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Crohn’s disease (CD) is a chronic inflammatory disease of the gastrointestinal tract which can affect any part of the intestine. A frequent manifestation of CD is the formation of perianal fistulas which can greatly affect patient’s quality of life due to continuous pain, abscess formation and malodorous discharge from the fistula causing skin irritation. Nowadays, a wide range of medical and surgical therapies for perianal fistulizing CD is available. However, achieving complete fistula healing is often a long process preceded by multiple relapses during the treatment. Currently, mesenchymal stromal cells (MSCs) have gained much interest as a potential therapeutic option for inflammatory disorders, including fistulizing CD, because they possess immunosuppressive and tissue regenerative properties. Therefore, in this thesis the focus is on the effects of current treatment strategies on fistula healing, the safety, feasibility and efficacy of locally injected MSCs in patients with perianal fistulizing CD, and the mechanisms by which systemically and locally injected MSCs attenuate colitis in experimental mouse models.

CURRENT TREATMENT OPTIONS FOR PERIANAL FISTULIZING CD

In most patients treatment of perianal fistulas is necessary and drugs, surgery or a combination of both is required to achieve fistula closure. Until now, anti-tumor necrosis factor (TNF) agents are the most effective medical treatment for the induction and maintenance of fistula remission. In combination with temporary antibiotics higher response rates are observed compared to monotherapy with anti-TNF. However, durable remission rates of initially healed fistulas are low after treatment with anti-TNF agents and surgery is often inevitable. Success rates of the different surgical options such as fibrin glue, fistula plug, mucosal advancement flap (MAF) and ligation of the intersphincteric fistula tract (LIFT) range from 38 - 71%. The majority of the included patients in these trials had perianal fistulas based on cryptoglandular disease. Perianal fistulas as a manifestation of CD have a different etiology than those caused by cryptoglandular disease and often evolve into in complex fistulas with presence of rectal inflammation. Therefore, the actual success rates of surgery for the closure of perianal fistulas may be lower in case of CD. Indeed, in the cohort of 232 patients with perianal fistulas based on CD more than three quarters of the patients had complex fistulas (chapter 3). After the conventional treatment strategies, simple fistulas healed significantly more often compared to complex fistulas which is in line with previous published papers. In addition, we observed that complex fistulas relapsed more often than simple fistulas after a median follow-up of 10 years resulting in a disappointing durable remission rate of only 37%. Interestingly, therapy with anti-TNF did not result in significantly higher fistula closure rates compared to patients who did not receive anti-TNF agents. In this retrospective study patients treated for perianal fistulizing CD from
1980 – 2000 were included and followed until 2010. Infliximab was available from 1999 in the hospital possibly resulting in a delayed time to treatment in patients with treated for perianal fistulas before 1999.

MESENCHYMAL STROMAL CELLS

A new experimental approach in the treatment of perianal fistulizing CD is cellular therapy with mesenchymal stromal cells (MSCs). MSCs have immunomodulatory and tissue regenerative capacities making them a potential new treatment option for perianal fistulizing CD. MSCs are present in the stroma of almost all solid organs and in the bone marrow, and are easily isolated and expanded in culture. An advantage of MSCs is their relatively immunological inertness meaning that allogeneic MSCs of healthy donors can be used creating the possibility of an ‘off-the-shelf’ treatment potential. In chapter 4 we describe the results of our early phase II randomized, double-blind, placebo-controlled clinical trial evaluating the use of allogeneic bone marrow-derived MSCs in the treatment of perianal fistulizing CD. Only patients refractory for the conventional treatment strategies were included. Local administration of MSCs additional to a standardized surgical treatment was safe and feasible. Interestingly, higher fistula healing rates were observed when a low dose of MSCs was given (1x10^7 or 3x10^7) compared to a high dose (9x10^7) or placebo. A similar inverted dose response was observed in both an experimental model and clinical trial when MSCs were injected in an ischemic heart.\textsuperscript{17,18} Whether immunogenicity resulting in faster clearance of the cells and/or a lower survival and function of a high dose of MSCs is the explanation for this inverted dose response has to be elucidated in further research.

