Beneficial Effect of Mechanical Stimulation on the Regenerative Potential of Muscle-Derived Stem Cells Is Lost by Inhibiting Vascular Endothelial Growth Factor

Sarah A. Beckman, William C.W. Chen, Ying Tang, Jonathan D. Proto, Logan Mlakar, Bing Wang, Johnny Huard

Objective—We previously reported that mechanical stimulation increased the effectiveness of muscle-derived stem cells (MDSCs) for tissue repair. The objective of this study was to determine the importance of vascular endothelial growth factor (VEGF) on mechanically stimulated MDSCs in a murine model of muscle regeneration.

Approach and Results—MDSCs were transduced with retroviral vectors encoding the LacZ reporter gene (lacZ-MDSCs), the soluble VEGF receptor Flt1 (sFlt1-MDSCs), or a short hairpin RNA (shRNA) targeting messenger RNA of VEGF (shRNA _VEGF MDSCs). Cells were subjected to 24 hours of mechanical cyclic strain and immediately transplanted into the gastrocnemius muscles of mdx/scid mice. Two weeks after transplantation, angiogenesis, fibrosis, and regeneration were analyzed. There was an increase in angiogenesis in the muscles transplanted with mechanically stimulated lacZ-MDSCs compared with nonstimulated lacZ-MDSCs, sFlt1-MDSCs, and shRNA _VEGF MDSCs. Dystrophin-positive myofiber regeneration was significantly lower in the shRNA _VEGF-MDSC group compared with the lacZ-MDSC and sFlt1-MDSC groups. In vitro proliferation of MDSCs was not decreased by inhibition of VEGF; however, differentiation into myotubes and adhesion to collagen were significantly lower in the shRNA _VEGF-MDSC group compared with the lacZ-MDSC and sFlt1-MDSC groups.


Key Words: angiogenesis ■ muscular dystrophies ■ stem cells ■ vascular endothelial growth factor

Duchenne muscular dystrophy is an inherited neuromuscular disorder that affects 1:3500 live male births and is caused by a lack of the membrane-stabilizing protein, dystrophin. Without dystrophin, muscle fibers experience periods of pathological degeneration and regeneration and subsequent loss of membrane integrity. Duchenne muscular dystrophy is the most severe form of muscular dystrophy, exhibiting progressive weakness leading to respiratory or cardiac failure and premature death. Stem cell therapy is a promising treatment for numerous disorders, including degenerative muscle diseases, such as Duchenne muscular dystrophy, heart failure, liver failure, bone degeneration, and so on; however, the low survival of transplanted cells and their decreased differentiation capacity into desired cell types hinder positive therapeutic outcomes. Many benefits of stem cell transplantation are attributed to paracrine effects, including increased angiogenesis, decreased fibrosis, immunomodulation, and secretion of survival and stem cell recruitment factors. Vascular endothelial growth factor (VEGF) is a critical paracrine factor whose main function is to promote angiogenesis by improving cellular survival, inducing proliferation, and enhancing migration and invasion of endothelial cells. Recent evidence, however, suggests that VEGF has effects on other cell types as well, such as regulating cardiac, myoblast, podocyte, and hematopoietic stem cell survival, bone differentiation, neurogenesis, and the stimulation of skeletal muscle regeneration. Muscle-derived stem cells (MDSCs) are a stem/progenitor cell population obtained using a modified preplate technique, which have long-term proliferation, self-renewal, and multilineage differentiation capabilities. Our previous work showed that murine MDSCs were superior to myoblasts in generating dystrophin-positive myofibers after transplantation into the hearts and skeletal muscles of dystrophic mice. MDSCs repaired the heart after myocardial infarction to a greater extent than myoblasts and demonstrated a higher level of VEGF expression in the MDSC graft. In dystrophic skeletal muscle, the transplantation of MDSCs expressing VEGF resulted in increased angiogenesis in the engraftment area; conversely, the transplantation of MDSCs expressing the soluble receptor for VEGF, sFlt1 (sFlt1-MDSCs), demonstrated decreased angiogenesis in the engraftment area. Similarly, in...
ischemic cardiac muscle, treatment with sFlt1 abrogated the regenerative capacity of these cells. Increasing VEGF levels in muscle cells before transplantation by ex vivo gene therapy increased levels of angiogenesis in the recipient muscle, reduced the death of donor cells, and improved engraftment of donor cells; however, excessive levels of VEGF can cause disorganized vascularization and have deleterious effects, such as the formation of angiomas or edema. Thus, there is a critical level of VEGF required for optimal tissue regeneration.

