Fibroblasts as an Alternative to Mesenchymal Stem Cells with Successful Treatment and Immune Modulation in EAE Model of Multiple Sclerosis

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Abstract

The immune modulatory potential of mesenchymal stem cells (MSCs) is well known and is the basis for multiple clinical trials in treatment of autoimmune conditions. Unfortunately, MSCs are relatively rare, difficult to expand in culture, and methods of obtaining MSCs are complicated and expensive. In contrast, fibroblasts are found in copious amounts in various tissues, are a robust cellular population, and can be cultured without need for costs associated with culture media. Previous studies by our group and others have demonstrated fibroblasts possess regenerative activities. In the current study we demonstrated: a) fibroblasts inhibit mixed lymphocyte reaction; b) suppress T cell activation; c) inhibit DC maturation; and d) stimulate T regulatory (Treg) cell formation. Importantly, administration of fibroblasts in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis resulted in disease inhibition, which was abrogated upon depletion of Treg cells. This data, combined with existing clinical safety data on fibroblast administration, supports the clinical translation of fibroblast-based therapies for multiple sclerosis.

Background

Multiple sclerosis (MS) is a T cell mediated autoimmune disorder targeting the myelin sheath which insulates neuronal bodies. The condition presents either as relapse remitting, which as the name suggests, undergoes periods of spontaneous remission, or primary progressive disease, in which the disease advances without remission. It is known that remission is associated with reduction of pathogenic T cells and upregulation of number and activity of T regulatory (Treg) cells (1), while progression is associated with augmentation of myelin-reactive T cells and suppression of Treg activity and number (2). Current treatments for MS include non-specific immune modulators such as Avonex, Copaxone, Rebif, Tysabri and Campath (3). These approaches possess various degrees of toxicity, adverse reactions associated with non-specific immune suppression, as well, display limited efficacy against primary progressive disease.

An ideal treatment for MS would possess the ability to suppress immunological aspects of the disease, while concurrently stimulating regeneration of injured neural tissues. Immunologically, it is believed that Treg cells play an important part of controlling disease progression. In addition to association between Treg and natural disease remission, studies have shown that efficacy of various clinically used treatments of MS associated with induction of Treg activity. Indeed, this has been shown in the case of interferon beta (4-10), as well as Copaxone (11-18), based interventions. Furthermore, animal studies have shown that adoptive administration of Treg cells in models of MS results in disease remissions (19-29). Alternatively, depletion of Tregs utilizing antibody or gene mediated approaches results in acceleration of the disease (30). Therefore, an ideal treatment for MS would possess ability to immune modulate through manipulation of the Treg compartment. Another aspect of MS is the neurodegeneration which occurs as a result of immunologically mediated demyelination. This demyelination results in progressive loss of neurons, which at the clinical level is observed as neurologically mediated deterioration. Chemical and other stimulators of endogenous neural stem cells have been shown to restore myelination in animal models, as well as reversing the pathology (31-52). This suggests it may be feasible to induce repair of tissues which have already been damaged by the immune system using agents which stimulate either oligodendrocyte function thereby promoting remyelination, as well as agents which stimulate endogenous neural stem cells, in order to regenerate neurons which have been damaged.

One approach which has been suggested to concurrently elicit regeneration while slowing down, or reversing immunological abnormalities in multiple sclerosis has been the utilization of cellular based therapies. While “stem cells” originally were believed to function therapeutically by restoring injured tissues, newer data demonstrated therapeutic activity correlates with generation of growth factors and/or antiapoptotic factors by stem cells. Indeed the mesenchymal stem cell type of therapies have been demonstrated to possess multiple immunological functions including: a) ability to suppress T cell activation (53-57); b) ability to suppress activation of NK cells (58-61); c) propensity to induce generation of Treg cells (62-64); and d) efficacy in a wide variety of models of autoimmune disease including the EAE model multiple sclerosis, autoimmune uveitis (65), and the collagen induce arthritis (CIA) model of rheumatoid arthritis (66). In addition to immune modulation, it has been demonstrated that MSCs possess the ability to regenerate neural tissue in models of MS, in part through stimulation of endogenous neural stem cells. Although MSCs possess multiple exiting properties, practical translation has been somewhat poor, with results obtained clinically only marginally effective. In addition, MSCs are

difficult to isolate in bone marrow and adipose MSCs. They also require highly invasive extraction techniques.