When 3x10^7 MSCs were locally administered, 85.7% of the fistulas healed already 6 weeks after the injection. Previously published phase I and II trials showed fistula closure rates of 69-82%.\textsuperscript{19-23} However, comparison of the results of these studies is difficult as cell source, cell number and time of primary endpoint and evaluation were not similar. In addition, in some trials fibrin glue was added to the local MSC treatment and multiple injections of MSCs were given when fistulas were not healed after some weeks. Furthermore, the location of injection of MSCs differed between the studies. Therefore, standardization of the treatment procedures is of utmost importance to be able to reliably compare the effect of local MSC therapy observed in different clinical trials. In chapter 5 we propose standardized operating procedures (SOPs) for the classification of perianal fistulizing CD, the surgical intervention, the local therapy administration and follow-up based on the procedures which we applied in our above described clinical trial.
MSCS: MODE OF ACTION

Although encouraging results have been obtained in almost all performed clinical trials on perianal fistulizing CD, the exact mode of action of MSCs is only partially known. Until now, only one animal model of perianal fistulas resembling human perianal fistulizing CD, has been described.24 However, only approximately 5% of these mice spontaneously developed perianal fistulas making this model unsuitable for evaluation of the mechanisms by which MSCs induce immunosuppression in perianal fistulizing disease. Fistulas usually arise at the site of distal intestinal inflammation and the effect of MSC treatment at the site of disease initiation still needs further exploration. Therefore, we induced experimental colitis in mice using dextran sulphate sodium (DSS) or 2,4,6-trinitrobenzenesulfonic acid (TNBS) to examine the mode of action of MSCs. Introduction of DSS to the drinking water for 7 days is toxic to the gut epithelial cells and breaks the mucosal barrier resulting in diarrhea, weight loss and visible fecal blood. This model is useful when studying the innate immune system as the adaptive immune system does not play a major role in establishing DSS-induced colitis.25 On the other hand, TNBS induces colitis via a Th1-mediated immune response resembling CD. TNBS is diluted in ethanol which affects the integrity of the mucosal barrier. TNBS then haptenizes the colonic microflora to become immunogenic resulting in segmental ulcerations with influx of macrophages and lymphocytes.25

MSCs can alter cytokine production of distinct immune cells in order to induce an anti-inflammatory milieu both in vitro and in vivo, however, whether or not MSCs need to be in contact with these immune cells to educate them to become tolerant or that the release of soluble factors without cell-to-cell contact is their main mechanism of inducing immunosuppression, is still under debate.26-29 In addition, various studies showed that MSCs are not intrinsically immunosuppressive and that priming with proinflammatory cytokines might be necessary to induce their full immunosuppressive capacities. However, ‘over’ or ‘under’ priming can result in enhancement of the proinflammatory response.30-32 In chapter 6 we elaborate on the importance of the time of MSC administration in an experimental colitis model. Only severe colitis was attenuated after MSC injection indicating the importance of an initiated/ongoing inflammation for MSCs to become immunosuppressive. The study described in chapter 7 we confirm these observations as MSCs only alleviated experimental colitis when injected 5 hours after the establishment of acute colitis, but not when mice had a ‘too’ severe colitis. In addition, attenuation of colitis was accelerated when MSCs were pretreated in vitro with interferon-gamma (IFNγ) and TNFα to become i/tMSCs before injection in colitic mice. We hypothesize that MSCs have to be licensed in vitro with proinflammatory cytokines to gain their full immunomodulatory capacities when administrated in vivo. In addition, migration promoting vascular cell adhesion molecule
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results suggest that the combination of migration and the upregulation of anti-inflammatory factors iNOS and TSG-6 is, at least partially, the mode of action of MSCs in experimental colitis. In addition, MSCs were observed to form spherical shaped cell aggregates, i.e. spheroids, at the serosal site of the distal colon. Both macrophages and T cells were observed within these spheroids suggesting that MSCs were in contact with these immune cells. In addition, the MSC spheroids formed a niche by producing collagen. Similar observations were recently made by an Italian group. The aggregated MSCs they observed produced TSG-6 that attenuated colitis, e.g., by upregulating iNOS expressing regulatory macrophages. Taken together, MSCs appear to form spheroids that produce collagen to create their own niche which possibly increase their immunosuppressive capacities. In that niche MSCs are in contact with distinct immune cells possibly resulting in upregulation of anti-inflammatory factors iNOS and TSG-6. Increasing migration to the inflamed colon by prestimulation with proinflammatory cytokines may accelerate attenuation of colitis as increased amounts of MSCs at the place of inflammation may result in the formation of more spheroids. We confirmed that in vitro generated MSC spheroids, resembling the MSC spheroids observed in vivo, are able to alleviate experimental colitis, as described in chapter 8. In this model we infused MSC spheroids locally via enema in order to evaluate the effect of MSC administration directly at the place of inflammation.