An alternative method to increase VEGF secretion by the cells is through mechanical stress (MS). VEGF secretion of MS-MDSCs was significantly greater than nonstimulated (NS) MDSCs in experiments comparing the 2 groups. MS improved transplantation outcomes in the injured heart compared with nonstimulated stem cells and has been shown to be a potent stimulus for angiogenesis and cell fate decisions. MS may represent an effective method to precondition stem cells to increase VEGF secretion and improve cardiac regeneration capacity, without the deleterious effects of VEGF overexpression.

We hypothesized that augmented angiogenesis after transplantation of MS-MDSCs was a result of increased VEGF secretion. Therefore, in the current study, we examined the importance of VEGF secretion by blocking VEGF through 2 different approaches: (1) transduction of MDSCs with sFlt1, which binds to and blocks VEGF secreted within the extracellular space by both donor and host cells, or (2) transduction of MDSCs with short hairpin RNA (shRNA) that specifically targets and degrades only VEGF (shRNA_VEGF MDSCs) mRNA transcribed by the donor cells. This approach allowed us to compare both the overall reduction of VEGF within the host tissue (sFlt1-MDSCs) and the specific reduction of VEGF secretion from only the donor cells (shRNA_VEGF MDSCs). We found that MS of lacZ-MDSCs significantly increased angiogenesis in the engraftment area, which was not observed in the mechanically stimulated sFlt1-MDSCs or shRNA_VEGF MDSCs. Transplantation of shRNA_VEGF MDSCs resulted in fewer dystrophin-positive myofibers than the other groups, and there was a corresponding decrease in the in vitro myogenic differentiation and adhesion capacity of shRNA_VEGF MDSCs. Taken together, our results support the hypothesis that VEGF is integral and critical to the increased angiogenesis observed after transplantation of MS-MDSCs into dystrophic skeletal muscle and that inhibiting VEGF secretion in the transplanted MDSCs results in loss of the beneficial effects of MS on the regenerative potential of MDSCs.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

In Vitro VEGF Secretion and Expression of Mechanically Stimulated MDSCs

Mechanically stimulated MDSCs were genetically modified to express either the soluble VEGF receptor (sFlt1-MDSCs) or shRNA to VEGF (shRNA_VEGF-MDSCs) to block VEGF secretion. Control MDSCs were transduced to express the lacZ reporter gene (lacZ-MDSCs). VEGF secretion from transduced cells was evaluated in vitro after 24 hours of MS. VEGF secretion of lacZ-MDSCs, measured by ELISA, was significantly increased after MS (Figure 1A; n=4; *P<0.05). There was no statistical difference in VEGF secretion between the sFlt1-MDSC and shRNA_VEGF MDSC groups, regardless of MS (Figure 1A); however, their VEGF secretion levels were lower than the lacZ-MDSCs (Figure 1A). Intracellular VEGF expression was also examined by Western blot analysis (Figure 1B–1E). We found an increase in intracellular VEGF levels from mechanically stimulated lacZ-MDSCs compared with NS lacZ-MDSCs (Figure 1B), which was significant when normalized to GAPDH (Figure 1C; n=3; *P<0.05). As expected, we found that under normal conditions, intracellular VEGF levels were decreased by sFlt1 or shRNA-VEGF expression (Figure 1D). Furthermore, after stretch, we found that the sFlt1 or shRNA-VEGF expression diminished stretch-induced VEGF increases (Figure 1E).

Inhibition of VEGF Secretion Increases Tissue Fibrosis in Dystrophic Muscle

We next aimed to determine the effect of blocking VEGF on MDSC transplantation in dystrophic muscle. Three hundred thousand cells from each of the 6 treatment groups were injected into the gastrocnemius muscles of mdx/scid mice. These mice are a model of muscular dystrophy that are both dystrophin deficient and are immunocompromised, and a useful model for cell transplantation. Muscle regeneration is often hindered by the formation of fibrotic tissue. To address how VEGF secretion and MS of MDSCs might affect fibrosis levels and muscle regeneration after cellular transplantation into dystrophic tissue, fibrosis levels were determined by Masson trichrome staining, and regeneration was examined by hematoxylin and eosin staining. Fibrosis levels were higher in muscles transplanted with sFlt1-MDSCs or shRNA_VEGF-MDSCs compared with lacZ-MDSCs, independent of MS (Figure 2G; n=6; *P<0.05). The percentage of centrally nucleated myofibers (regenerating myofibers) was significantly reduced in the muscles transplanted with MDSCs where VEGF was blocked (Figure 2H; n=6; *P<0.05). These results are consistent with our previous findings where we found that blocking VEGF in dystrophic tissue, by transplanting sFlt1-MDSCs, led to increased levels of fibrosis, which further suggests that VEGF produced by the transplanted MDSCs reduces fibrotic scarring in the recipient mice and increases muscle regeneration.

