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ORIGINAL ARTICLE

Treatment of enterocutaneous fistula in Crohn's Disease with adipose-derived stem cells: a comparison of protocols with and without cell expansion

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Abstract
Background Expanded adipose-derived stem cells (ASC) have been shown to be effective in treating Crohn's patients with enterocutaneous fistulas. It is possible that unexpanded cells corresponding to the stromal vascular fraction (SVF) may also be effective.
Materials and methods A subpopulation of patients from a previous proof-of-concept phase I study with enterocutaneous fistulas received autologous expanded ASCs. The same selection criteria for inclusion were applied to patients who underwent SVF implantation to treat enterocutaneous fistulas. After tract curettage, cell suspensions (either SVF cells from lipoaspirate or expanded ASCs) were injected into the tract walls, and the fistulous tract was sealed with fibrin adhesive (with or without cells).
Results In the series that received ASCs, four fistulas could be evaluated, and cure was achieved in three out of four cases. In the series that received SVF cells, four fistulas were evaluated, with cure achieved in one out of four cases.
Conclusions Although a comparison of case series cannot be considered firm evidence, a therapeutic protocol that uses expansion prior to implantation does seem to be more effective than one that uses SVF cells directly from a lipoaspirate sample.

Keywords Cell therapy · Adipose stem cells · Enterocutaneous fistula

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Introduction

Enterocutaneous fistulas associated with Crohn's disease are an extremely challenging problem since many patients do not respond to available treatments [1]. Such fistulas and their recurrence are a very distressing complication that significantly reduces the quality of life of affected patients. Despite a clear therapeutic need, few clinical trials have been performed with novel therapies [2].

Currently, the most accepted treatment is a monoclonal antibody, infliximab [1]. The efficacy of infliximab maintenance therapy after a successful induction phase was investigated in a randomized study of patients with enterocutaneous fistulas [3]. However, only 7% of infliximab-treated patients and 15% of placebo-treated patients had enterocutaneous fistulas, and the outcomes were not analyzed by fistula type. It is therefore difficult to assess the efficacy of infliximab in this type of fistula.

Mesenchymal stem cell therapy represents another possible approach in fistulas, and fatty tissue from liposuction procedures is an attractive source of such stem cells [4, 5]. Previously, we have reported the use of cultured (expanded) adipose-derived mesenchymal stem cells (ASC) in the treatment of a patient with rectovaginal fistula and in the treatment of different types of fistula, including enterocutaneous fistulas, in a phase I study [6, 7].

A series of patients with enterocutaneous fistulas were treated in a phase I study; we used cells from the stromal vascular fraction (SVF), that is, ASCs without expansion. In both processes, we used the same protocol to obtain the SVF.

Materials and methods

We report an observational study with series of patients with Crohn's enterocutaneous fistulas who had not responded to medical treatment and in whom traditional surgery had failed. All procedures were performed in the University Hospital La Paz, Madrid, by the same surgeon and in the same laboratory. Both the phase I therapeutic protocol with expanded ASCs and the SVF protocol were approved by the ethics committee of the University Hospital La Paz in accordance with Spanish law; all patients signed a detailed informed consent form prior to any interventions, as well as a consent for publication. We conducted this study in accordance with the ethical standards of the Committee on Human Experimentation of the University Hospital La Paz and in accordance with the ethical standards of the Helsinki Declaration of 1975.

Stromal vascular fraction from lipoaspirate

Between 80 and 100 ml of lipoaspirate was obtained from plastic surgery. The raw lipoaspirate was washed extensively with equal volumes of sterile phosphate-buffered saline (GIBCO BRL, Paisley, UK) to remove cells, saline, and local anesthetic. The lipoaspirate was digested with type I collagenase (GIBCO BRL) at 0.075% final concentration in saline solution at 37°C for 45 min to release the cellular fraction. Dulbecco's modified Eagle's medium (GIBCO) with fetal bovine serum (10% *v*/*v*) was then added to inactivate the collagenase; the resulting cell suspension was centrifuged for 10 min at 250×*g* and the cell pellet was washed with phosphate-buffered saline, centrifuged again, and treated with ammonium chloride 160 mM at room temperature for 10 min to lyse any remaining erythrocytes. The cells were then washed, filtered through 40-μm nylon mesh, and suspended in sterile Ringer-lactate solution (Griffols S.A., Barcelona, Spain) for immediate implantation without expansion (SVF). No additional supplements were added to the ASC culture in the manufacturing process. With regard to the SVF fraction, the cells were never cultured. Cell viability was confirmed with trypan-blue (Sigma, St Louis, MO, USA), and it was always higher than 95%.

