine whether these graft-derived factors facilitated healing in a completely paracrine fashion, injured vessels were wrapped in a cell impermeable membrane prior to transplantation (supporting information Figure S4A). An identical pattern of repair over time was observed in these vessels (supporting information Figure S4E), when compared to grafted vessels without a membrane (supporting information Figure S3G–J) suggesting that omentally-derived paracrine factors play a modest but appreciable role in vessel healing.

3.5 T β 4 promotes incorporation of graft cells into vessel walls and accelerates healing

Because our *in vitro* data demonstrated T β 4 promotes mesothelial cell proliferation, migration, and differentiation, we investigated whether its application promoted incorporation of omentum-derived progenitors into injured vessels *in vivo*. Agarose beads were soaked in soluble T β 4 and transplanted onto injured vessels along with graft preparations (Figure 5H). In striking contrast to vessels grafted without T β 4 treatment, these vessels had CFDA-labeled graft cells that were fully incorporated into the outer most and

middle layers of the tunica media of arteries 10 days post injury (Figure 7A–C). These integrated cells expressed early and late smooth muscle markers including α sma (Figure 7D, G), smMHC (Figure 7E, H), and Cald1 (Figure 7F, I). 56% of cells in the outer most layer and 42% of cells in the middle tunica media layer of these vessels were composed of CFDA-labeled cells (supporting information Figure S5). None of the CFDA-labeled cells were found in the subendothelial smooth muscle or endothelial layer. The efficacy of T β 4 to mobilize graft cells was remarkable as the only cases where graft cells did not incorporate into the vessel were those in which the graft became post-operatively displaced (3 of 12 vessels assayed at day 10). In all cases where the graft remained in position (9 of 12 vessels at day 10), CFDA-labeled progenitor cells integrated into the vessel wall.

As T β 4 mediated incorporation and differentiation of graft cells into injured vessel walls, we next determined whether this accelerated the healing time of these vessels. T β 4 treatment stimulated endothelial replacement as early as 2 days post injury and accelerated repopulation of the tunica media, with total smooth muscle replacement by 10 days, rendering these vessels indistinguishable from controls (supporting information Figure S2O–Q). This accelerated repair was not due to T β 4 treatment alone, as application of T β 4-soaked beads alone had a healing time course that returned to that observed with non-stimulated omental grafts (supporting information Figure S2K–N). Therefore, the significantly accelerated healing observed with T β 4-treated grafts was due to the ability of T β 4 to mobilize omental cells for repair. These data demonstrate that T β 4 potentiated the cellular contribution of omental grafts that ultimately led to marked acceleration of vessel repair.

3.6 T β 4-treated omentum-grafted vessels are functionally similar to uninjured vessels

Reacquisition of vessel function is the most important aspect in therapeutic intervention. Therefore, uninjured vessels along with injured and repaired vessels were analyzed with myography as previously described (Reese et al., 2009a) (Figure 8A). Vessels were challenged with the following vasoactive mediators: KCl, the synthetic thromboxane receptor agonist U46619, acetylcholine, and phenylephrine. Intra-luminal diameters were recorded for each challenge and tracings for each individual vessel were generated. Representative tracings for each vessel type are shown in Figure 8C–F. Changes in intra-luminal diameter were quantified in Figure 8B.

Exposure of an uninjured control vessel (Figure 8C) to KCl, which acts by depolarizing smooth muscle cells, induced 25% constriction from resting baseline dimensions. In response to U46619, the lumen diameter constricted 80%. When the endothelium dependent vasodilator acetylcholine was added to vessels that were pre-constricted with U46619, the vessels dilated 45%. The addition of phenylephrine, a direct acting vasoconstrictor, produced 35% constriction of the lumen diameter. These constriction and relaxation responses were considered as the baseline vasoactive responses.