Fibroblasts are a fundamental cell type in wound healing and have been previously demonstrated to possess some similarities to MSCs including CD73, CD90, and CD105 markers, as well as orthodox differentiation ability into bone, cartilage and adipose tissue. Although not studied in multiple sclerosis, a previous study explored whether fibroblasts isolated from skin may suppress the host immune response in a model of autoimmune arthritis. It was found that fibroblasts possessed the capacity to inhibit in vitro the proliferation of T lymphocytes. Fibroblasts also secrete modulatory molecules, such as prostaglandin E2 and nitric oxide, similar to MSCs. To assess their role in vivo, the collagen-induced arthritis model was used, and showed that similar to MSCs the intravenous injection of fibroblasts efficiently suppressed clinical signs of arthritis and delay of disease onset. This effect was associated with reduced inflammation as reflected by biological parameters and increased levels of IL-5, IL-10 and IL-13 in the spleens of treated mice (67). The CybroCell™ product is a clinical grade fibroblast population which has been demonstrated to possess preclinical and clinical efficacy in treatment of degenerative disc disease. In the current paper we assessed whether fibroblasts are superior immune modulators to MSCs, as well as assessed their therapeutic ability in an animal model of EAE.

Materials and Methods

Animal Model of Multiple Sclerosis

Adult male Lewis rats (9–10 weeks of age, 200–250 g) were assigned to four groups: normal control group (n = 12), BM-MSC group (n = 12), Adipose MSC group (n = 12), and Fibroblast group (n=12). Experimental autoimmune encephalomyelitis (EAE) was induced by subcutaneous injection of guinea pig spinal cord homogenate (GPSCH) emulsified at a 1:1 ratio with complete Freund adjuvant (CFA) containing heat killed *Mycobacterium tuberculosis*. Each rat received an intraperitoneal injection of 300 ng Pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 ml distilled water immediately after the subcutaneous injection and again 48 hours later. Cells were injected at a concentration of 1 million cells per rat intravenously subsequent to administration of GPSCH. The clinical manifestations of EAE were assessed daily until the time of sacrifice. Disease severity was scored on a 5-point scale: 0 = no signs, 1 = partial loss of tail tonicity, 2 = loss of tail tonicity, 3 = unsteady gait and mild paralysis, 4 = hind limb paralysis and incontinence, and 5 = moribund or death. Disease scoring was performed by pathologists blinded to treatment conditions.

Antibody Depletion and Cytokine Assessments

Depletion of Treg cells was performed using goat anti-rat CD25 antibody by administration of the antibody every second day intraperitoneally. Confirmation of Treg depletion was performed by flow cytometric assessment. When >75% depletion of CD25 cells at 7 days post EAE induction was accomplished (confirmed by different epitope targeting antibody), animals were considered “depleted”. For assessment of cytokine levels, blood was drawn from the tail vein and IL-10 and IL-17 levels were assessed from plasma using Enzyme Linked Immunosorbent Assay (ELISA). Assessments were made at days 0, 18, and 31.

Fibroblasts

Rat dermal fibroblasts were obtained from ATCC and propagated in Optimem media using 10% FCS and pen-strep in fully humidified atmosphere. In some experiments human fibroblasts were utilized and grown under similar conditions.

Mixed Lymphocyte Reactions

Dermal fibroblasts were obtained from ATCC and maintained in DMEM media with 10% FCS in a fully humidified environment, with penicillin/streptomycin mixture and non-essential amino acids. Cells were harvested at 75% confluence by trypsinization and plated with immature dendritic cells at day 5 of DC maturation. Cells were plated in 12 well plates with 100,000 fibroblasts per 1,000,000 DC, 500,000 fibroblasts per 1,000,000 DC and 1,000,000 fibroblasts per 1,000,000 DC. After 48 hours of culture cells were extracted and CD40 expression was assessed by flow cytometry.