Proangiogenic Effect of MS Is Abolished When VEGF Is Blocked

Two weeks after transplantation, dystrophin-positive myofibers (green) and CD31-positive capillaries (red) were immunohistochemically detected (Figure 3A–3F). Dystrophin is a marker for donor-derived regenerating myofibers, and CD31 (or platelet endothelial cell adhesion molecule) is a marker for donor-derived regenerating myofibers, and CD31 (or platelet endothelial cell adhesion molecule) is a marker for the donor-derived regenerating myofibers, and CD31 (or platelet endothelial cell adhesion molecule) is a marker for donor-derived regenerating myofibers, and CD31 (or platelet endothelial cell adhesion molecule) is a marker.
for endothelial cells. Within the engraftment area, the MS lacZ-MDSC group had significantly greater CD31 structures compared with all other groups (Figure 3G; n=6; *P<0.05). In addition, all 4 groups that had VEGF blocked had significantly less CD31 structures compared with the control lacZ-MDSCs (Figure 3G; n=6; &P<0.05). Angiogenesis distal to the engraftment area was analyzed to further examine the paracrine effects that the implanted cells imparted. Similarly, all 4 groups that had VEGF blocked exhibited significantly fewer CD31-positive structures in areas distal to the engraftment site compared with the NS and MS lacZ-MDSC groups (Figure 3H; n=3; *P<0.05). To take into account variability in the level of skeletal muscle regeneration, we also quantified the number of CD31-positive structures adjacent to dystrophin-positive muscle fibers. This ratio was significantly greater in the MS-MDSC transplantation group compared with NS-MDSC and both the MS and NS sFlt1-MDSC groups (Figure 3I; n=6; *P<0.05). There was no difference in the CD31/dystrophin ratio between MS and NS sFlt1-MDSCs (Figure 3I). There was also no difference in the CD31/dystrophin ratio between NS and MS shRNA_VEGF MDSCs; however, this ratio was significantly greater than the ratios of the other groups (Figure 3I; #P<0.05). This is likely because of the fact that the dystrophin-positive fiber engraftment is significantly reduced in the shRNA_VEGF--transduced MDSC group. Overall, this suggests that the proangiogenic effect of MS on MDSCs is abrogated when VEGF is blocked, as there was no difference in angiogenesis between the stimulated and nonstimulated groups, but there was an increase in angiogenesis after MS in the control group.

**Figure 1.** Secreted and intracellular vascular endothelial growth factor (VEGF). A, VEGF secretion was analyzed with ELISA and normalized to percent nonstimulated (NS) lacZ-MDSC. VEGF secretion was significantly increased after mechanical stimulation (MS; n=12; *P<0.05). B, Intracellular VEGF in the NS and MS lacZ-MDSC groups is shown. C, Intracellular VEGF levels in lacZ-MDSCs were significantly increased after MS (n=3; *P<0.05). D, Intracellular VEGF in the NS lacZ-MDSC, sFlt1-MDSC, and short hairpin RNA (shRNA)_VEGF MDSC groups was analyzed with Western blot. GAPDH is shown as loading control. E, Intracellular VEGF in MS lacZ-MDSC, sFlt1-MDSC, and shRNA_VEGF MDSC groups is shown with GAPDH as loading control. lacZ-MDSC indicates that muscle-derived stem cells were transduced with retroviral vectors encoding the LacZ reporter gene; and sFlt1-MDSC, muscle-derived stem cells were transduced with retroviral vectors encoding the soluble VEGF receptor Flt1.

**Dystrophin-Positive Fiber Engraftment Is Reduced After Transplantation of shRNA_VEGF-MDSCs, But Not in sFlt1-MDSC Transplanted Muscles**

To further investigate whether the innate myogenicity of MDSCs was influenced by the inhibition of VEGF secretion, we quantified the regenerating dystrophin-positive myofibers within the engraftment area. Dystrophic muscles implanted with shRNA_VEGF-MDSCs had significantly reduced dystrophin-positive myofiber regeneration (NS: 9±1, MS: 7±1) compared with lacZ-MDSCs (NS: 38±4, MS: 34±3) and sFlt1-MDSCs (NS: 45±6, MS: 52±5); this was independent of MS (Figure 3J; *P<0.05). Furthermore, dystrophin-positive fiber engraftment was significantly reduced after transplantation of shRNA_VEGF-MDSCs, but not sFlt1-MDSCs,
sugesting that internal autocrine VEGF signaling may play a role in MDSC engraftment into dystrophic muscle.

**shRNA_VEGF Transduction Does Not Decrease MDSC Proliferation or Motility**

The decrease in the number of dystrophin-positive myofibers observed after transplantation of MSDCs lacking VEGF could possibly be a result of a failure of proliferation, an impairment of adhesion/migration, or a defect in the myogenicity of the engrafted cells. To further investigate the underlying mechanisms, we examined the in vitro proliferative, migratory, and myogenic capacities of these cells.