Stem cell expansion and preparation for implantation

The SVF cells were seeded at $2–3\times10^4$ cells/cm^2 and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (GIBCO) and 1% ampicillin/streptomycin without additional supplements at 37°C under 5% CO_2 atmosphere. Once approximately 80% confluence had been reached, cells were detached by trypsinization (trypsin: EDTA; GIBCO) and replated at $2–3\times10^4$ cells/cm^2. Expansion cycles (passages) of the cells were repeated up to three times until reaching the required number of cells for implantation. In two cases, the cells were then frozen until use. Morphologic determinations and phenotypic analysis by flow cytometry and immunohistochemistry were performed during expansion of cells [7]. Mycoplasma contamination was evaluated in the ASCs using MycoAlert Mycoplasma Detection Kit (Cambrex, East Rutherford, NJ, USA). With respect to SVF, they were not evaluated due to their high heterogeneity. At least 1 week before the intervention was scheduled, expanded ASCs were thawed and cultured. In all cases, these ASCs were then washed with phosphate-buffered saline, trypsinized, and centrifuged. The cells were then resuspended in Ringer-lactate solution. Cell viability was confirmed with trypan-blue (Sigma) and was always greater than 95%. The suspensions were used immediately.

Treatment procedure

1. Deep curettage of the tract
2. Injection of the cell suspension using a long thin needle in the walls of the tract, depositing half of the total cell dose. Injection was very superficial, and no deeper than 2 mm.
3. Half the cells were resuspended in the thrombin component of a fibrin glue kit (Tissucol® Duo 2.0; Baxter, Madrid, Spain) before combining the kit's two components. The track was then sealed.
4. The area of the external opening was covered with gauze.

Treatment outcomes

One of the fistulas in one patient could not be assessed because she presented with acute sepsis associated with a new enterovesical fistula that required emergency resection of the treated area. This event was not considered related to the therapeutic protocol.

Follow-up schedule

Weekly follow-up was scheduled for 8 weeks after surgery. Patients were considered healed when total epithelialization of the external opening was evident after 8 weeks, regardless of prior observations. Thereafter, patients attended a follow-up approximately every month for at least 12 months and for up to 48 months. No ultrasound or magnetic resonance imaging examinations were used in this evaluation.

Results

Patients

Six patients were included in this study; all of them signed the corresponding informed consent for the different treatments. The range of the patients' age was between 36 and 48 years. The patient's baseline clinical features (Vienna classification Crohn's disease [8]), age at diagnosis, comedication, etc., are shown in Table 1.

Treatment

Two patients of this series corresponded to a subgroup from a previous phase I trial [7]. These patients were treated with expanded ASCs for five enterocutaneous fistulas. The remaining patients were treated for four enterocutaneous fistulas with SVF (Table 1).

Clinical results

Of the four assessable treatments with expanded ASCs, three (75%) were successful, with complete re-epithelization of the external opening at week 8 (Table 1). Notably, a recto-vaginal fistula in one of these patients had not healed with