In contrast to these controls, 2-day post injury vessels were found to be unresponsive to all of the pharmaceutical challenges (Figure 8D). This is not surprising considering that no viable cells are present in the tunica adventitia, media, or intima at this time point (supporting information Figure S2B). Similarly, 10-day post injury vessels were only modestly reactive to vasoactive stimuli (Figure 8E). While these vessels are more reactive than vessels at 2 days post injury, they are significantly less reactive than uninjured control vessels (Figure 8B). This is likely due to the fact that at this time point, only the endothelial cells and one of the three layers of smooth muscle cells are present (supporting information Figure S2D). Strikingly, vessels receiving T β 4-treated grafts were functionally indistinguishable from uninjured control vessels 10 days after injury (Figure 8F). These data support the hypothesis that omentum-derived progenitors differentiated into smooth muscle

cells in these repaired vessels and are functionally indistinguishable to native vascular smooth muscle cells (Figure 8B).

4. Discussion

For over a century, clinicians have successfully used human omentum to treat a variety of conditions (Cannaday, 1948). While this procedure prevents infection and promotes revascularization, little was known regarding the foundation underlying these healing properties. Reports suggested that activated omentum secretes pro-angiogenic and chemotactic factors to promote blood and lymphatic vessel formation (Litbarg et al., 2007; Singh et al., 2009). Our results confirm and elaborate on these findings. When grafts were transplanted onto an injured vessel, they provided paracrine factors and differentiated into smooth muscle cells but did not integrate into the vessel wall. We also know from our previous studies that embryonic serosal mesothelium contributes the majority of vasculogenic cells to the developing gut vasculature (Wilm et al., 2005). In light of these findings, we hypothesized that if omental progenitor cells could be mobilized to incorporate into damaged tissue, the power of this therapy would be greatly expanded.

T β 4 was a likely candidate to facilitate mobilization of these cells as it directs differentiation and migration of adult epicardial progenitors (Smart et al., 2007). The efficacy of T β 4 in a clinical setting is also promising as therapies involving its use are currently being developed to treat a variety of injuries (Crockford, 2007; Philp et al., 2003a; Sosne et al., 2001). Here we show T β 4 directs omentum-derived progenitors to proliferate, migrate, and differentiate into vascular lineages. These outcomes mirror the effect of T β 4 addition to epicardial cells (Smart et al., 2007) (Figure 2,3). Furthermore, the previously identified molecular mechanism underlying T β 4 activation of AKT signaling (Bock-Marquette et al., 2004) is conserved in omental cells (Figure 4). Importantly, we show T β 4 can be applied to omental grafts to mobilize progenitor cell integration into the media of injured vessels as well as increase the paracrine response of the graft (supporting information Figure S3). This novel methodology produced repaired vessels whose function was indistinguishable from uninjured controls over a significantly reduced time course. These studies establish the principle that omental grafts can be potentiated by molecular factors in order to maximize therapeutic outcomes.

Understanding how to tailor omental progenitor responses to specific tissue injuries will have a broad impact on an array of clinical fields including gastroenterology, neurology, and cardiovascular medicine. For example, endothelial progenitor cells have been exclusively used in cell-based therapies to treat vascular injuries (Asahara et al., 1997; Gulati and Simari, 2009; Krenning et al., 2009; Kumar and Caplice, 2010; Mund et al., 2009). While the therapeutic benefit of these cells is evident in animal models, their efficacy in humans is not as robust (Mund et al., 2009). As we have demonstrated that T β 4-treated mesothelial progenitors can trans-differentiate into endothelial cells as well as smooth muscle precursors, future omental cell-based therapies may enhance the success rate of such procedures.

However, not all processes associated with the mobilization of omentum are positive. Omentum is intimately associated with abdominal adhesions, a major complicating factor in post-surgical recovery (occurring in greater than 90% of all open abdominal surgeries (Liakakos et al., 2001)) and female infertility (Bruggmann et al. 2010). The very cells that mediate these debilitating injuries are those identified in a reparative role here. Future studies to elucidate both these positive and negative properties of omentum-based progenitors as well as identify other molecular factors that either promote reparative or repress deleterious characteristics will be of interest to basic scientists and clinicians alike.