Previous reports showed increased oxidative stress in dystrophic muscle compared with wild-type controls. Consequently, we examined the proliferation and motility of the MSDCs under normal and oxidative stress culture conditions. Proliferation medium supplemented with H2O2 was used to model oxidative stress. Cells were monitored for 60 hours on a live cell imaging system, and cell number was quantified every 12 hours. The proliferation of shRNA_VEGF MDSCs in normal media was significantly greater than sFlt1-MDSCs but not significantly different from the lacZ-MDSCs (Figure 4A; n=12; *P<0.05). The proliferation of MSDCs under oxidative stress was not affected by blocking VEGF; however, in the MS sFlt1-MDSC and MS shRNA_VEGF groups, motility was decreased after exposure to oxidative stress conditions (Figure 4C; *P<0.05; n=12 per group). This suggests that the shRNA_VEGF MSDCs do not have a defect in their in vitro proliferative abilities, but that the combination of blocking VEGF, MS, and oxidative stress reduces MDSC motility.

**Blocking VEGF Decreases MDSC Migration**

Next, we examined the effect of MS and blocking VEGF on the migration and adhesion of MSDCs. A wound healing assay was performed where MSDCs were grown until confluent and then scratch-wounded with a pipette. We analyzed percent wound closure after 18 hours of incubation and found that all MS groups tended to have slower wound healing compared with NS groups, particularly in the shRNA_VEGF MDSC group (Figure 4D; n=6; &P<0.05 between NS and MS shRNA_VEGF MDSCs). Blocking VEGF secretion in general also decreased wound healing, because all groups had significantly slower migration capacities compared with the NS lacZ-MDSC group (Figure 4D; *P<0.05), which suggests that blocking VEGF decreases MDSC migration time.

**In Vitro Adhesion and Myogenic Differentiation of MSDCs Are Impaired by shRNA Silencing VEGF, But Not by sFlt1 Expression**

Adhesion was analyzed by plating MSDCs on collagen-coated dishes and then rinsing away nonadherent cells after 30 minutes. The remaining attached cells were detected by crystal violet staining. There was significantly lower adhesion in the
MS shRNA_VEGF MDSC group compared with all other groups (Figure 5A; n=12; *P<0.05). The NS shRNA_VEGF group also had significantly lower adhesion than all groups, except for the MS lacZ-MDSC and MS shRNA_VEGF MDSC groups (Figure 5A; n=12; &P<0.05). Blocking VEGF with shRNA decreased the adhesion of MDSCs, which was augmented after mechanical stretch.

To determine the myogenic capacity of these cells, we compared the ability of genetically modified MDSCs mechanically stimulated or nonstimulated to form myotubes in low-serum differentiation media for 5 days. Subsequently, we fixed and stained cells for fast skeletal myosin heavy chain and quantified their myogenic index, that is, the number of fast skeletal myosin heavy chain–positive nuclei normalized to the total number of nuclei. We observed drastically fewer fast skeletal myosin heavy chain–positive myotubes formed by shRNA_VEGF-MDSCs compared with the other groups (Figure 5B; n=15; *P<0.05). Altogether, these results suggest that the low dystrophin-positive engraftment of shRNA_VEGF MDSCs may be the result of a deficiency in myogenesis, possibly related to an autocrine reduction of VEGF, because there was no deficiency in the myogenesis of the sFlt1-MDSCs.

Discussion

Stem cell therapy has enormous potential for tissue regeneration; however, methods to harness stem cell potential and overcome obstacles, such as limited differentiation and survival of transplanted cells, are necessary. This study focused on understanding the underlying mechanisms of MS through VEGF blockade to further understand the function of stem cells in tissue repair and regeneration.

Cells are often subjected to MS in their native environment: heart beat, lung inflate, muscle stretch, and cells react to the resultant mechanical load. Mechanical stretch is a powerful stimulus for a broad spectrum of cellular responses, including growth, differentiation, motility, remodeling, and gene expression. Previous studies demonstrated that mechanically stimulated MDSCs expressed more VEGF and were able to repair the infarcted hearts of mice more effectively than nonmechanically stimulated MDSCs, which was attributed, at least in part, to an increase in angiogenesis in the peri-infarct area. In addition, blocking VEGF with sFlt1-MDSCs abrogated the benefits of MDSCs in both skeletal muscle regeneration and cardiac repair.