Generation of DC was performed by culturing monocytes in GM-CSF and IL-4 for 5 days according to the method of Inaba et al. and subsequently matured by addition of TNF-alpha on day 5 before the coculture. In some experiments LPS was added to the fibroblasts at a concentration of 5 ug/ml.

Results

Reduction of Multiple Sclerosis Pathology in the Experimental Allergic Encephalomyelitis (EAE) Model by Fibroblasts is Superior to Mesenchymal Stem Cells

Previous studies have demonstrated fibroblasts possess various similarities to mesenchymal stem cells (MSCs) including surface markers, morphology, and some aspects of immune modulation (68). In order to compare therapeutic activity between fibroblasts and MSCs, we utilized the rat model of multiple sclerosis termed experimental allergic encephalomyelitis (EAE). This model consists of immunization with xenogeneic spinal cord homogenate as a source of myelin basic protein (MBP). Also, utilizing a strong adjuvant to break self-tolerance, it combines with pertussis toxin to permeabilize the blood brain barrier. For comparison, MSCs were utilized from adipose and bone marrow sources.

All cells were administered at a concentration of 1 million cells, intravenously, the same day as administration of xenogeneic spinal cord homogenate. As seen in Figure 1, BM-MSCs possessed the least EAE inhibitory activity, while adipose-MSCs possessed slightly stronger inhibitory activity. Fibroblasts induced potent reduction of disease progression, with no clinical symptomology on day 24, in contrast, both BM and adipose derived MSCs treated groups had pathology through completion of experiments at day 31.

Figure 1: Superior Inhibition of EAE Pathology by Fibroblasts Compared to MSC

In Vitro Mechanisms of Fibroblast Immune Modulatory Activity

While previous studies showed immune modulatory activities of fibroblasts, including ability to suppress mixed lymphocyte reaction, direct suppression of autoantigen specific responses has not been demonstrated to our knowledge. This is fundamentally important due to the qualitative differences between an alloantigen-specific immune reaction, in which numerous T cell clones are responding, compared to antigen-specific reactions in which only one or a number of T cell clones are expanding. Accordingly, T cells specific to myelin basic protein were generated by in vitro stimulation with antigen presenting cells and interleukin-2 administration. An increasing number of fibroblasts were shown to dose-dependently inhibit proliferation of antigen specific reactive T cells (Figure 2a). Additionally, utilizing a similar system, a question was contemplated whether fibroblasts can elicit generation of T regulatory cells (Treg). It is known that Treg cells are capable of suppressing autoimmunity in a variety of situations, leading to the question of whether fibroblasts are capable of inducing generation of this cell type. As seen in Figure 2b, increasing numbers of FoxP3 cells were observed in MPB-reacting T cells when fibroblasts were added.

Figure 2: Fibroblasts Modulate Antigen-Specific T cell Responses

Fibroblasts Block IL-17 Inflammatory Pathway

It is known Treg cells are reciprocal cells to Th17 cells, and it is also known the Th17 CD4 T cell subsets plays a major role in pathology of EAE as well as numerous other autoimmune disorders in man and mice (69-79). Accordingly, we assessed the IL-17 mediated pathway in vitro and in vivo. Fibroblasts dose dependently suppressed macrophage production of TNF-alpha subsequent to toll-like receptor (TLR-4) stimulation with lipopolysaccharide (Figure 3a). Given that IL-17 is a hallmark of pathogenic Th17 cells, which are upstream of TNF-alpha producing cells in the immunological cascade, we assessed in vitro whether IL-17 was reduced by fibroblasts in response to IL-6 induction. We observed a dose dependent inhibition of IL-17 production

Figure 3: Fibroblasts Block IL-17 Pathway in Vitro and in Vivo

in vitro (Figure 3b). In order to assess in vivo relevance of these findings, we sought to determine levels of IL-17, as well as its reciprocal cytokine IL-10. This is made by Treg cells and is known to activate Treg cells by assessing levels of these cytokines from the peripheral blood of rats undergoing EAE at days 0, 18 and 31. Quantification of cytokines was performed in rats who received control saline, fibroblasts, BM-MSCs, and adipose MSCs. Similar changes were observed in vivo with suppression of IL-17 and stimulation of Treg cells (Figure 3c and 3d).