This study is the first to demonstrate that omentum can directly contribute reparative progenitor cells to injured tissues upon treatment with T β 4, thus revealing a more powerful therapeutic potential than that of its current clinical application. The ability of adult mesothelial cells to repopulate injured vessel walls is reminiscent of embryonic serosal mesothelium providing vasculogenic cells to developing blood vessels. Previous work also highlighted the potential of these same mesothelial cells to develop into non-vascular cell types occupying a variety of niches in various organs (Wilm et al., 2005). It is possible that the potential to generate these non-vascular lineages is retained in adult mesothelial structures and can be stimulated by other molecular factors in ways similar to that which we have shown here for vascular lineages. With knowledge of the cellular contribution of omentum to injured tissues coupled with the finding that these reparative cells are amenable to chemical potentiation, a myriad of therapeutic interventions are possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all members of the Bader lab for technical support and scientific advice. We also thank the Vanderbilt DNA Resources Core for assistance with the quantitative RT-PCR studies. This work was supported by an American Heart Association Greater Southeast Affiliate post-doctoral fellowship 0825385E to ELS, 1R01DK070741-01 to DMB, and HL077395 and HL096967 to JR.

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Figure 1. Murine omentum is mesothelial in nature but can differentiate into smooth muscle and endothelial cell lineages

Native murine omentum consists of a membranous portion (A, white asterisk) surrounded by a ribbon of adipose tissue (A, black asterisk) that contains vasculature (A, arrow). Cells in the membranous portion of the omentum stained positively for the mesothelial markers Wt1 (B) and cytokeratin (D), while no cells were found to express the endothelial marker PECAM (C) or the smooth muscle marker α sma (E). Omental explants were cultured for 48 hours with and without the addition of T β 4. Untreated cells robustly expressed Wt1 (F, arrow) with some cells also expressing α sma (F arrowhead). In contrast, untreated cells did not express smMHC (J) or PECAM (L). When cultured with T β 4, omental explants robustly expressed α sma (G, I), with very few cells expressing Wt1 (G, arrow). These cells also expressed smMHC (K) and PECAM (M). If cultured for an additional 48 hours, these PECAM positive cells went on to form endothelial tubes (M, inset). Nuclei are marked with DAPI.

Figure 2. Tβ4 increases smooth muscle and endothelial cell marker expression

Quantitative real time RT-PCR was used to measure changes in gene expression in intact omentum and omental cells cultured for 48 hours with or without the addition of Tβ4. Untreated cultures had modest increases in smooth muscle markers compared to native intact omentum (A). However, cells treated with Tβ4 had significant increases in early and late smooth muscle markers as well as smooth muscle related transcription factors (A). Furthermore, Tβ4-treated cells had significantly increased expression of endothelial markers (B). In addition, control cultures of omental cells had a modest decrease in expression of the mesothelial marker Wt1 while cultures treated with Tβ4 had a significant decrease in Wt1 expression (B). Asterisks represent a statistically significant difference compared to the level of gene expression in the intact native omentum sample (n=5, $P < 0.01$). Error bars represent standard error of the mean.

Figure 3. Tβ4 promotes migration and proliferation in cultured omental cells

Omental cells were cultured on transwell filters with and without addition of Tβ4 and Wortmannin. The number of migrated cells was increased in cultures treated with Tβ4 (B) as compared to untreated control cultures (A). Addition of Wortmannin along with Tβ4 (C) inhibits migration below levels observed in control cultures. The fold change in the number of treated cells that migrated relative to the number of control cells that migrated is quantified (G). Proliferation was measured using an antibody specific for phosphorylated Histone H3 (pHH3). Omental cells treated with Tβ4 had more cells staining positive for pHH3 (E, arrows) than untreated control omental cells (D, arrow). Cells treated with Wortmannin along with Tβ4 (F) were no more proliferative than control cultures. The percent of pHH3 positive cells compared to total nuclei in control, Tβ4-treated, and Tβ4+Wortmannin-treated cultures is quantified (H). Asterisks represent a

Figure 4. Tβ4 increases expression of ILK and phosphorylated AKT in cultured omental cells
 Omental cells were cultured with and without addition of Tβ4 and Wortmannin. Cells treated with Tβ4 have increased cytoplasmic expression of ILK (B, arrow) compared to control cells (A). Addition of Wortmannin inhibits Tβ4-induced ILK expression (C). Non-specific nuclear staining as reported by the manufacturer is evident in all groups (A–C arrowhead). Similarly, Tβ4-treated cells have increased expression of phosphorylated AKT (E, arrow) compared to control cells (D), although some positive staining is detected in these cells (D, arrow). Addition of Wortmannin inhibits Tβ4-induced phosphorylation of AKT (F).