Figure 3. Angiogenesis and dystrophin-positive myofiber engraftment muscle-derived stem cells (MDSCs) were transplanted into the gastrocnemius muscles of mdx/SCID mice. Two weeks later, CD31 and dystrophin expression were quantified. Green staining represents dystrophin, and red staining represents CD31. A–F, Representative pictures of transplantation of nonstimulated (NS)-MDSCs, mechanical stimulation (MS)-MDSCs, NS sFlt1-MDSCs, MS sFlt1-MDSCs, NS short hairpin RNA (shRNA)_vascular endothelial growth factor (VEGF) MDSCs, and MS shRNA_VEGF MDSCs, respectively. Scale bar, 0.05 mm. G, CD31 was quantified in the engraftment area, and there was increased CD31 expression after mechanical stimulation of lacZ-MDSCs (n=6; *P<0.05 to all other groups), as well as increased CD31 expression in the NS lacZ-MDSC group compared with the MS and NS sFlt1-MDSC and shRNA_VEGF MDSC groups (&P<0.05). H, CD31 distal to the engraftment area was also quantified, and there was increased CD31 expression in the lacZ-MDSC groups compared with the other groups (n=3; *P<0.05). I, The CD31/dystrophin ratio was quantified, and this was increased in the MS lacZ-MDSC group compared with the NS lacZ-MDSC group and the sFlt1-MDSC group (n=6; *P<0.05). There was also an increased ratio in the NS lacZ-MDSC group compared with the sFlt1-MDSC group (&P<0.05). The CD31/dystrophin ratio was increased in the shRNA_VEGF MDSC group compared with all other groups (#P<0.05). J, Quantification of dystrophin-positive myofiber engraftment. The NS and MS shRNA_VEGF MDSC transplantation groups had significantly less dystrophin-positive engraftment than all other groups (n=6; *P<0.05 to NS/MS MDSC, NS/MS sFlt1-MDSC). lacZ-MDSC indicates MDSCs were transduced with retroviral vectors encoding the LacZ reporter gene; and sFlt1-MDSC, MDSCs were transduced with retroviral vectors encoding the soluble VEGF receptor Flt1.
Unlike gene therapy where excessive continuous VEGF expression may cause disorganized vascular structures and is consequently detrimental to tissue repair,22,23 mechanical loading physiologically preconditions MDSCs and therefore avoids the unwanted side effects seen in MDSCs genetically engineered to express VEGF. These studies demonstrated that MS induced the MDSCs to secrete an appropriate level of VEGF, which greatly enhanced the tissue repair process.

In the current study, we examined the vital role that VEGF plays in MDSC-mediated tissue regeneration by inhibiting both host and donor-derived VEGF by using sFlt1-transduced MDSCs. We also looked at the effect of reducing only the donor-derived VEGF by using shRNA_VEGF-transduced MDSCs that specifically target only the donor cell VEGF mRNA. sFlt1 binds VEGF with the same affinity and specificity as the full-length receptor but does not initiate signaling as it is not cell-associated because of its lack of a tyrosine domain. sFlt1 both sequesters VEGF and heterodimerizes with receptors, blocking VEGF signaling in 2 ways.15,38 VEGF is expressed uniformly in adult murine skeletal muscle30; therefore, sFlt most likely reduces the concentration of total VEGF in the extracellular space but does not completely abrogate its effect.

Previous studies have shown a decrease in fibrosis and muscle necrosis with sustained VEGF secretion using gene or cell therapy40; however, when MS-MDSCs were transplanted into an infarcted heart they did not significantly affect fibrosis levels compared with the transplantation of NS-MDSCs.37 We examined collagen formation in muscles implanted with sFlt1-MDSCs, shRNA_VEGF-MDSCs, or lacZ-MDSCs and found increased fibrosis formation in all the groups where VEGF was blocked. This was independent of MS and seems to be associated with the overall reduction of VEGF levels. In addition, we did not observe a decrease in fibrosis in the mechanically stimulated groups, which was consistent with our previous results in the heart.37 This could be attributed to the transient and reversible increase in VEGF secretion stimulated via the mechanical preconditioning of cells which may not have been sufficient to affect the long-term remodeling process.

Angiogenesis is a critical aspect of tissue repair, and VEGF is a potent growth factor involved in this process.8 We observed that when lacZ-MDSCs were mechanically stimulated, their potential to promote angiogenesis increased compared with the nonstimulated lacZ-MDSCs. However, when VEGF secretion was blocked with sFlt1 or shRNA, there was no difference in angiogenesis between the MS and NS groups. This finding suggests that the favorable response to MS is abrogated, regardless of the method used to block VEGF, and that VEGF secretion by the donor MDSCs is a significant factor affecting the increase in angiogenesis observed after MDSC
transplantation. Interestingly, more CD31-positive vasculature was observed distal to the site of cell engraftment, possibly as a result of the disruption of local vasculature by the needle at the injection site.