Inhibition of Dendritic Cell Maturation by Fibroblasts

The fundamental importance of dendritic cells (DCs) cannot be overstated in both activation and suppression of immune responses. Mature DCs are uniquely capable of activating naïve T cells through their high and dense expression of membrane-bound costimulatory molecules such as CD40, CD80, and CD86 and the soluble costimulatory signal IL-12. Immature dendritic cells possess ability to induce immunological tolerance through their high levels of co-inhibitory molecules such as IL-10 and PD-L1 (80-94). Given that fibroblasts are associated with post-injury healing, and that upregulation of immune inhibitory molecules is known to occur in the healing phase of injury, we sought to investigate whether DC maturation was altered by fibroblasts using a co-culture system. Suppression of TNF/LPS induced DC maturation was observed in terms of downregulation of CD40, CD80, CD86, and IL-12, along with upregulation of the inhibitory molecules IL-10, IL-1RA, and PD-L1 (Figure 4a-g). In accordance with the in vivo data demonstrating superior activity of fibroblasts to MSCs, fibroblasts were superior to bone BM and adipose derived MSCs at suppressing DC maturation (Figure 5a-g).

Figure 4: Fibroblasts Inhibit DC Maturation

Figure 5: Fibroblasts are Superior to MSC at Inhibiting DC Maturation

Depletion of Treg Results in Abrogation of Protection from EAE Pathology by Fibroblasts

Given the findings that fibroblasts reduce generation of IL-17 cytokine in response to IL-6 in vitro and inhibition IL-17 in vivo while stimulating IL-10, the potential of fibroblasts inhibiting EAE through a Treg dependent manner was considered. This possibility is further strengthened by our findings that fibroblasts stimulate generation of Treg in vitro. Accordingly, a series of experiments was conducted in order to determine whether antibody mediated depletion of Treg would alter efficacy of fibroblasts at reduction of EAE pathology. As seen in Figure 6, reduction of EAE pathology in response to fibroblast administration was significantly abrogated in animals in which Treg were depleted but not in isotype control immunized animals.

Figure 6: Depletion of Treg Abrogates Therapeutic Effect of Fibroblasts on EAE Pathology

- ◇ Control
- Fibroblast + AntiCD25 every 6 days
- △ Fibroblast + AntiCD25 every 3 days
- × Fibroblast

Regenerative Effects of Fibroblasts on EAE Pathology

Although numerous immune modulatory agents have been explored in the context of EAE as a preclinical model for translational development, relatively little work has been performed in examining the possibility of agents which stimulate regeneration of tissue subsequent to injury. Accordingly, we examined the ability of neuronal remyelination by assessing whether the number of remyelinated neurons increased subsequent to treatment. As seen in Figure 7a, an increase in remyelination was observed, which was more profound with fibroblasts as compared to BM and adipose derived MSCs. Additionally, slices of the dentate gyrus revealed cells possessing the proliferating cell nuclear antigen (PCNA), which is a marker of cellular proliferation (Figure 7b). These data suggest that fibroblasts reduce pathology of EAE not only by stimulation of inhibitory immune responses, primarily such as Treg and immature/tolerogenic DC, but also fibroblasts mediate a regenerative effect.

Figure 7: Regenerative Effects of Fibroblasts in EAE Model

Discussion

Regenerative therapy for MS has historically been limited to stem cell approaches. Autologous hematopoietic stem cell use post-immune ablation has demonstrated some level of efficacy, however cost and potential adverse reactions of full-immune compromise make this treatment difficult to implement on a large scale. The use of MSCs has demonstrated promise in animal models, but, clinical responses have been mediocre. The cost of culturing MSCs, as well as difficulties in acquisition and quality control represents hurdles in the commercialization of these cells. In the current paper we demonstrate superior efficacy of fibroblasts to MSCs in terms of reducing progression of disease pathology, stimulating Treg formation, inhibiting IL-17 and augmenting IL-10.