Table 1 Summary of clinical features, treatments applied and outcome

Sex	Age (years)	Disease classification[a]	Years from diagnosis	Comedication[b]	Fistula site	Implant #	Passage no./% ASCs in culture	Culture time (days)/ process time (hours)	No. of cells ($\times 10^6$)	Outcome
Expanded ASC-treated patients										
F	40	L3B3A1	16	CyA CPFx	Middle line	2	1	9	9.0	Healed
F	40	L3B3A1	16	CyA CPFx	Periumbilical	3	1	7	8.0	NA[c]
F	41	L3B3A1	17	CyA CPFx	Suprapubic	5	2	8	3.5	Healed
F	41	L3B3A1	17	CyA CPFx	Right lower quadrant	9	3	12	15.0	Healed
M	41	L2B3A1	13	Aza	Right lower quadrant	7	2	16	30.0	Not healed
SVF-treated patients										
F	44	L1B3A2[d]	4	Not specific[e]	Periumbilical	1	5	3.10	5.5	Not healed
F	48	L2B3A1	24	Not specific[f]	Periumbilical	3	2	3.30	176.0	Healed
M	36	L2B3A1	11	Aza	Middle line	4	3	2.50	30.0	Not healed
F	41	L2B3A1	23	GC Aza CPFx	Left lower quadrant	5	3	3.10	20.0	Not healed

ASC adult stem cells, *Aza* azathioprine, *CPFx* ciprofloxacin, *CyA* cyclosporine A, *GC* corticoid, *NA* not assessed, *SVF* stromal vascular fraction
[a] According to Vienna classification: localization, behavior, age [8]
[b] Medication was not modified during the procedure and for at least 8 weeks after surgery
[c] Not included in final analysis because implant area was resected before the end of follow-up (see text)
[d] Clinical diagnosis without histopathological verification
[e] Dolantin, lorazepam, escitalopram, calcium
[f] Desloratadine, inhaled budesonide and formoterol, magnesium

similar treatment. In the SVF-treated group, one out of the four fistulas treated with SVF had healed after 8 weeks (25%). No adverse reactions were reported in any of the transplants performed.

Follow-up

Patients were followed up for a minimum of 1 year. The reported efficacy outcomes at week 8 were maintained at the 1-year visit. No adverse reactions were reported during the follow-up period. Unhealed patients will receive the standard surgical care until a possible ASC treatment is available.

Discussion

Implantation of expanded ASCs is a novel and promising therapy for fistulizing Crohn's disease. In a phase I proof-of-concept study, healing was obtained at 8 weeks in six out of the eight assessable fistulas [7]. In the subpopulation with enterocutaneous fistula considered in this study, three out of the four fistulas treated had healed at the end of initial follow-up.

A therapeutic procedure that used the cells from the so-called SVF before expansion would be simpler, but given the lower concentration of real ASCs (2–5% of the total according to our characterization of the SVF, 1–2% according to Daniels [9]), such an approach might not be as effective. In an attempt to treat Crohn's enterocutaneous fistula, in four patients with enterocutaneous fistula, we injected SVF cells according to the same protocol as in the phase I study, with the exception that the SVF cells were used without expansion [7]. It is noteworthy that only one of the four fistulas treated using the SVF protocol healed compared to three of the four with the expanded ASC protocol.

Although this is not a randomized controlled trial, the surgical team was the same, and every effort was made to ensure that the procedures were done in similar conditions. In addition, the number of cases compared is small, further undermining the validity of the comparison. Nevertheless, the findings do point to better outcomes with the expanded ASC protocol and could justify further investigation. It might not also be coincidence that the only patient with healing treated with cells from the SVF was the one with at least five times more cells (and, hence, five times more ASCs) implanted than the other patients. Otherwise, the better quality of expanded cells could be an important factor.

The mechanism of action of ASCs in this setting is unknown, although in an autoimmune disorder such as Crohn's disease the immunosuppressive action of ASCs might play a role. McIntosh et al. reported that ASCs significantly suppressed T-cell proliferation in a mixed lymphocyte reaction [10]. No such effect occurred for the SVF fraction without expansion, an observation that might explain why expanded ASCs are more effective than the SVF if, indeed, immunosuppression is implicated in the mechanism of action. The same authors also reported that allogeneic SVF cells, unlike expanded ASCs, were immunogenic in that they elicited a T-cell proliferative response.

In conclusion, although a comparison of a case series cannot be considered firm evidence, these data can build a basis for future controlled trials to understand the advantages of using expanded ASCs vs SVF.

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