Although VEGF is well known as a mediator of angiogenesis, there is increasing evidence suggesting that it plays an important role in other cellular functions, including skeletal muscle regeneration. For example, intramuscular administration of recombinant adeno-associated virus-VEGF into the mdx mouse was shown to promote skeletal muscle regeneration and enhance muscle function.40 Also, Deasy et al21 demonstrated that MDSCs expressing VEGF had greater numbers of centrally nucleated fibers compared with control MDSCs. In the current study, we demonstrated that blocking VEGF resulted in decreased numbers of centrally nucleated fibers.

VEGF has also been shown to prevent the death of donor cells; when the hind limb muscles of mice were pretreated with VEGF before myoblast transplantation, it resulted in a reduction of donor cell death and improved cellular engraftment.16 Furthermore, Arsic et al17 observed that VEGF promoted the fusion of myogenic cells to form myotubes and protected the cells from undergoing apoptosis.

In this study, we observed an effect on the differentiation of transplanted MDSCs when VEGF was decreased with shRNA. There were significantly fewer dystrophin-positive myofibers in the shRNA_VEGF-MDSC transplantation groups compared with the lacZ-MDSC and sFlt1-MDSC groups, indicating that VEGF produced by the transplanted cells is important for their function and capacity to regenerate myofibers in dystrophic muscle. This result is in accordance with previous studies that showed that VEGF-null embryonic stem cells had a reduced capacity to differentiate into skeletal muscle,41 which indicates that VEGF had an effect on autocrine myogenic differentiation. Furthermore, C2C12 cells transduced with AAV-sFlt1 had a reduction in their in vitro myotube formation capacity compared with controls;41 however, in another study, C2C12 cells treated with VEGF or a small molecule to block receptor tyrosine kinase activity showed no difference in myotube differentiation capacity.11 After VEGF blockade, we examined the myogenic differentiation capacity.
in vitro and found that shRNA_VEGF-MDSCs formed fewer myotubes than lacZ-MDSCs and sFlt1-MDSCs, which is consistent with our in vivo findings. Taken together, these results indicate a significant role for VEGF signaling in myogenic differentiation and muscle regeneration.

VEGF has been shown to act in an autocrine manner and has a protective/survival effect on many cell types, including endothelial cells, embryonic stem cells, hematopoietic stem cells, and myoblasts. When hematopoietic stem cells were treated with small-molecule inhibitors that blocked internal VEGF receptor signaling, their colony formation was significantly decreased, but treatment with sFlt1 did not have an effect. Autocrine VEGF signaling was also shown to be critical for vascular homeostasis. When VEGF was conditionally knocked out in endothelial cells, there was an increase in endothelial cell death with no reduction in serum VEGF, indicating that paracrine VEGF levels could not compensate for the lack of VEGF within the endothelial cells. The fact that shRNA_VEGF-MDSCs had a reduced capacity for myogenic differentiation but sFlt1-MDSCs had normal differentiation indicates that paracrine VEGF signaling by MDSCs contributed to their decreased capacity for differentiation into dystrophin-positive myofibers.

In conclusion, we demonstrated that inhibiting VEGF secretion by MDSCs blocked the beneficial effects imparted by mechanical preconditioning of these cells before cell transplantation, which resulted in a reduction in the cells’ differentiation capacity in vitro and in vivo, as well as a reduction in the cells’ angiogenic capacity within the transplantation area. This study highlights the importance of donor-derived VEGF in MDSC-mediated muscle regeneration and suggests a potential role of internal autocrine VEGF signaling in MDSC myogenic differentiation. Future studies should involve exploring the mechanisms of these signaling pathways and dissecting out the differences between paracrine and autocrine VEGF signaling by MDSCs.

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Disclosures

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References

Mechanical stimulation (MS), a powerful stimulus for cell growth, differentiation, and motility, was reported to increase secretion of vascular endothelial growth factor (VEGF) and augment the effectiveness of muscle-derived stem cells (MDSCs) for tissue repair. Nevertheless, the importance of VEGF as a key mediator of the effects of MS has never been explored. This study investigated the role of VEGF in MS-MDSCs.

Angiogenesis was increased in muscles transplanted with MS-MDSCs compared with nonstimulated MDSCs and VEGF-blocked MDSCs (with the soluble VEGF receptor, sFlt1, for extracellular VEGF or short hairpin RNA for intracellular VEGF). Myofiber differentiation/regeneration and angiogenesis were significantly lower in the short hairpin RNA_VEGF-MDSC group compared with the control MDSC and sFlt1-MDSC groups (forced alignment of mesenchymal stem cells from murine skeletal muscle by the preplate technique). Nevertheless, the role of angiogenesis of MS on MDSC-mediated muscle repair are lost. These results have important implications for enhancement of cellular functions before and cell adhesion were significantly lower in the short hairpin RNA_VEGF-MDSC group compared with the control MDSC and sFlt1-MDSC groups, suggesting a novel autocrine VEGF-mediated effect on MDSC differentiation. We conclude that by inhibiting VEGF, beneficial effects of MS on MDSC-mediated muscle repair are lost. These results have important implications for enhancement of cellular functions before transplantation.
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The beneficial effect of mechanical stimulation on the regenerative potential of MDSCs is lost by inhibiting VEGF.