Figure 5. Carotid artery injury and omental graft transplantation model

Mouse carotid arteries were isolated and injured *in vivo* using a liquid nitrogen cryo-spray device. Uninjured control (A, C) and injured (B, D) arteries were sectioned and stained with hematoxylin and eosin or an αsma antibody. In the control (A, C), nuclei (arrows) are visible throughout the vessel and αsma positive cells are evident in the vessel wall (C). In contrast, nuclei are absent from the injured connective tissue and vessel wall (B, D, arrows), while residual αsma expression is visible in necrotic myocytes (D). Immediately following injury, an omental graft (E, arrow) labeled with the fluorescent vital dye CFDA (F) was transplanted onto the injured site (E–G). For some experiments, Tβ4-soaked agarose beads were placed on the injured vessel (H, arrow) before being wrapped in a graft (inset in H).

Figure 6. Omentum-derived cells can differentiate into smooth muscle cells in vivo

After 2 days, graft cells (A, arrow) are adjacent to the vessel wall (A, arrowhead) and retain cytokeratin expression (B) that co-localizes with the CFDA label (C). Graft cells (D, arrow) do not express α sma (E, arrow). While α sma expression is detected in the vessel wall (E, arrowhead), α sma expression does not co-localize with CFDA labeled cells (F). After 14 days, graft cells express smooth muscle markers (G–O). α sma is expressed in the vessel wall (H, arrowhead) and in the graft (H, I arrow). Similarly, smMHC is expressed in the vessel wall (K, arrowhead) and in the graft (K, L arrow). Furthermore, Cald1 is expressed in the vessel wall (N, arrowhead) and in the graft (N, O arrow). Additional faint green coloring in the vessel wall is due to auto-fluorescence of the elastin and collagen present in the elastic laminae. Nuclei are marked with DAPI. Vessel lumens are marked with asterisks.

Figure 7. Addition of Thymosin β 4 to omental grafts promotes incorporation of omentum-derived cells into the tunica media of the vessel

Vessels were injured and CFDA labeled omental grafts were applied in conjunction with agarose beads soaked in T β 4 (visible in B, E, H, asterisks). Vessels were isolated 10 days post injury, sectioned, and stained with antibodies specific for the smooth muscle markers α sma, smooth muscle myosin heavy chain (smMHC), or Caldesmon (Cald1). CFDA labeled cells (A, B, C, arrows) were found in the tunica media of the vessel and co-localized with α sma (D, G), smMHC (E, H), and Cald1 (F, I) positive cells. Insets are magnified views. Nuclei are marked with DAPI. Faint additional green coloring in the vessel wall is due to autofluorescence of the elastin and collagen present in the elastic laminae. (n=12)

Figure 8. Tβ4 treated omental grafted vessels are functionally equivalent to uninjured vessels
 Vessels were isolated and mounted in perfusion chambers (A) to perform myography studies. Vessels were inflated to a pressure of 100mm Hg (1) and challenged with vasoconstrictors including KCl (2), the thromboxane agonist U46619 (3), and phenylephrine (PE) (5) as well as the vasodilator acetylcholine (AcH) (4). Intra-luminal diameters were recorded (C–F). Baseline tracings for uninjured control vessels were established (C). Injured vessels isolated 2 days post injury were non responsive (D). Injured vessels isolated 10 days post injury were modestly responsive (E). In contrast, the response of injured vessels that received a Tβ4-treated omental graft (F) was statistically indistinguishable from uninjured vessels (C). Luminal diameters are quantified in panel B. Asterisks represent a statistically significant difference compared to the uninjured control value (n=6, P<0.01). The cross represents a statistically significant difference compared to the uninjured control value (n=6, P<0.05). Error bars represent standard error of the mean.