Utilization of fibroblasts as a substitute for MSCs has been previously proposed by our group based on similar phenotypic and morphological characteristics between the two cell types. Previously published data supporting that fibroblasts, similar to MSCs, possess ability to differentiate along the orthodox pathway, such as chondrocytes, adipocytes, and osteocytes. In addition to studies, which demonstrated efficacy of fibroblasts for differentiating into chondrocytes in vivo and generating improvement in animal models of degenerative disc disease, an independent group reported similar findings. This strongly supports development of fibroblast-based products as an alternate to MSCs. The recent FDA IND clearance to initiate clinical trials using fibroblasts for treatment of patients with degenerative disc disease, is further support for this. The demonstration of superior inhibition of multiple sclerosis-like pathology using fibroblasts as compared to MSCs cannot be accounted for by different passage numbers since these were equalized between groups. Furthermore, an increased efficacy using adipose derived MSCs compared to bone marrow was shown. Previous studies which have suggested superior immune modulatory effects of adipose MSCs compared to BM MSCs. While fibroblasts used in the experiments were dermal derived, the possible optimization of therapeutic activity by choosing fibroblasts from other sources is an intriguing question which is currently under investigation.

Mechanistically, the suppression of IL-17 production in vitro and in vivo appeared to be related to efficacy of fibroblasts. Other studies have suggested that MSCs possess ability to suppress IL-17, however, in these experiments fibroblasts were markedly superior. Some studies have shown wound healing is associated with potent immune modulation, accordingly, given the potent role of fibroblasts in wound healing, cytokine mediated immune modulation activity of fibroblasts appears to be quite potent. Other soluble factors have not been examined but are the topic of current investigation. One particular area warranting further examination is whether fibroblasts are mediating therapeutic effects by secretion of exosomes. Our preliminary data supports the ability of exosomes to modulate some aspects of the regenerative process. Indeed, future studies may seek to optimize therapy by administration of exosomes together with cellular therapy.

It has previously been demonstrated by us (WPM and TEI) that immature DC give rise to tolerogenic DC which in turn cause activation of more Treg. This “inhibitory feedback loop” demonstrated to be responsible for maintaining allograft tolerance in the B6 to BALB/c cardiac heterotopic transplant model. Accordingly, it may be feasible to imagine that fibroblasts are giving rise to immature, or “tolerogenic” DC, which in turn cause generation of Treg cells. Indeed, the data described supports the fundamental role of Treg in fibroblast-mediated reduction for EAE pathology resulting in depletion of the cell loss of therapeutic activity. Future studies to combine Treg cells with fibroblast administration and/or tolerogenic DC are underway. This gives rise to the concept that the fibroblast may not only serve as a

monotherapy for treatment of MS, but may also increase efficacy of existing cell therapies for this condition. Furthermore, certain drugs on the market are believed to function, *intra alia*, through stimulation of Treg cells. The combination of fibroblasts with drugs such as copaxone and interferon beta are being investigated.

Endogenous neural stem cells have been reported to accelerate repair of various neurological injuries including stroke, traumatic brain injury, and EAE. The possibility of stimulating endogenous neural stem cells using external approaches has been investigated with some success using approaches such as hCG administration. Recently administration of MSCs has been shown to enhance proliferation of endogenous neural stem cells and may have been a mechanism of action for some types of cellular therapy. The current study demonstrated fibroblasts can enhance neural stem cells, which offers the possibility of repairing injured tissue. Additionally, our data demonstrated augmentation of myelination subsequent to treatment. Whether this is result of *de novo* regeneration of oligodendrocytes from progenitor cells, or whether activation of existing oligodendrocyte cells is still under investigation. This data is particularly interesting in light of a publication by Nessler et al. who showed no effect of MSCs on cuprizone induced demyelination, which is non-immune mediated (95).

In conclusion, we describe the novel ability of fibroblasts to reduce autoimmunity in the EAE model of multiple sclerosis. The possibility of utilizing less expensive fibroblasts as a superior type over MSCs for therapeutic use is will lead to further development and testing.

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