Sarah A. Beckman, William CW Chen, Ying Tang, Jonathan D. Proto, Logan Mlakar, Bing Wang, Johnny Huard.

Supplemental Figure

Supplemental Figure I: Schematic of the vectors used in this study.
Materials and Methods:

Animal studies: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. As such, all animal studies conformed to the Guide for the Care and Use of Laboratory Animals as published by the National Institute of Health.

MDSC isolation: Mice were sacrificed by isofluorane overdose followed by cervical dislocation and the skeletal muscles were removed. As previously described, the pre-plate technique was then performed to isolate MDSCs from the skeletal muscle of three week old C57BL mice (Jackson, Bar Harbor, ME). MDSCs were cultured in proliferation media (PM) containing Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS, Invitrogen), 10% horse serum (HS), 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical, Westbury, NY).

MDSCs transduced with a retrovirus carrying the LacZ reporter gene, sFlt1 or shRNA to VEGF: A retroviral vector containing a modified LacZ gene with a nuclear localization sequence has been used previously in our laboratory. The retroviral vector encoding human sFlt1 (Invitrogen) containing a retro-backbone of pCLX driven by the hCMV/LTR promoter was derived from pLXSN (Clontech, Mountain View, CA) as described previously. Based on a retro-CL vector named retro-CLB4G, we removed the BMP4 gene and IRES-GFP cassettes by digesting with Bgl II and Not I enzymes. The purified retro-backbone was filled by the Klenow enzyme for ligation with an insert size of 1.7 kb from the recombinant adeno-associated viral vector digested by the same enzymes and also filled by the Klenow enzyme. This insert comprises of the not only the human U6 driving shRNA cassette that targets to the 198 – 216 region of mouse VEGF mRNA (Ambion #240535, Grand Island, NY), but also contains the CMV promoter controlling the ZsGreen reporter gene to monitor virus transduction efficiency (supplementary Figure 1). The detailed sequence of mVEGF/shRNA includes a 19 bp sense strand (5’-CGAGATAGAGTACATCTTC), 9 bp loop (5’-TTCAAGAGA), and a 19 bp antisense strand (5’-GAAGATGTACTCTATCTCG). To ensure the expression of shRNA-VEGF, transduced cells were sorted to homogeneity (>99% purity) by flow cytometry based on their ZsGreen expression.

Mechanical Stimulation: MDSCs were cultured on bioflex plates-- flexible 6-well culture plates coated with collagen type I (105 cells/mm², Flexcell Intl. Corp, Hillsborough, NC). After 12 hours of culture, an FX-4000T strain unit subjected the cells to 10% equibiaxial strain with a 0.5 Hz sine wave for 24 hours. Control MDSCs were cultured on the same plates without strain. The mechanical stimulation parameters were chosen based on previous studies which indicated an increased VEGF secretion under these conditions in vitro.

VEGF secretion: MDSCs were plated in PM at a density of 105 cells/mm² in 6-well collagen type I coated plates. Prior to collection, the media was switched to DMEM with 1% penicillin/streptomycin for 24 hours. Enzyme-linked immunosorbent assays (ELISAs) for mouse VEGF (R&D Systems, Minneapolis, MN) were performed according to the manufacturer’s instructions and as previously described. VEGF levels were normalized to cell number.
**Western Blot:** Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (#9806, CellSignaling Technology, Inc., Danvers, MA) supplemented with protease (P8340) and phosphatase inhibitors (P5726 and P0044, 1:100, Sigma-Aldrich, St. Louis, MO) and quantified using the Bio-rad Protein Assay Kit 2 (#500-0002, Bio-Rad, Hercules, CA). Membranes were incubated with polyclonal rabbit anti-VEGF (Ab46154, Abcam, Cambridge, MA, USA, 1:1000) at 4°C overnight in 5% milk or bovine serum albumin (BSA) in Tris buffered saline with Tween-20 (TBST). Following washing in TBST, membranes were incubated with the secondary antibody, HRP-conjugated polyclonal goat anti-rabbit (#31460, Thermo Fisher Scientific, Rockford, IL). In order to ensure equal loading, membranes were probed with mouse HRP-conjugated rabbit anti-GAPDH (ab9482, Abcam) or stained with Ponceau S (P7170, Sigma-Aldrich). All Western blots were run under reducing conditions to dissociate multimer complexes.