FUTURE PERSPECTIVES

The results described in this thesis demonstrate the importance of the moment of MSC administration in experimental mouse models. An ongoing inflammation seems to be of importance to prime MSCs towards an immunosuppressive phenotype subsequently attenuating colitis. Indeed, the recently published phase III trial on autologous adipose-derived MSCs as a treatment for complex cryptoglandular perianal fistulas (which excluded patients with CD) showed no beneficial effects of MSCs added to fibrin glue compared to
fibrin glue alone, possibly because the pathogenesis of cryptoglandular fistulas is not based on a chronic inflammatory disease such as CD.\textsuperscript{36} In contrast, we observed that if mice had a ‘too’ severe colitis, administration of MSCs did not have a therapeutic effect on the disease. The latter suggests that in human severe perianal fistulizing CD should possibly not be treated solely with MSCs. On the other hand, in our clinical trial, which resulted in a fistula healing rate of 85.7\% 6 weeks after local injection of 3x10\(^7\) allogeneic bone marrow-derived MSCs, as described in chapter 4, only patients with active perianal fistulizing CD refractory to conventional treatment strategies were included indicating that only patients with severe fistulas joined the study. Since biologicals such as infliximab, adalimumab and vedolizumab, are very expensive, studies to determine the long-term fistula healing rate after local MSC administration are needed. In addition, trials evaluating the most effective donor source (autologous vs allogeneic; bone marrow-derived vs adipose-derived; young donor vs old donor), MSC cell dose, frequency and interval of MSC administration, and treatment strategy (injection into the wall or into the lumen of the fistula; with or without addition of fibrin glue) are warranted. Currently, the department of Gastroenterology and Hepatology at the Leiden University Medical Center plans to initiate a trial using 3x10\(^7\) allogeneic bone marrow-derived MSCs to evaluate the efficacy of this treatment in a larger group of patients with refractory perianal fistulizing CD. In addition, patients with refractory rectovaginal fistulas will probably be included. Furthermore, a multicenter, randomized, double-blind, placebo-controlled phase III trial to evaluate the safety and efficacy of allogeneic adipose-derived MSCs as a treatment for complex perianal fistulizing CD has been conducted by TiGenix. The results of this study are expected to be published at the end of 2015.

In all published trials MSCs were locally injected in perianal fistulas, probably to ensure a high dose of MSCs at the place of inflammation with a lower risk of systemic adverse events compared to intravenous (i.v.) injection. In addition, perianal fistulas are more easily accessible for local treatment compared to luminal CD. Until now, probably because of the latter reason, MSCs were only given i.v. in trials treating patients with luminal CD. Recently, Forbes et al.\textsuperscript{37} performed an open-label study evaluating the efficacy of allogeneic bone marrow-derived MSCs in moderate to severe refractory CD. Four i.v. infusions of 2x10\(^6\) cells/kg at weekly intervals were given to 15 patients. 53\% of the patients were in clinical remission 3 weeks after the last injection of MSCs and 80\% of the patients showed a clinical response to the treatment. In contrast, however, the phase I study from our group using a single dose of autologous bone marrow-derived MSC in CD was found to be relatively ineffective.\textsuperscript{38} Several studies have shown that the number of MSCs that specifically home to the site of inflammation after systemic injection is low.\textsuperscript{39,39,41} Therefore, in our preclinical study, as described in chapter 8, we infused MSCs locally via an enema at the site of
inflammation in mice with DSS-induced colitis. Although intraluminal infusion of MSCs resulted in attenuated colitis, we did not observe MSCs in the mucosa when the colons were histologically evaluated after sacrifice. Currently, we are examining if MSCs injected endoscopically into the wall of the inflamed colon leads to alleviated disease in mice with DSS-induced colitis. We hypothesize that by injecting MSCs into the wall of the colon, these cells will engraft into the mucosa and possibly enhance their efficacy at the site of inflammation. The next step would be to evaluate the safety, feasibility and preliminary efficacy of endoscopically injected MSCs into the inflamed areas of the colon in patients with localized luminal disease.

As described in chapter 7, prestimulation of MSCs with proinflammatory cytokines to gain their full immunomodulatory abilities resulted in accelerated attenuation of TNBS-induced colitis compared to non-prestimulated MSCs. Recently, a case report on the treatment of a patient with refractory CD with IFN-γ-prestimulated MSCs was published. The patient received twice 2×10^6 cells/kg. Although the authors describe that the infusions were well tolerated, the patient had an exacerbation of CD 10 days after the second infusion. Several studies have suggested that MSCs can become antigen presenting cells when primed with IFN-γ by upregulating MHC class II. The possibility that these IFN-γ-MSCs had become immunogenic is not discussed in this case report except for the fact that an increased percentage of natural killer cells was found in the peripheral blood of the patient after the second infusion indicating lysis of MSCs by these cells. Methods to enhance the immunomodulatory abilities of MSCs other than by priming them with proinflammatory cytokines, must be investigated. Recently, MSCs were observed to spontaneously aggregate into spheroid-like structures after systemic infusion (chapter 7). In vitro, clustering of MSCs resulted in enhanced expression of immunomodulatory proteins such as PGE2 and TSG-6 which might lead to improved therapeutic efficacy. Therefore, more preclinical studies to investigate role of MSC aggregation in establishing the immunosuppressive capacities of MSCs should be conducted.

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