**Cell transplantation:** For each replicate, 300,000 MDSCs in 20µl PBS were transplanted into the gastrocnemius muscle of 4-6 week old male mdx/scid mice (Jackson, Bar Harbor, ME). Six muscles per group (NS lacZ-MDSC, MS lacZ-MDSC, NS sFlt1-MDSC, MS sFlt1-MDSC, NS shRNA_VEGF MDSC, MS shRNA_VEGF MDSC) were used for analysis. Before transplantation the mice were anesthetized with 2-4% isoflurane in O₂. After 2 weeks, the mice were sacrificed by isoflurane overdose followed by cervical dislocation and muscles were collected, flash frozen in liquid-nitrogen cooled methyl-2-butane and cryosectioned at a thickness of 10µm, as previously described.4

**Histology and Immunohistochemistry**

Masson's Modified IMEB Trichrome (IMEB, San Marcos, CA) staining, which stains collagen (blue) and muscle (red), was performed according to the manufacturer’s guidelines following a protocol previously described by our research group.3,10,11 The sections were assessed for the percent area of collagen in 3 sections per muscle which were normalized to total muscle area using CellProfiler image analysis software (www.cellprofiler.com). Briefly, each image was unmixed to generate a “blue” and “red” grayscale image. These images were then thresholded to identify the appropriate areas and measured.12,13 Percent central-nucleation was determined by H&E staining which was performed according to a previously described protocol.4 The number of CD31 positive cells in the engraftment area was determined by staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma, St. Louis, MO) and goat anti-rat Alexafluor 594 secondary antibody (1:300, Invitrogen). Dystrophin was stained with rabbit anti-dystrophin (1:300; Abcam, Cambridge, MA ) and donkey anti rabbit Alexafluor 488 (1:300, Invitrogen). Endothelial cells within the dystrophin positive and distal areas were determined by counting the number of CD31 positive cells per 60X high powered field in 3 fields per muscle using image J software (NIH, Bethesda, MD).

**Proliferation and motility:** MDSCs were plated in PM at a density of 10 cells/mm² in a 24-well collagen type-I coated plate. Twenty-four hours later, the media was switched to PM, or PM containing 250µM hydrogen peroxide (H₂O₂). The plates were placed onto a live cell imaging system (LCI, Kairos Instruments, LLC, Pittsburgh, PA), and bright field images were taken every 10 minutes over a 60 hour period in 3 locations per well.14 Images were analyzed with ImageJ software. Cell proliferation was determined by counting the number of cells present in the bright
field images at 12 hour intervals, and cell numbers were normalized. Cell motility was analyzed using ImageJ plugin Manual Tracking, and 3 cells were analyzed per well for total distance traveled.

**Wound Healing (Migration) Assay:** MDSCs were plated in PM at a density of $10^5$ cells/mm$^2$ on 12 well collagen type-I coated plates until confluent. A single straight wound was created in the center of each well by scratching the cell monolayer with the tip of a sterile 5 ml serologic pipette. Plates were washed twice with PBS and subsequently placed on the LCI system described above in PM. Each population was cultured in duplicate wells and photographed (3 pictures per well) every 10 minutes for 18 hours. The entire assay was repeated independently 3 times. LCI images were analyzed using Image J. The distance between cells on either side of the wound at wound creation ($D_0$) and end point ($D_1$) were randomly measured 3 times per picture. Migration rate (%) was calculated according to the following equation: $((D_0 - D_1)/D_0)\times100$.

**Differentiation:** MDSCs were plated at a density of 50 cells/mm$^2$ on 24-well collagen type-I coated plates. Twenty-four hours later, the media was changed to DMEM + 2% FBS. At five days, the plates were stained with mouse anti-fast skeletal myosin heavy chain antibody (fsMHC; 1:400, Sigma,) and 4’, 6-diamidino-2-phenylindole (DAPI, for nuclei). The percentage of nuclei in fsMHC positive myotubes compared to total DAPI positive nuclei was quantified to assess myotube formation.

**Adhesion:** MDSCs were plated at a density of 316 cells/mm$^2$ on 96-well collagen type-I coated plates. After 30 minutes cells were washed with ice cold PBS then fixed in cold methanol for 10 minutes. Adherent cells were stained with 0.5% Crystal Violet for 20 minutes and then rinsed in tap water and air dried. Cells were solubilized in methanol and absorbance at 540 nm was determined on a spectrophotometer. The absorbance obtained from wells in the absence of cells was subtracted from all data points.

**Microscopy:** Fluorescence and bright field microscopy were performed using either a Nikon Eclipse E800 microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix Imaging, Cheektowaga, NY) or a Leica DMIRB inverted microscope with a Retiga digital camera and Northern Eclipse software.

**Statistical analysis:** The means and standard errors were calculated for all measured values, and statistical significance between the groups was determined by a 1-way ANOVA (SPSS 20 IBM). In the event of a significant ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (Student-Neuman-Keuls or Games-Howell).


